

DRUG TARGETING AND STIMULI SENSITIVE DRUG DELIVERY SYSTEMS

Edited by
Alexandru Mihai Grumezescu

Pharmaceutical Nanotechnology Series

Drug Targeting and Stimuli Sensitive Drug Delivery Systems

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Drug Targeting and Stimuli Sensitive Drug Delivery Systems

Edited by

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**William
Andrew**
Applied Science Publishers

William Andrew is an imprint of Elsevier
The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States

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British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-813689-8

For Information on all William Andrew publications
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Publisher: Matthew Deans

Acquisition Editor: Laura Overend

Editorial Project Manager: Thomas Van Der Ploeg

Production Project Manager: Debasish Ghosh

Cover Designer: Greg Harris

Typeset by MPS Limited, Chennai, India

Contents

List of Contributors	xix
Series Preface: Pharmaceutical Nanotechnology	xxv
Preface	xxvii

CHAPTER 1 Treatment strategies in cancer from past to present 1

*Hatice Yildizhan, Nezehat Pinar Barkan, Seçil Karahisar
Turan, Özerk Demiralp, Fatma Duygu Özel Demiralp,
Bengi Uslu and Sibel A. Özkan*

1.1	The Importance of Diagnosis in Cancer Therapy	1
1.2	Traditional, Well Proven Therapies.....	3
1.2.1	Surgery	3
1.2.2	Radiotherapy	6
1.2.3	Hormone Therapy	8
1.2.4	Chemotherapy	8
1.3	Targeted Therapies and Immunotherapy	17
1.3.1	Why Immunotherapy is the Magician in Cancer Therapy?.....	18
1.3.2	Well Known Immunotherapeutic Agents and Their Advantages/Disadvantages.....	19
1.4	Smart Role of the Nanotechnology in Cancer Treatment.....	27
1.4.1	Brief List of Studies for Usage of Nanotechnology in Cancer Treatment	28
1.5	Conclusion	30
	References.....	31
	Further Reading	37

CHAPTER 2 Nanotechnology-based drug delivery systems: Challenges and opportunities..... 39

*Deepti Sharma, Navneet Sharma, Mallika Pathak,
Paban K. Agrawala, Mitra Basu and Himanshu Ojha*

2.1	Introduction	40
2.1.1	What is Nanotechnology?.....	40
2.1.2	Nanotechnology Research Fields.....	40
2.1.3	Drug Delivery to Targets	41
2.2	Fundamentals of Nano-Targeted Drug Delivery	44
2.2.1	Shape and Size	45
2.2.2	Realistic Concerns with Nanodrug Delivery	53

2.3	Evaluation of Nanotechnology for Industrial Applications	54
2.3.1	Research Pipeline	55
2.3.2	Clinical Trials.....	58
2.3.3	Developmental Questions	58
2.3.4	Assessment of Marketed Products and Technologies	61
2.3.5	Techniques for Further Development of Nanodrug Delivery	63
2.3.6	Nanotoxicology	64
2.4	Conclusion	68
	References.....	68

CHAPTER 3 Nanotechnology applications in drug controlled release..... 81

*Analía Simonazzi, Alicia G. Cid, Mercedes Villegas,
Analía I Romero, Santiago D. Palma
and José M. Bermúdez*

3.1	Introduction	82
3.1.1	Nanotechnology in Drug Delivery	84
3.1.2	Targets for Drug Delivery	86
3.1.3	Advantages of Nanoparticles.....	87
3.1.4	Drug Release From Nanoparticles.....	88
3.2	Nanotechnology-Based Drug Delivery Systems	89
3.2.1	Fullerenes	89
3.2.2	Solid Lipid Nanoparticles.....	91
3.2.3	Routes of Administration of Solid Lipid Nanoparticles.....	93
3.2.4	Liposomes	94
3.2.5	Microemulsions	95
3.2.6	Nanoemulsions	96
3.2.7	Dendrimers	97
3.2.8	Carbon Nanomaterials	98
3.2.9	Magnetic Nanoparticles	98
3.3	Nanoparticles Preparation Methods.....	99
3.3.1	Solvent Evaporation Method	99
3.3.2	Polymerization Method.....	100
3.3.3	Coacervation or Ionic Gelation Method.....	100
3.3.4	Spontaneous Emulsification or Solvent Diffusion Method	100
3.3.5	Double Emulsion and Evaporation Method	101
3.3.6	Salting Out Method.....	101
3.3.7	Precipitation Method.....	102

3.3.8	Miniemulsion	102
3.3.9	Microemulsion	103
3.3.10	Interfacial Polymerization.....	104
3.3.11	Controlled/Living Radical Polymerization.....	105
3.3.12	Desolvation.....	105
3.3.13	Supercritical Fluid Technology	106
3.3.14	Spray-Drying	106
3.4	Limitations/Toxicology of Nanomedicines	106
3.5	Future Trends for Nanomaterials in Drug Delivery	107
	References.....	108
CHAPTER 4	Target-specific delivery: An insight.....	117
	<i>Prasoon Pandey and Neelam Balekar</i>	
4.1	Targeting.....	118
4.1.1	Background	118
4.1.2	Concept and Rationale	120
4.1.3	Target Identification.....	120
4.1.4	Levels of Targeting.....	122
4.2	Site-Specific Delivery	124
4.2.1	Receptors.....	124
4.2.2	Carriers	126
4.2.3	Ligands	140
4.3	Challenges and Obstacles.....	140
4.4	Gene Delivery	141
4.4.1	Introduction	141
4.4.2	Approaches for Gene Therapy.....	142
4.5	Gene Modification.....	143
4.5.1	Replacement Therapy	143
4.5.2	Corrective Gene Therapy.....	143
4.6	Gene Transfer Techniques	144
4.6.1	Physical Techniques.....	144
4.6.2	BIOLOGICAL (Viral Vectors).....	146
4.6.3	CHEMICAL (Nonviral Gene Delivery).....	146
4.7	Gene Transfer to Specific Cell Lines	146
4.7.1	Somatic Gene Therapy.....	146
4.7.2	Germline Gene Therapy.....	147
4.7.3	Delivery Systems in Gene Therapy.....	147
4.7.4	Candidate Diseases	149
4.7.5	Problems Associated With Gene Therapy.....	149
4.8	Conclusion	150
4.9	Future Perspective	150
	References.....	151

CHAPTER 5	Stimuli-responsive nanosystems for drug-targeted delivery	155
	<i>Carla M. Lopes, Pedro Barata and Rita Oliveira</i>	
5.1	Introduction	155
5.2	Stimuli-Responsive Nanosystems	160
5.2.1	Physical Stimuli-Responsive Nanosystems	160
5.2.2	Chemical Stimuli-Responsive	171
5.2.3	Dual and Multistimuli Responsive Nanosystems	177
5.3	Conclusions and Future Perspectives	193
	Acknowledgments	194
	References	194
CHAPTER 6	Stimuli sensitive ocular drug delivery systems	211
	<i>Francisco J. Otero-Espinar, Anxo Fernández-Ferreiro, Miguel González-Barcia, José Blanco-Méndez and Asteria Luzardo</i>	
6.1	Introduction	211
6.2	Anatomical Characteristics of the Eye	212
6.2.1	Barriers to Topical Ocular Drug Delivery	215
6.2.2	Classic Routes of Ophthalmic Administration	215
6.3	Hydrogel as Drug Delivery Systems	217
6.3.1	Stimuli-Sensitive Hydrogels	218
6.3.2	Properties of In Situ Hydrogels: Studies of Stimuli Response	222
6.3.3	Stimuli-Responsive Hydrogels as Ophthalmic Drug Delivery Systems: Drug-Loading Efficiency and Drug Release	229
6.4	Other Pharmaceutical Systems for the Modification of Drug Loading and Release Properties	231
6.5	New Technologies for Determining Ocular Residence Time and Ocular Biodistribution of Hydrogels	242
6.6	Toxicity and Safety of Polymers Used in Stimuli Gel System: In Vitro, Ex Vivo and In Vivo Assays	246
6.7	Effect of the Hydrogels on the Corneal Regeneration Capacity	247
6.8	In Vivo Studies in Animals and Effectiveness of Drugs When They are Incorporated in the Hydrogels	248
6.9	Studies of Drug Penetration and Promotion	249
	References	251

CHAPTER 7	Stimuli-sensitive nanomaterials for antimicrobial drug delivery	271
	<i>Smritilekha Bera and Dhananjoy Mondal</i>	
7.1	Introduction	271
7.2	Physicochemical Behavior of Nanoparticles on Drug Property Improvement	273
7.2.1	Influence of Particle–Particle Interaction at Nanoscale to Antibacterial Activity	274
7.3	Mechanism of Action of Antimicrobial Nanoparticles	276
7.4	Type of Antimicrobial Nanoparticles	278
7.5	Effect of Stimuli on Drug Delivery	279
7.5.1	Effect of Temperature and pH on Antimicrobial Nanoparticles.....	280
7.5.2	Effect of Light on Antimicrobial Nanoparticle.....	283
7.5.3	Effect of the Enzyme on the Antimicrobial Nanoparticle	291
7.5.4	Effect of Magnet on Antimicrobial Nanoparticle	293
7.6	Conclusions and Future Prospects	296
	Acknowledgment	296
	References.....	297
CHAPTER 8	Stimuli-responsive micelles: A nanoplatform for therapeutic and diagnostic applications	303
	<i>Hema A. Nair, Gopal Singh Rajawat and Mangal S. Nagarsenker</i>	
8.1	Introduction	304
8.2	Properties of Polymeric Micelles.....	304
8.2.1	Size	305
8.2.2	Critical Micelle Concentration and Micelle Stability	305
8.2.3	Ability to Avoid Uptake by the Mononuclear Phagocytic System	305
8.2.4	Core-Shell Structure.....	306
8.3	Structural Aspects of Micelles and Micelle-Forming Amphiphiles	307
8.3.1	Micelle Shape and Morphology	307
8.3.2	Amphiphilic Block Copolymers Used in Fabrication of Micelles.....	308
8.4	Micelles in Drug Delivery Applications.....	309
8.5	Micelles in Targeted Drug Delivery.....	311
8.6	Stimuli-Responsive Micelles.....	312
8.6.1	pH-Responsive Micelles	313

8.6.2	Thermoresponsive Micelles	318
8.6.3	Photoresponsive Micelles	323
8.6.4	Miscellaneous.....	329
8.7	Diagnostic Applications	330
8.7.1	Nuclear Imaging.....	331
8.7.2	Magnetic Resonance Imaging.....	333
8.7.3	X-ray Computed Tomography.....	334
8.7.4	Fluorescence-Based Imaging	335
8.8	Conclusion	335
	References.....	336

CHAPTER 9 Design of targeting peptides for nanodrugs for treatment of infectious diseases and cancer 343

Sanja Glisic and Veljko Veljkovic

9.1	Introduction	344
9.2	Targeting.....	344
9.2.1	Passive Targeting	345
9.2.2	Active Targeting	346
9.3	Targeting Ligands	348
9.3.1	Monoclonal Antibodies.....	348
9.3.2	Antibody Fragments.....	349
9.3.3	Nanobodies as Targeting Therapeutics and Targeting Ligands	349
9.3.4	Aptamers	350
9.3.5	Proteins.....	350
9.3.6	Peptides	351
9.4	Nanomedicine for Targeted Delivery of Natural Compounds.....	353
9.4.1	Paclitaxel Nanodelivery Systems	353
9.4.2	Peptide-Targeted Paclitaxel-Loaded Nanoparticles	354
9.5	Nanomedicine and Infectious Diseases	355
9.6	Identification of Peptide-Based Targeting Ligands.....	356
9.6.1	Selection of Targeting Peptides From Biological and Chemical Libraries.....	356
9.7	Computational Design of Peptide Ligands.....	358
9.7.1	The Concept of Long-Range Intermolecular Interactions in Biological Systems	359
9.8	Nanoinformatics, Databases and Targeting Information.....	369
9.9	Conclusion	370
	Acknowledgments	371
	List of Abbreviations.....	371

References.....	371
Further Reading	379

CHAPTER 10 Targeting drugs to cell and organ using nanoparticles 383

Mayuri V. Gurav and Satish B. Bhise

10.1	Introduction	384
10.1.1	General Introduction on Nanoparticles	384
10.1.2	Cell and Organ Targeting Using Nanoparticles	384
10.1.3	Intracellular Targeting Using Nanoparticles	384
10.2	Characteristics of Nanoparticles Affecting Drug Targeting	385
10.2.1	Particle Size	385
10.2.2	Surface Properties of Nanoparticles	385
10.2.3	Drug Release	386
10.3	Modifying Nanoparticles for Cell, Organ Specific Targeting.....	386
10.4	Nanoparticles Targeting Cells.....	387
10.4.1	Nanoparticles Targeting Tumor Cells	387
10.4.2	Nanoparticles Targeting Lymphocytes.....	391
10.4.3	Nanoparticles Targeting Neutrophils.....	392
10.4.4	Nanoparticles Targeting Cardiac Cells.....	394
10.5	Nanoparticles Targeting Organs	394
10.5.1	Nanoparticles Targeting Lungs.....	394
10.5.2	Nanoparticles Targeting Brain.....	396
10.5.3	Nanoparticles Targeting Vascular Endothelium	398
10.5.4	Nanoparticles Targeting Skin	399
10.5.5	Nanoparticles Targeting Intestinal Epithelium.....	402
10.5.6	Nanoparticles Targeting Inner Ear	403
10.6	Nanoparticles Targeting Protein	403
10.7	Nanoparticles in Diagnosis	404
10.7.1	Detection of Progressive Liver Disease	404
10.7.2	Detection of Chronic Kidney Disease	405
10.8	Conclusion	406
	References.....	406
	Further Reading	408

CHAPTER 11 Extracellular vesicles as a recipe for design smart drug delivery systems for cancer therapy 411

*Carmen V.F. Halder, Emanuella M.B. Fonseca,
Alessandra V. de S. Faria and Stefano P. Clerici*

11.1	Introduction	412

11.2	Extracellular Vesicles: Biogenesis and Composition.....	412
11.3	Influence of Calcium, pH, and Hypoxia in the Biogenesis of Extracellular Vesicles	416
11.4	Extracellular Vesicle Biochemical and Biological Characterization.....	417
11.4.1	Exosome/Ectosome Isolation: Differential Centrifugation, Polymer Precipitation, Antibody-Coupled Magnetic Beads, and Microfluidic.....	417
11.4.2	Extracellular Vesicle Characterization	419
11.5	Methods for Loading Cargo in Extracellular Vesicles.....	421
11.5.1	Loading of Cells Before Extracellular Vesicle Isolation.....	422
11.5.2	Loading of Extracellular Vesicle After Isolation.....	422
11.6	Extracellular Vesicle Mimetics.....	423
11.7	Extracellular Vesicles Uptake Into Recipient Cells	424
11.7.1	Participation of Lipid Rafts	424
11.7.2	Clathrin-Mediated Endocytosis	424
11.7.3	Caveolin-Dependent Endocytosis	425
11.7.4	Macropinocytosis	425
11.7.5	Phagocytosis.....	425
11.8	Assessing Extracellular Vesicle Uptake Into Recipient Cells	426
11.8.1	Labeling Membrane	427
11.8.2	Labeling Exosome Cargoes	427
11.8.3	Intravesicle Probe.....	428
11.9	Overview of Extracellular Vesicles for Therapeutic Delivery of Small Molecules, Proteins, and RNAs.....	428
11.9.1	Exosomes as Drug Delivery Platforms for Small Molecules	430
11.9.2	Exosomes as Drug Delivery Platforms for Proteins	431
11.9.3	Exosomes as Drug Delivery Platforms for RNAs	432
11.10	Extracellular Vesicles as Drug Delivery Platforms for Therapeutic Benefit in Clinical Trials	435
11.11	Concluding Remarks	436
	Acknowledgments	438
	References.....	438
CHAPTER 12	Advancements in exogeneous techniques for stimuli-sensitive delivery systems	447
	<i>Hemant K.S. Yadav, Alyazya Mohammed and Manar Dibi</i>	
12.1	Introduction	447

12.2	External-Stimuli-Responsive Systems	450
12.2.1	Magnetically Responsive Systems.....	450
12.2.2	Ultrasound-Responsive Systems.....	460
12.2.3	Electro-Responsive Drug Delivery Systems	465
12.3	Other Classes of Exogenous Stimuli-Responsive Systems.....	467
12.3.1	Light-Responsive Systems (Photo-Sensitive).....	467
12.3.2	Thermo-Responsive Systems.....	468
12.4	External Multistimuli-Responsive Drug Delivery Systems	471
12.4.1	External Dual Stimuli-Responsive Systems.....	471
12.5	General Phenomena in Delivering Anticancer Therapeutic Agents Via Stimuli-Responsive Drug Delivery Systems	472
12.6	Future Directions for Drug Delivery Systems that Response to External Stimuli	475
12.7	Conclusion and Perspectives.....	476
	References.....	476

CHAPTER 13 Ligand-directed tumor targeting with hybrid viral phage nanoparticles..... 483

Bhavin Dalal, Thomas J. Quinn, Lauren Foster, Melody Lin, Marissa Matthews and Brian Yuhan

13.1	Introduction	484
13.1.1	Early Foundations of Gene Therapy	484
13.1.2	Clinical Applications of Gene Therapy.....	484
13.2	Vectors.....	485
13.2.1	Nonviral Vectors	485
13.2.2	Viral Vectors	486
13.3	Targeted Gene Therapy.....	490
13.4	Ligand-Directed Nanoparticles	490
13.5	Advantage of Targeted Gene Therapy With Bacteriophage	491
13.6	Hybrid Adeno-Associated Virus/Phage Vector.....	491
13.7	Construction of Targeted Adeno-Associated Virus/Phage Vectors for Eukaryotic Cell Transduction.....	495
13.8	Clinical Potential of Adeno-Associated Virus/Phage.....	496
13.9	Molecular-Genetic Pet Imaging Using RGD-AAVP-HSVtk	497
13.10	Targeting Tumor Vasculature With RGD-Targeted Adeno-Associated Virus/Phage Vector-Mediated Tumor Necrosis Factor- α	499
13.11	RGD-Targeted Adeno-Associated Virus/Phage Vector-Mediated Tumor Necrosis- α In Vitro Assay	500
13.12	Preclinical Trial in Small Animal Model With RGD-Adeno-Associated Virus/Phage-Tumor Necrosis Factor- α	501

13.13	Preclinical Trial in Large Animal Model With RGD-Adeno-Associated Virus/Phage-Tumor Necrosis Factor- α	502
13.14	Octreotide-Targeted Adeno-Associated Virus/Phage Vector-Mediated Tumor Necrosis- α In Vitro Assays.....	503
13.15	Preclinical Trial in Small Animal Model With Oct-Adeno-Associated Virus/Phage-Tumor Necrosis Factor- α	503
13.16	Toxicity and Preclinical Safety of Adeno-Associated Virus/Phage-Tumor Necrosis Factor- α	504
13.17	Combination Adeno-Associated Virus-Mediated Gene Therapy With Radiation Therapy	505
13.18	Preclinical Evaluation of Adeno-Associated Virus/Phage-Tumor Necrosis Factor With Radiation Therapy in Metastatic Melanoma Model	506
13.19	Future Directions.....	507
	References.....	511

CHAPTER 14 Delivering miRNA modulators for cancer

treatment 517

Marilene Estanqueiro, Helena Vasconcelos, José Manuel Sousa Lobo and Helena Amaral

14.1	Introduction	517
14.2	The Therapeutic Potential of miRNA Modulators in Cancer	525
14.3	MicroRNA Modulators Delivery	526
14.3.1	Principal Barriers	526
14.3.2	Viral Vectors	527
14.3.3	Nonviral Vectors	533
14.4	Clinical Trials and Future Perspectives	548
14.5	Conclusions	549
	References.....	550

CHAPTER 15 Oral controlled and sustained drug delivery systems: Concepts, advances, preclinical, and clinical status 567

Gaganjot Kaur, Jasleen Grewal, Kiran Jyoti, Upendra K. Jain, Ramesh Chandra and Jitender Madan

15.1	Introduction	568
15.1.1	Overview and Classification of Oral Controlled Release Dosage Forms.....	568
15.1.2	Overview of Oral Sustained Release Dosage Forms ...	569
15.2	Ideal Properties of Drugs for Oral Controlled and Sustained Drug Delivery	570

15.2.1	Physicochemical Properties	570
15.2.2	Biological Properties.....	572
15.3	Polymers for Fabrication of Oral Modified Drug Delivery Systems.....	573
15.3.1	Polymers for Oral Matrix Systems	574
15.3.2	Polymers for Oral Membrane Controlled Systems	576
15.3.3	Polymers for Bioadhesive Controlled Release Systems.....	577
15.3.4	Stimuli-Responsive Polymers.....	578
15.4	Oral Controlled Release Dosage Forms	581
15.4.1	Design and Concepts in Oral Controlled Release Dosage Forms.....	581
15.4.2	Biopharmaceutics and Preformulation Considerations in Oral Controlled Release Dosage Forms	584
15.4.3	Coating Systems for Oral Controlled Release Systems.....	588
15.4.4	Challenges and New Technologies in Oral Controlled Release Drug Delivery	590
15.4.5	Quality by Design Approach in Oral Controlled Release Drug Delivery	597
15.4.6	Advantages and Disadvantages of Controlled Release Drug Delivery Systems	599
15.5	Oral Sustained Release Dosage Forms	600
15.5.1	Design and Fabrication of Oral Sustained Release Systems.....	600
15.5.2	Potential Advantages and Limitations of Oral Sustained Release Dosage Forms	604
15.5.3	Key Formulation Considerations in Oral Sustained Release Dosage Forms.....	605
15.5.4	Coating Systems for Oral Sustained Release Dosage Forms	607
15.6	Preclinical and Clinical Status of Oral Controlled and Sustained Release Dosage Forms	616
15.7	Conclusion.....	616
	References.....	616
	Further Reading	626
CHAPTER 16	Hydrogels: From simple networks to smart materials—advances and applications	627
	<i>Shweta Sharma, Ankush Parmar and S.K. Mehta</i>	
16.1	Introduction	628

16.2	Hydrogels in a Historical Perspective: From Simple Networks to Smart Materials	631
16.2.1	First Generation Hydrogels.....	631
16.2.2	Second Generation Hydrogels	633
16.2.3	Third Generation Hydrogels	638
16.2.4	Fourth Generation: Smart Hydrogels	642
16.3	Properties of Hydrogels.....	644
16.3.1	Factors Affecting Swelling of Hydrogels.....	644
16.3.2	Dynamics of Swelling.....	644
16.3.3	Mechanical Properties.....	645
16.3.4	Cytotoxicity and In Vivo Toxicity	645
16.3.5	Miscellaneous Properties	646
16.4	Classification of Hydrogels.....	647
16.4.1	Hydrogels Based on Properties.....	649
16.4.2	Stimuli-Responsive Hydrogels	649
16.5	Synthesis of Hydrogels	650
16.5.1	Chemically Cross-Linked Hydrogels.....	650
16.5.2	Physically Cross-Linked Hydrogels	658
16.6	Hydrogels in Drug Delivery.....	660
16.7	Conclusion	662
	List of Abbreviations.....	663
	References.....	664

CHAPTER 17 Recent advances in understanding of blood–brain tumor barrier (BTB) permeability mechanisms that enable better detection and treatment of brain tumors..... 673

Divya Khaitan, Polluru L. Reddy, Divya S. Narayana and Nagendra S. Ningaraj

17.1	Introduction	674
17.1.1	Blood–Brain Barrier Permeability	674
17.1.2	Blood–Brain Tumor Barrier Permeability.....	675
17.1.3	Biochemical Modulation of the Blood–Brain Tumor Barrier	676
17.2	Functional Ion Channels on Blood–Brain Tumor Barrier	677
17.3	Nanomedicines and Nanoimaging	678
17.4	Assessment of Brain Tumor, Tumor Vasculature and Permeability.....	678
17.4.1	Clinical Applications of Perfusion Magnetic Resonance Imaging	680

17.5	Nanotechnology for Improved Brain Tumor Detection and Treatment.....	682
17.6	Summary.....	684
	Acknowledgments	685
	References.....	685
	Further Reading	688
CHAPTER 18	Polymeric nanocarriers for site-specific gene therapy	689
	<i>Geeta Arya, R. Mankamna Kumari, Nikita Sharma, Nidhi Gupta, Ramesh Chandra and Surendra Nimesh</i>	
18.1	Introduction	689
18.2	Site-Specific Gene Targeting	691
	18.2.1 Antibody Mediated Targeting.....	692
	18.2.2 Aptamers	692
	18.2.3 Other Ligands.....	693
18.3	Polymeric Nanoparticles	693
	18.3.1 Chitosan.....	694
	18.3.2 Polyethylenimine.....	696
	18.3.3 Poly (Lactic-co-Glycolic) Acid	697
	18.3.4 Poly-L-Lysine.....	699
	18.3.5 Cyclodextrins	700
	18.3.6 Dendrimers.....	701
	18.3.7 Polymethacrylate.....	703
18.4	Clinical Trials of Polymeric Nanocarriers for Gene Therapy.....	705
18.5	Future Perspectives of Gene Therapy.....	707
	References.....	707
	Further Reading	714
CHAPTER 19	Cyclodextrin-based polymeric nanosystems	715
	<i>Nazlı Erdoğan, Gamze Varan, Cem Varan and Erem Bilensoy</i>	
19.1	Introduction	715
19.2	Cyclodextrins.....	716
	19.2.1 Natural Cyclodextrins and Cyclodextrin Derivatives ...	717
	19.2.2 Amphiphilic Cyclodextrins	721
19.3	Amphiphilic Cyclodextrin Nanoparticles	724
19.4	Polymeric Nanoparticles Incorporating Cyclodextrins	730
19.5	Cyclodextrin-Based Polymeric Nanomedicines	733
19.6	Polymer-Drug Conjugates.....	734

19.7	Supramolecular Hydrogels.....	736
19.8	Polymer-Modified Drug Carriers.....	737
19.9	Conclusion.....	739
	References.....	740
CHAPTER 20 Lipid nanoparticles: In vitro and in vivo approaches in drug delivery and targeting 749		
	<i>Yi Lu, Jianping Qi and Wei Wu</i>	
20.1	Introduction.....	750
20.2	Preparation of Lipid Nanoparticles.....	752
20.2.1	High-Pressure Homogenization.....	752
20.2.2	Microemulsion Technique.....	753
20.2.3	Solvent Emulsification Evaporation Technique.....	753
20.3	In Vitro Characterization.....	754
20.3.1	Routine Characterization of Lipid Nanoparticles.....	754
20.3.2	Polymorphism and Degree of Crystallinity.....	757
20.3.3	Assessment of Coexisting Structures.....	759
20.3.4	In Vitro Lipolysis.....	759
20.4	In Vivo Characterization.....	760
20.4.1	Pharmacokinetics and Bioavailability.....	760
20.4.2	Biodistribution.....	761
20.4.3	In Vivo Imaging.....	762
20.5	Models for Specific Administration Route.....	766
20.5.1	Evaluation of Lymphatic Absorption.....	766
20.5.2	Dermal Delivery.....	767
20.5.3	Pulmonary Delivery.....	769
20.6	Cellular Models.....	770
20.6.1	Endocytosis.....	770
20.6.2	Translocation.....	772
20.7	Conclusion.....	773
	Acknowledgments.....	774
	References.....	774
	Index.....	785

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Series Preface:

Pharmaceutical Nanotechnology

Due to its immense applicative potential, nanotechnology is considered the leading technology of the 21st century. The science and engineering of nanometer-sized materials is currently employed for the development of numerous scientific, industrial, ecological, and technological fields. Biology, medicine, chemistry, pharmacy, agriculture, food industry, and material science are the main fields which have benefited from the great technological progress developed in nanoscience.

In the pharmaceutical field, nanotechnology has revolutionized traditional drug-design concept and the art of drug delivery. The idea of a highly specific nanoscale drug for the targeted therapy of diseases is now considered a feasible treatment for severe health conditions.

Some scientists believe that the pharmaceutical domain has been reborn by the important contribution of nanotechnology. The field of pharmaceutical nanotechnology has the potential to offer innovative solutions for all diagnosis, therapy, and prophylaxis domains. Application of nanotechnology tools in pharmaceutical research and design is likely to result in moving the industry from a “blockbuster drug” model to “personalized medicine.” The current main focus of clinicians is to treat patients individually, not their general diagnosed diseases, which are usually difficult to diagnose or incorrectly diagnosed. There are compelling applications in the pharmaceutical industry where suitable nanotechnology tools can be successfully utilized. By designing and modifying drugs at nanoscale, pharmaceutical nanotechnology could be useful not only for the development of completely new therapeutic solutions, but also to add value to existing products. This possibility opens perspectives of success for pharmaceutical companies in existing markets, but also for new markets.

Scientists have manifested an impressive interest on the field of pharmaceutical nanotechnology research in recent years. However, we face today a true dilemma of data unavailability, due to the multitude of existing information which can be highly inaccurate and contradictory. This is because of the lack of an efficient model for sorting the plethora of nanotechnology tools and information that exists, and strategically correlate those with potential opportunities into different segments of pharmaceutical research and design.

This series is trying to cover the most relevant aspects regarding the great progress of nanotechnology in the pharmaceutical field and to highlight the currently emerging trend of pharmaceutical nanotechnology towards the personalized medicine concept.

The 10 volumes of this series are structured to wisely offer relevant information regarding basic concepts and also to reveal the newest approaches and perspectives in pharmaceutical nanotechnology.

Nanoscale Fabrication, Optimization, Scale-Up and Biological Aspects of Pharmaceutical Nanotechnology, introduces the readers into the amazing field of nanoscale design. Also, this volume facilitate understanding of the biological requirements of nanostructured pharmaceutical formulations for advanced drugs.

In *Design and Development of New Nanocarriers*, the most recent progress made on the field of nano-delivery is discussed. Modern nanostructured drug carriers employ innovative solutions for the detection and treatment of various diseases in a personalized and efficient manner.

Design of Nanostructures for Theranostics Applications, highlights the impressive impact of nanotechnology in the development of combined diagnosis and therapy concept: theranostics.

Design of Nanostructures for Versatile Therapeutic Applications, offers a dynamic solution for immune modulation, treatment of diseases by natural-based products and infection control, while employing nanostructured solutions to achieve top results.

Nanostructures for the Engineering of Cells, Tissues and Organs: From Design to Applications, is a highly investigated and debated field; tissue engineering, is dissected through this volume. Here is shown how nanotechnology has advanced research and applications in the manipulation and engineering of cells and tissues *in vitro*.

Organic Materials as Smart Nanocarriers for Drug Delivery, deals with the specific world of organic nanomaterials, revealing their wide applications, types, and advantages in drug delivery.

In the volume entitled: *Inorganic Frameworks as Smart Nanomedicines*, the main focus is to discuss the variety and properties of inorganic nanostructures for therapy and drug delivery in the context of improved personalized medicine.

Lipid Nanocarriers for Drug Targeting, deals with recently developed lipid nanostructures and the advances made in drug targeting.

Drug Targeting and Stimuli-Sensitive Drug Delivery Systems, dissects smart stimuli-responsive nanosystems employed to specifically detect various biochemical conditions and control the release of drugs.

Fullerens, Graphenes and Nanotubes: A Pharmaceutical Approach, reveals major findings made on widely applied drug-design nanosystems, namely fullerenes, graphenes and nanotubes. The impact of these nanostructures in pharmaceutical research is highlighted.

All 10 volumes are nicely illustrated and chapters are organized into a logical manner to be accessible to a wide audience. The series is a valuable resource of new and comprehensive scientific proof on the intriguing and emerging field of pharmaceutical nanotechnology, which could be of a great use for scientists, engineers, pharmaceutical representatives, clinicians, and any non-specialist interested user.

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Preface

Drug Targeting and Stimuli Sensitive Drug Delivery Systems

This book reviews the recent progress in the field of drug targeting and stimuli-sensitive drug delivery systems. The book covers innovative approaches related to stimuli-responsive nanomaterials with single or multiple responses to physiological or other external stimuli. Concepts, advances, and preclinical and clinical status of different drug delivery systems are presented.

The book, *Drug Targeting and Stimuli Sensitive Drug Delivery Systems*, contains 20 chapters, prepared by outstanding researchers from China, United States, UAE, Brazil, Serbia, Spain, Portugal, Argentina, India, and Turkey.

Chapter 1, Treatment strategies in cancer—from past to present, prepared by Hatice Yildizhan et al., gives an up-to-date overview about targeted therapies that act by affecting the pathways responsible in cell growth, division, and spread of cancer cells. Following scientific and technological developments, new applications have arisen, such as gene-therapy, stem cell therapy, immunotherapy, and nanotechnology-derived therapies. Combination of chemotherapeutic with nanoparticles is a novel approach that is being used in the treatment of cancer.

Chapter 2, Nanotechnology-based drug delivery systems: challenges and opportunities, by Deepti Sharma et al. undertakes a detailed investigation of various factors that influence nanoparticles-based drug delivery systems, such as operational efficiency, safety, scaling up, and cost aspects.

Chapter 3, Nanotechnology applications in drug controlled release, prepared by Analía Simonazzi et al. presents a variety of nanodrug delivery systems such as nanoemulsions, lipid or polymeric nanoparticles, and liposomes investigated as functional drug carriers for treating a wide range of therapies, most notably cardiovascular defects, autoimmune diseases, and cancer. Designed nano-sized devices or drug carriers provide various advantages for effective drug delivery.

Chapter 4, Target specific delivery: an insight, prepared by Prasoon Pandey and Neelam Balekar, presents novel approaches in drug delivery strategy that has a known target, which may be organs, tissue, cells, or structures inside of cells (i.e. gene).

Chapter 5, Stimuli-responsive nanosystems for drug-targeted delivery, prepared by Carla M. Lopes et al. gives an overview of the most significant physical and chemical stimuli-responsive nanosystems and elucidates their current and relevant applications in controlled and targeted drug delivery attending different routes of administration.

Chapter 6, Stimuli sensitive ocular drug delivery systems, by Francisco O. Espinar et al. focuses on some new representative areas of ophthalmic drug delivery research: use of pharmaceutical nanosystems, and especially smart polymers. Stimuli-sensitive hydrogel systems show a markedly reversible sol-gel phase transitions in response to physiological stimuli (temperature, pH and presence of ions in organism fluids, enzyme substrate) or other external (electric current, light).

This kind of materials can improve the drug bioavailability by increasing the residence time of the formulation in the eye surface.

Chapter 7, Stimuli-sensitive nanomaterials for antimicrobial drug delivery, prepared by Smritilekha Bera and Dhananjoy Mondal covers recent studies in the development of stimuli-sensitive nanomaterials for antimicrobial drug delivery in the presence of light, pH, magnet, enzymes, and other endogenous and exogenous agents, which can activate the nanostructure to release the drug molecules at the target site avoiding obstacles and cytotoxicity en route.

Chapter 8, Stimuli-responsive micelles: a nanoplatform for therapeutic and diagnostic applications, prepared by Hema A. Nair et al. presents a detailed review of the various classes of stimuli-responsive micelles, the strategies in their design and the current advancement with respect to their drug delivery and diagnostic applications.

Chapter 9, Design of targeting peptides for nanodrugs for treatment of infectious diseases and cancer, prepared by Sanja Glisic and Veljko Veljkovic is an overview a new concept of the long-range intermolecular interactions for recognition and targeting between drugs and therapeutic targets. This concept was used for the development of the bioinformatics platform for design of targeting peptides for delivering antimicrobial and anticancer nanodrugs to therapeutic targets.

Chapter 10, Targeting drugs to cell and organ using nanoparticles, prepared by Mayuri V. Gurav and Satish B. Bhise gives a general overview about nanoparticulate systems which have great biomedical and pharmacological potentials, being able to convert poorly soluble, poorly absorbed, and labile biologically active substances into promising deliverable drugs. The core of this system can enclose a variety of drugs, enzymes, genes and is characterized by a long circulation time due to the hydrophilic shell which prevents recognition by the reticular-endothelial system. To optimize this drug delivery system, greater understanding of the different mechanisms of biological interactions, and particle engineering, is still required.

Chapter 11, Extracellular vesicles as a recipe for design smart drug delivery systems for cancer therapy, prepared by Carmen V.F. Halder et al. focuses on the recent progress and challenges in the engineering of exosomes as drug delivery vehicles.

Chapter 12, Advancements in exogeneous techniques for stimuli-sensitive delivery systems, prepared by Hemant K.S. Yadav et al. discusses stimuli-responsive nanocarriers that control the bio-distribution and effect of drugs in response to specific stimuli, specifically magnetic field, ultrasound intensity, and electric pulses.

Chapter 13, Ligand-directed tumor targeting with hybrid viral phage nanoparticles, prepared by Bhavin Dalal et al. presents recent progress in the development of hybrid viral phage nanoparticles that represents a novel approach to overcome limitations and demonstrates great flexibility and has potential for a wide array of applications. This utility is achieved by the ability to modify the hybrid genome to target any receptor with delivery of any transgene.

Chapter 14, Delivering miRNA modulators for cancer treatment, prepared by Marilene Estanqueiro et al. aims to describe the role of miRNAs in cancer pathology and their response to therapy, then reports the potential therapeutic role of miRNA modulators in cancer treatment and finally explores the applicability of different nanocarriers in the delivery of these molecules.

Chapter 15, Oral controlled and sustained drug delivery systems: concepts, advances, preclinical, and clinical status, prepared by Gaganjot Kaur et al. presents recent progress on controlled and sustained release dosage forms as well as techniques and technology used in development of modified drug delivery systems. In addition, recently preclinically and clinically analyzed oral-modified drug delivery systems have also been summarized.

Chapter 16, Hydrogels: from simple networks to smart materials—advances and applications, prepared by Shweta Sharma et al. highlights the synthesis, properties, and classification of hydrogels on the basis of response and activities. A special attention has been given to the developing trend of hydrogels from a historical perspective, from simple networks to smart materials. Examples of injectable hydrogels for therapeutic agent delivery have also been detailed. The chapter specifically describes the progress that has been made in the field of hydrogels for biomedical applications in the past 50 years, starting from the pioneering work of Wichterle and Lim in the 1960s.

Chapter 17, Recent advances in understanding of blood–brain tumor barrier (BTB) permeability mechanisms that enable better detection and treatment of brain tumors, prepared by Divya Khaitan et al. reviews recent studies on biology of blood–brain tumor barrier permeability regulation in brain tumors. The contributors also discussed nanomedicines, multimeric molecular imaging strategies, including development of peripheral benzodiazepine receptors (PBR) ligand-PK11195 for better metastatic brain tumor detection. Such agents with fluorophores or therapeutics encapsulated in nanospheres are useful in detection of brain tumors, treatment, and monitoring.

Chapter 18, Polymeric nanocarriers for site-specific gene therapy, prepared by Geeta Arya et al., explored the strategies and advances made towards the development of polymeric nanocarriers in order to achieve site-specific gene delivery.

Chapter 19, Cyclodextrin-based polymeric nanosystems, prepared by Nazlı Erdoğan et al. gives an up-to-date overview about nanosystems based on natural and amphiphilic cyclodextrins. In addition, recent studies on cyclodextrin-based nano-sized drug delivery systems were summarized.

Chapter 20, Lipid nanoparticles: in vitro and in vivo approaches in drug delivery and targeting, prepared by Yi Lu et al. gives a brief introduction to the principle preparation techniques of lipid nanoparticles, while focusing on the in vitro and in vivo characterization methods. Furthermore, evaluation models for specific administration routes as well as cellular models are included.

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Treatment strategies in cancer from past to present

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CHAPTER OUTLINE

1.1 The Importance of Diagnosis in Cancer Therapy	1
1.2 Traditional, Well Proven Therapies	3
1.2.1 Surgery.....	3
1.2.2 Radiotherapy.....	6
1.2.3 Hormone Therapy	8
1.2.4 Chemotherapy	8
1.3 Targeted Therapies and Immunotherapy	17
1.3.1 Why Immunotherapy Is the Magician in Cancer Therapy?	18
1.3.2 Well Known Immunotherapeutic Agents and Their Advantages/ Disadvantages	19
1.4 Smart Role of the Nanotechnology in Cancer Treatment	27
1.4.1 Brief List of Studies for Usage of Nanotechnology in Cancer Treatment ..	28
1.5 Conclusion	30
References	31
Further Reading	37

1.1 THE IMPORTANCE OF DIAGNOSIS IN CANCER THERAPY

Cancer is known as a kind of genetic disease and can be heritable or directly caused by severe alterations in genomic DNA. Past decades' research shows us that cancer cannot be generalized for all patients, since there are single nucleotide polymorphism (SNP) factors and heterogenic natures of the tumor. At this point, recent research focused on the idiopathic therapies with the help of high-throughput technologies. This includes sequencing technologies (genomic, transcriptomic, proteomics and metabolomics) and bioinformatic applications. High-throughput applications have vital importance for diagnosis, assessing tumor heterogeneity, classifying molecular signatures of the patient, and therefore has

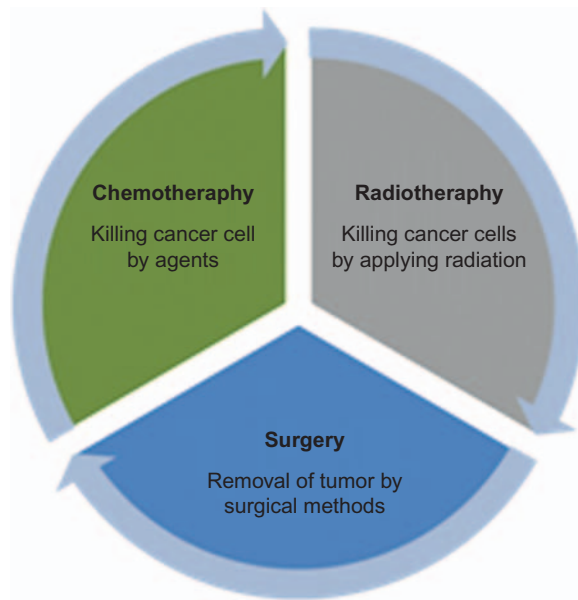
additional vital importance for the therapy determination and efficacy. Diagnosis is also part of treatment but this chapter would not contain detailed diagnosis strategies.

Even if it is not a good idea to categorize cancer in one way, there are several traditional ways for the classification of cancer. In this section, we cover how physicians decide to use which drugs for the purpose of patient treatment. But to emphasize it can be used again, physicians generally do not assign cancer as one disease; they must consider that each patient has his/her specialized disease. But with the traditional knowledge of cancer, it is identified in several subgroups.

By based on all this knowledge, the physician considers whether the patient has cancerous tissue or not. The next question is how it can be treated in these conditions with the safest drugs. Treatment period is also of vital importance; the patients progress must be followed by clinicians to get a route for future treatment strategies can also be used. At the first stage of treatment, the tumor is categorized as follows:

- cancerous tissue is indolent (or not),
- tumor is aggressive (or not), it has potential to metastasize (or not),
- identification of tumor grade.

The therapy must be planned by considering different factors, such as prime location of tumor, metastatic region, patient properties (age, gender, health status, etc.), stage of tumor or the cell type in the heterogenous tumor tissue. In addition to that, the therapy must be organized by a clinician and a pathologist, radiation oncologist, radiologist and nuclear medicine specialists, all must participate in the therapy of the patient. The success of the treatment must aim for local-regional control of the disease, prevention of future repetition of metastasizes, improving the life quality of the patient, and to improve long-term survival chances for patient. Possible cancer treatments can be summarized as: surgery, chemotherapy, radiation therapy, immunotherapy, and palliative care. These therapies can be applied alone or in combination, depending on the status of the patient. As expected, to decide which therapy would be applied has vital importance, especially for the patient's life. During the treatment, numerous side effects can be observed. The most threatening side effect is to increase the incidence of secondary cancer formation. There can be other side effects, which will be explained further in this chapter. Gene expression arrays which are specific expression for a specific biological phenomena, often referred to as the key analytical tools to identify the biomarkers. In addition to that, bioinformatic applications are also very important applications to identify genes, whose expression correlates with a specific biological phenotype, drug responsiveness, or prognosis. After deciding the advantage/disadvantage chart of the therapy, the treatment can then be started by the clinicians.

**FIGURE 1.1**

Traditional well known therapies widely used in cancer therapy. These therapies can be applied as collection or alone, depending on the cancer type and other factors.

1.2 TRADITIONAL, WELL PROVEN THERAPIES

This chapter mainly focuses on ongoing fastidiously selected studies about cancer therapy. The main outline of this chapter is the timeline, from traditional applications to up-to-date studies. Traditional well proven therapeutic applications are not point out in detail. These studies are explained in short and studies are summarized in [Fig. 1.1](#).

1.2.1 SURGERY

1.2.1.1 *Surgery to treat cancer*

1.2.1.1.1 Brief outline of the surgical application in cancer therapy

Surgery has been used for many years as the main treatment to remove cancer tissue away from the body. The earlier a lesion is found, the easier it is to remove it. It also plays an important role for diagnosis and staging.

If cancer has spread from the primary site to other tissues of the body, surgery cannot usually cure it. With some types of cancer, surgery can help people to live for a long time and may sometimes lead to a cure.

1.2.1.2 Methods used in conventional surgery

1.2.1.2.1 Diagnostic

The surgeon removes the tumor and some normal tissue close to the lesion to have a clear margin. This procedure is called biopsy.

There are generally two main types of surgical biopsies.

1. Incisional biopsies: removal of a suspicious area for examination;
2. Excisional biopsies: removal of total lesion.

In some instances, like malignant melanoma, a wide margin must be excised with the primary lesion in order to decrease the risk of local recurrence. Until relatively recently, this involved removal of 5 cm of normal-appearing skin around the lesion, as well as the soft tissue down to, and including, muscle fascia (Hussussian, 2010).

At the head and neck area, in addition to standard incisional and excisional biopsies, fine needle aspiration cytology has become established as a minimally invasive technique with high sensitivity and specificity in a variety of lesions (Larson et al., 1984).

After a biopsy, the tissue is sent to pathology laboratory to examine. This gives more information about the cancer. The results helps the doctor plan further treatments, such as chemotherapy or radiotherapy.

1.2.1.2.2 Staging

Staging surgery is performed to find out the tumor size and where the tumor has spread. In the course of this surgery, the surgeon often removes some lymph nodes close to the cancer to find out if it has spread. Treatment is changed by the stage.

1.2.1.2.3 Curative or primary surgery

Curative (primary) surgery is usually preferred if the cancer is located in only one body part. It is quite possible that all of the cancerous tissues can be removed in these operations, so surgery is the main treatment strategy in these cases (Chang et al., 2007; Silberman and Silberman, 2009). It may sometimes be combined with other procedures such as chemotherapy or radiotherapy (Kim et al., 2016).

1.2.1.2.4 Surgery to debulk cancer

Debulking surgery is applied to remove some of the cancer tissue. If taking out the entire tumor is not available without damaging the adjacent tissues or organs, a debulking procedure is performed (Piura and Glezerman, 1989).

1.2.1.2.5 Palliative surgery

If there are problems such as disability, pain, or discomfort caused by advanced cancer, palliative surgery can be performed. This surgery is not applied as a cancer treatment, but only used to reduce the cancer-related disorders and improve

the cancer patients' welfare. Sometimes certain cancers are more likely to cause bleeding; in this circumstances palliative surgery is an option to stop bleeding (Zhou et al., 2016).

1.2.1.2.6 Preventive (prophylactic) surgery

Preventive (prophylactic) surgery is performed to remove a suspicious tissue or a lesion that is likely to evolve to become a cancer. This surgery can be applied without any sign of cancer. An organ or a tissue can be removed from the person who has a genetical predisposition and involve in a high risk group for a specific type of cancer. This type of surgery is applied as a precaution to reduce cancer risk.

1.2.1.3 Reconstruction

If you have part of your body removed due to malignant tumor, it might be possible to have reconstructive surgery. The remaining part is recreated by using other body tissues with flap and graft operations or with prosthesis. In the past decade alone, there have been significant improvements in the quality of life of these patients in consequence of the improvements in one-stage reconstruction with musculocutaneous flaps and microvascular free-tissue transfers (Demiralp et al., 2014).

Examples of reconstructive surgery include breast reconstruction after a mastectomy and surgery to restore a person's appearance and function after surgery to the head and neck area.

1.2.1.4 Types of Surgery

Conventional surgery requires large incisions through the skin, muscle, and other available body layers.

Newer surgical techniques are less invasive than conventional surgery. By using different types of surgical instruments and technology, the processes may cause less pain and reduce the amount of recovery time:

- Laparoscopic surgery: A laparoscope is a flexible, slender tube, which is inserted through an incision to view the body inside. This surgical procedure can be used for abdominal and gynecologic cancers.
- Cryosurgery: Cryosurgery uses liquid nitrogen, CO₂, or argon spray to freeze and destroy atypical cells. This technique can be preferred in the treatment of precancerous states, in skin, cervix, and genital areas (McCarty and Kuhn, 1998).
- Laser surgery: This is a type of treatment in which powerful beams of light are used to cut through tissue. This technique can be used to destroy or shrink tumors like basal cell carcinoma, cervical, vaginal, esophageal, and nonsmall cell lung cancer.
- Mohs micrographic surgery: It is a surgical technique for the removal of certain skin cancers by taking special horizontal sections of skin tissue and

examining them under a microscope. Mohs micrographic surgery (MMS) is a therapeutic option for the treatment of malignant cutaneous neoplasia. The main indications for micrographic surgery are relapsed, aggressive tumors in anatomic areas with high rates of recurrence or in areas where it is necessary to spare healthy tissue ([Chagas and Santana Silva, 2012](#)).

1.2.2 RADIOTHERAPY

Traditional treatment contain three steps: chemotherapy, radiation, and surgery. They can be applied alone or in combination to get better results for the patient health. The use of radiation such as high-energy beams, X-rays, gamma rays, electron beams, or protons, to cure diseases is called radiotherapy. The discovery of X-rays in 1895 led to the usage of radiation in medical applications, such as investigation and diagnosis (X-rays) and treatment (radiotherapy). This therapy is chosen when the location of cancer cells is only in one part of the body, which is also means this therapy can be applied to patients who have nonmetastatic cancer. However, this does not mean radiation cannot be applied to the type of cancer which is spread through the whole body. In these situations, it can be applied to kill cancer cells just in one part of the whole.

Radiotherapy destroys unwanted cancer cells in the treatment site by damaging DNA. The goal of this treatment is to apply the highest doses to cancer cells, while administering the lowest doses to the adjacent healthy cells. Radiation can be given alone or combine with other treatments, such as surgery or chemotherapy. This combined procedure may help primary treatments be more effective.

Radiation applications can be used for cancer treatment, reducing the size of the tumor, controlling the tumor growth, and also for decreasing the pain. Radiotherapy is used before (neoadjuvant) or after (adjuvant) other treatments. The aim is to make the main treatment more effective.

1.2.2.1 Types of Radiotherapy

There are several different types of radiotherapy and ways to give it, which can be used to treat cancer; the common types are three-dimensional (3D) conformal radiotherapy and intensity modulated radiotherapy (IMRT).

- Image-guided radiotherapy (IGRT)
- 3D conformal radiotherapy
- Intensity-modulated radiotherapy
- volumetric-modulated arc radiotherapy (VMAT)
- stereotactic ablative radiotherapy (SABR)

Three-dimensional conformal radiotherapy the radiation beam to the cancer cells. The main advantage for 3D conformal radiotherapy treatment is to give a high dose of radiotherapy to the cancer cells and reduce the radiotherapy dose for normal healthy cells ([Rahman et al., 2016](#)).

IMRT shapes the radiation rays very close to the cancer tissue. It can also vary the strength of the radio spectra so that different areas of the body get different doses of radiation. This may help reduce the amount of radiation given to normal cells around the cancer tissue. VMAT is a type of IMRT. It focuses the radiation on the cancer cells and the main advantages of this radiotherapy treatment is the short treatment time period. It may also help to reduce the risk for surrounding organs, reducing side effects by using low radiation (Halperin et al., 2013).

SABR is a new type of radiotherapy. It is also known as stereotactic body radiotherapy. The main one is Cyberknife, which is a type of SABR. SABR delivers a higher dose of radiotherapy; the applied dose is higher, usually three to five treatments over 1–2 weeks is adequate for different kinds of cancer. It is important that this treatment is carried out very accurately to reduce the risk of damage to healthy tissues from the high dose of radiation. There are also some risks for some of the cancer types, such as in pancreatic cancer treatments and a risk of damaging the stomach or bowel from high doses of radiation, which could cause side effects (Jain et al., 2013).

Another application of the SABR is fitted onto the patient's head. This system is called Gamma Knife (Cheah et al., 2016). The limitation of the Gamma Knife is mainly in its usability outside the head, although the new generation of Gamma Knife can treat tumors up to C2 vertebra level.

All the systems are used in conjunction with a stereotactic frame fixed onto the skull of the patient. Firstly, 3D coordinates of the tumor should be determined by the stereotactic frame, and the radiation delivered accurately to the target tissue. Small circular collimators fitted at the end of the treatment head as tertiary collimators were used for narrowing the beam of X-rays from a conventional linear accelerator. This system is also requires a stereotactic frame to be fixed onto the patient's head and used invasive or minimally invasive procedures. These methods of conformal dose delivery to the target tissue reduce radiation dose to the surrounding normal tissues (Sawarkar et al., 2015).

1.2.2.2 Main objective of radiotherapy

Radiation therapy is one of the most common treatments for cancer and mainly aims to damage DNA of the cell by applying energy, photons, or charged particles.

Radiotherapy destroys unwanted cancer cells in the treatment site by damaging the DNA. Radiation can be given alone or combine with other treatments, such as surgery or chemotherapy. This combined procedure may help your primary treatment more effective.

Radiation oncologists may apply radiation to cure or reduce the tumor size, to control the tumor growth or to relieve pain. Radiotherapy is used before (neoadjuvant) or after (adjuvant) other treatments. The aim is to make the main treatment more effective.

Table 1.1 Commonly Used Drugs Which Are Used in “Hormone Therapy” Main Formulations and Their Functions (DeVita et al., 2008)

Formulations in Hormone Therapy	Mode of Action
Tamoxifen	Tamoxifen blocks estrogen and cell surface receptor interactions
Aromatase inhibitors	Prevents the reaction which is resulted in changes from androgen to estrogen hormone
Luteinizing hormone blockers	Drugs that stop the ovaries making estrogen or progesterone
Anti androgens	Stop testosterone and prostate cancer cell surface receptors
Gonadotrophin releasing hormone blocker	Interfere with testosterone production from testicles

1.2.3 HORMONE THERAPY

Hormones are natural components of all living organisms. In humans, they are synthesized by glands and these natural products are also used in medical treatment for different purposes. As discussed in this book, the main aim of cancer treatment is to get rid of malignant cells from the body. In addition to surgery, radiotherapy, and chemotherapy, hormone therapy is one of the contrary methods of therapy. Like other kinds of treatment, choice of treatment process with hormones or hormone antagonist depends on type, location, and stage of the cancer. Generally used hormone-based drug are listed in Table 1.1. Naturally, hormones are carried through body via the blood stream and they take important roles in controlling the differentiation and growing of cells and organs. Because of their effect on gene expression of the cells, hormone-targeted therapy is widely used in cancer treatment (Sweeney et al., 2015). Corticosteroids and glucocorticoids play an important role in the secondary management of cancer. Hormone therapy is mainly used in the treatment of hormone-source tissue cancers, such as breast, ovarian, prostate, endometrial, and kidney cancer (DeVita et al., 2008). In addition to these cancer types, hormone treatment can also have adverse effects on some other kind of cancer, such as B-cell non-Hodgkin lymphomas (Kuo et al., 2016). Hormone therapy can only be used in hormone sensitive type of cancers and generally aims to stop hormone synthesis and prevention of hormone-positive effect on cancer cells by interfering with their interactions (Brunton et al., 2005).

1.2.4 CHEMOTHERAPY

Chemotherapy can be identified as the use of chemically formulated drugs for the treatment of cancer and other diseases. Separate from surgery and radiation,

chemotherapy is used to treat cancer through the whole body, which makes it a valuable tool for treatment of metastatic cancer. In the last decade of the 20th century, researchers discovered how cancer is differentiated from normal cells. After that, cancer treatment verged to the direction of preventing cancerous pathways.

In this part, simple stages which can be used as one-size-fits-all approaches are summarized. The critical stages for drug discovery can be ordered as: characterization of target cancer cells by using high throughput approaches (genomics, proteomics, etc.); getting leading profiles of these cells, which can also be called biomarkers of cancerous process; create candidate chemical formulas; then make different cancer-specific assays (cell proliferation assay, mobility assays, or others that are specific for each cancer type) for each chemical. After making the selection of candidate drugs, apply in vivo assays for drugs and as the last few number of drugs can pass phase studies. As the last of these stages there would be just one chemical which can be tested by the FDA team. [Fig. 1.1](#) summarizes these challenging steps. These steps take nearly 20 years just for one chemotherapy drug.

At the chemotherapy drug discovery stage, computers are one of the valuable tools for researchers to minimize the biological application steps. The use of gene expression arrays and bioinformatics makes it possible to discover information about candidates genes.

1.2.4.1 Main logic of chemotherapeutic application

For both earlier discovered drugs and ongoing studies, one of the most important things in drug studies is to identify the effective target. The selection of a new target for cancer drug discovery is increasingly based on the strength of the evidence that it represents a dependence or vulnerability for a given stratified set of cancer patients, commonly defined by their molecular genetic status. The target can be classified as different subgroups. In this chapter, we classify them based on their role in the cancer process. The drug can aim to activate/deactivate the target and to disturb cancer cell proliferation or metabolic activity of the cell, such as protein synthesis, mobility of the cell; and act directly on DNA to achieve double-strand DNA breaks. These main steps in cancer chemotherapy will be explained in detail through this chapter.

1.2.4.2 Cell cycle inhibitor drugs

Cancer cell pathology is caused by their uncontrolled cell proliferation. Therefore, the first selected drug for treatment is to target cell cycle control. Uncontrolled cell proliferation is one of the hallmark of the cancer and that is why the cell cycle is still one of the most popular topics in cancer research, even if there is a special subgroup, named mitotomics. Since they are not cancer-cell specific agents, cell cycle studies are not come up but they have great potential to get approved from FDA, by usage in cancer patient treatments. To be able to control the cell cycle is double-sharped edge treatment way for now. However, there

are also some exceptions. As in the case of some cancer cells lack of double strand breaks checks which is normally functional in the normal cell. In normal cells, the system has been evolved in the direction of destroying the genomic instability, but in cancer cells this is not the case. This control mechanism is known as mitotic catastrophe and it is a kind of antiproliferative mechanism, such as apoptosis, senescence, and necrosis (Galluzzi et al., 2012; Vitale et al., 2011). This term was identified in 2012 and in recent research it is widely used, since destroyed mitotic catastrophe is represents a break point for cancer cell. Therefore, fixing these mechanisms can be a good option for the treatment of cancer. Even if this mechanism has not been fully understood, better understanding of the paths can create new opportunities to overcome the development of tumor tissue. The action mode of these drugs can be summarized as microtubule targeting agents, polymerizes/depolymerizers, mitotic spindle targets, mitotic checkpoint targets, mitotic exit inhibition mechanisms, centrosome disruption, and G2 checkpoint abrogation (Gee, 2015). Mitotic catastrophe is characterized by different studies, but the main idea is based on disappearing of the multinucleated cell form. In this chapter, docetaxel can be given as an example of a mitotic catastrophe action chemotherapeutic agent. Docetaxel has been used in the therapy of human breast cancer cell and small lung cancer cell; it is known as a microtubule stabilizing taxane agent (Crown and O’Leary, 2000). As a general rule, apoptosis has been known as the main mechanism for cancer treatment, but recent results show that we need more complex, multi-factorial solutions. In 2005, Morse and colleagues showed the docetaxel action mode by assessing the viability of cells after treatment, cell cycle check point, and micronucleated cell scoring. The results of this study show that this widely used chemotherapeutic agent action is based on fixing mitotic catastrophe (Morse et al., 2005).

As seen until this point, traditional chemotherapy drugs mainly focused on the genomic changes and checkpoints of the cancer cells to destroy tumor tissue. Yet it is not the best point since genomic change cannot be performed and is restricted, the main focus for chemotherapeutic drugs is focused on cancer cell proteins. For this purpose, researchers make detailed studies to acquire information about the cancer cell proteome, such as The Human Proteome Atlas project (“Cancer atlas—The Human Protein Atlas,” n.d.). Since there is much more knowledge about proteome than the genome, recent research targets cancer-specific critical proteins, rather than the genome.

1.2.4.3 Antimetabolitic agents

As discussed in the previous section, inhibition of cell proliferation is one of the most focused types of cancer therapy. During replication, cells must make double all components, such as DNA, RNA and other macromolecules. Therefore, a group of cancer drugs aims to interfere cell proliferation by acting directly on DNA replication. These drugs are called antimetabolites and can be explained as a group of chemical molecules that are able to block DNA or RNA synthesis

Table 1.2 Antimetabolites Can be Explained So; a Group of Chemical Molecules That Are Able to Block DNA or RNA Synthesis and the Below Table Summarize the FDA Approved a Subgroup of Antimetabolic Drugs (Parker, 2009)

Name of the Drug	Approval Year
5-aza-2'-deoxycytidine (decitabine)	2006
O ⁶ -Methylarabinofuranosyl guanine (nelarabine)	2005
2'-Fluoro-2'-deoxyarabineofuranosyl-2-chloroadenine (clofarabine)	2004
5-aza-cytidine (vidaza)	2004
N ⁴ -Pentyloxy carbonyl-5'-deoxy-5-fluorocytidine (capecitabine)	1998
2,2-Difluoro-2'-deoxycytidine (gemcitabine)	1996
2-Chloro-2'-deoxyadenosine (cladribine)	1992
Anabinofuranosyl-2-fluoroadenine (fludarabine)	1991
2'-Deoxycoformycin (pentostatin)	1991
5-Fluoro-2'-deoxyuridine (floxuridine)	1970
Arabinofuranosylcytosine (cytarabine)	1969
6-Thioguanine	1966
5-Fluorouracil	1962

(Henderson and Mandel, 1963). These drugs can act selectively, which means they can kill cancer cells selectively (but not totally), since the normal adult cells are mainly found in quiescent state, which means they are not replicating continuously (Parker, 2009). At this point antimetabolic agents (which are summarized in Table 1.2) can be called minimal toxic agents for the cancer suffer patient and they are part of an effective treatment. Many of the antimetabolic drugs act via the same paths and they are mainly used to treat leukemias, breast cancer, ovarian cancer, and where possible, other types of cancers.

As discussed previously, antimetabolites are a kind of cytotoxic agent and they are structurally similar to purines, pyrimidines or nucleosides, but are chemically different to their analog. The main logic behind these drugs is based on two ideas. The first is the inhibition of the key enzymes that are essential for DNA synthesis, therefore they inhibit replication of DNA and so impair cell proliferation. The other mode of action of these drugs is the incorporation of them instead of the nucleotide, causing double strand breaks or immature replication termination (Lieken et al., 2009; Vande Voorde et al., 2012). These drugs generally take a role in the S phase of cell proliferation. They can be assessed by the short half-time of the chemical therefore, their activity is dose- and delivery time dependent (Lind, 2011).

Antimetabolites are divided into different subgroups: antifolates (whose action mechanism is summarized in Fig. 1.2), antiprimidines, antipurines, thymidylate synthase inhibitors, and arabinosides.

1.2.4.4 Antibiotics like antitumor drugs

There are also other kinds of drugs which are sourced by microorganisms such as *Streptomyces* spp. These drugs are also known as anthracycline-based drugs. Even if they are isolated microorganisms, their mode of action are dissimilar to antibiotics. Indeed, there is no exact explanation how these drugs impede cancer cells, but they are widely used, especially in the treatment of ovarian and breast cancer, and Kaposi's sarcoma. The short list of these drugs are summarized in Table 1.3. Mode action of the antitumor drugs can be summarized as: through the intercalation of DNA, they can cause double or single strand DNA breaks, which

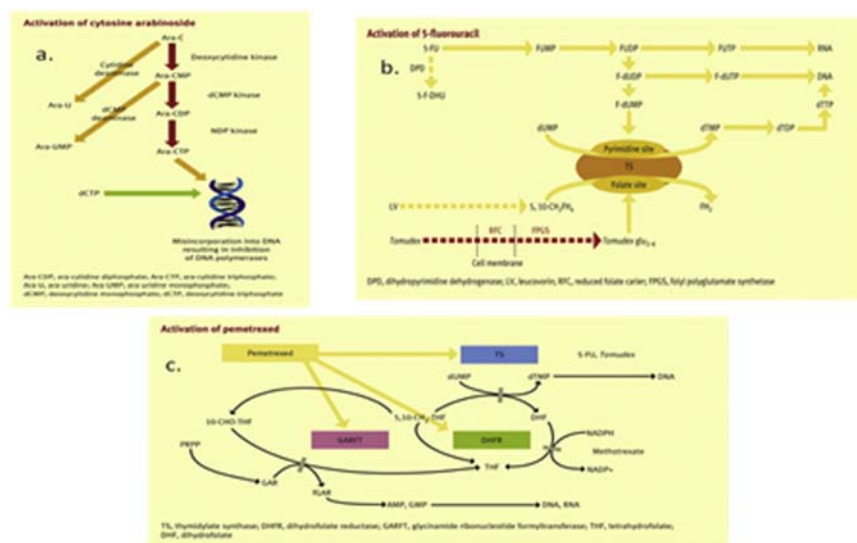


FIGURE 1.2

Antifolates (A), thymidylate synthase inhibitors (B), and arabinosides (C) samples and mechanisms.

Source: Reprinted with permission from Elsevier (Lind, M.J., 2011. *Principles of cytotoxic chemotherapy. Medicine (Baltimore), Oncology: Part 1 of 2, 39, 711–716*).

Table 1.3 Short List of FDA Approved Anthracycline Based Drugs

FDA-Approved Anthracyclines Antitumor Based Drugs	FDA-Approved Nonanthracyclines Based Antitumor Drugs
Daunorubicin	Actinomycin-D
Doxorubicin (Adriamycin)	Bleomycin
Epirubicin	Mitomycin-C
Idarubicin	Mitoxantrone

can start a reaction that can cause oxidative stress in the cancer cell. Doxorubicin-based formula drugs are used by clinician, such as Myocet and Doxil. Doxorubicin, which is the first encapsulated drug categorized as antitumor drug, is used in the treatment of solid tumors, transplantable leukemias, and lymphoma (Slingerland et al., 2012). Since its capsulation formula, this drug is thought to be a selective drug. In addition to its FDA-approved formula, doxorubicin-based drugs are searched for different delivery formulas to improve its selective-binding capacity. For example, antibody embedded liposomes (immunoliposomes) are one of the key topics for cancer treatment. The antibody-coated studies are valuable since, through identification of cancer cell-specific antibodies, these conjugated drugs can selectively bind cancer cells. This conjugation is generally observed at the end site of a PEG polymer (Schnyder and Huwyler, 2005). There is also other research focused on triggered release of drugs, studied to be able to get selective drugs for cancer treatment. The main basis of triggered released mechanisms is that, when the capsules encounter cancer cells (which has specific cancer cell biomarkers), it will open and release drugs (Coyne et al., n.d.). Otherwise, it will not open and the cytotoxic drug cannot getting by the normal cell. The trigger-starting tool can be thermo-sensitive, enzyme-sensitive or acid-triggered (Rivankar, 2014).

This kind of chemotherapeutic drugs are also tried to deliver in biological particles are also one of the most promising way for the local chemotherapeutic drug treatment. Doxorubicin is one of the drugs which has potential in this kind of therapy, because of its physicochemical parameters (Tonetti et al., 1991).

1.2.4.5 Different types of drug delivery

As discussed earlier, drug delivery has vital importance and it is one of the key topics. If these studies are successful, they will offer lots of advantages, in comparison to well-known dosage form methods. The main advantages of these promising approaches can be summarized as follows:

- better understanding and controlling of drug dosage (Benita and Donbrow, 1982),
- since encapsulation is prepared by enteric-coated dosage, they can be selectively absorbed by the intestine (Felt et al., 1998),
- Beside the mechanic difficulties, it would be easier and less complex to prepare the drug, with respect to compression into tablets or solution (Hombreiro Pérez et al., 2000; Passerini and Craig, 2002),
- even if encapsulation is not enough to protect drugs from the environmental conditions, encapsulation is better than tablet form of drugs. This technique can be supply drugs better protection from unbeneficial conditions such as humidity, oxygen, heat (Carrasquillo et al., 2001).

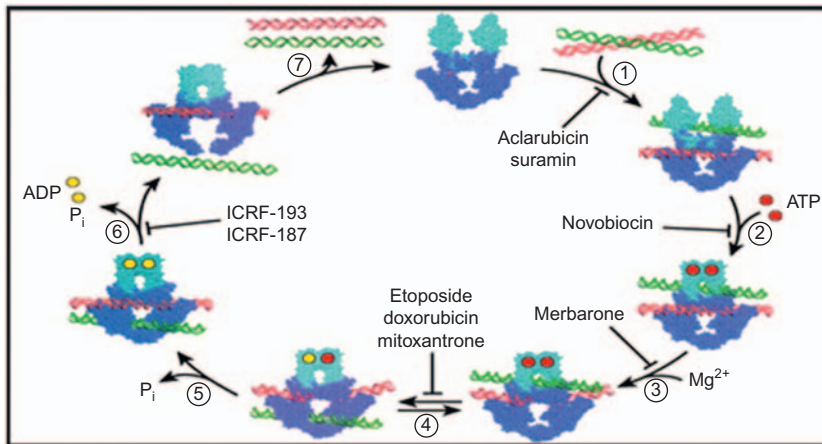
To summarize the advantages of the capsulated drugs; highly protected barrier for drug against the environmental conditions and the biological process which can lead the degradation of the drug. Since protection from the degradation and

triggered opening of the capsule, it is possible to control the time of release and keep the drug in the body for a long time, over months. This technique also can create an easier administration of the drug. Time control can also be performed by encapsulation technology, which will be vitally adverse for some kinds of disease treatment. Microparticulate drug delivery systems are an interesting and promising option when developing an oral controlled release system. As discussed, the biggest disadvantage of the chemical-based treatments is being unable to target the specific tumor cell. These drugs also affect normal cells and the results sometimes can be worse for patient. Hopefully, because of encapsulation technology, it would be possible to treat only unhealthy cell without any loss of the healthy cells (“Bioencapsulation_Innovations_2015_01.pdf,” n.d.; Carrasquillo et al., 2001; Lind, 2011; Wang and Hu, 2012).

1.2.4.6 Topoisomerase inhibitors

Other widely used drug groups contain topoisomerase inhibitors. Topoisomerase is a kind of enzyme that takes a role in the 3D structure of DNA; they catalyze the breaking and handling of the phosphodiester backbone of the DNA. They are important factors for DNA replication and transcription, since they supply DNA transition by cleavage or re-ligation reactions (Champoux, 2001). In cancer research, they are popular reagents, since it is thought that by blocking of the topoisomerase enzymes, it would be possible to block the ligation step of DNA during replication or transcription steps, would result in single- or double-strand DNA breaks. These breaks would disturb the integrity of genomic structure of cells and therefore apoptosis or necrosis mechanism would be activated. Fig. 1.3 illustrates topoisomerase inhibitor drugs active at different stages of the cell cycle.

Mammalian cells normally encode seven different topoisomerase enzymes. Four of the topoisomerase encoding genes encode type I enzymes while the other three parts encode type II topoisomerase enzyme. As indicated, topoisomerase enzymes are generally divided into two subgroups, according to which enzyme they act on. Type I topoisomerase is monomeric structured enzyme found in mammalian cells. This enzyme binds double-strand DNA and prevents torsional stress during replication or transcription cleavage of single strand DNA. Topoisomerase I inhibitors are mainly used in the treatment of colorectal and ovarian cancers. Topoisomerase I inhibitors include lamellarin D, irinotecan (CPT-11), topotecan, and camptothecin chemical drugs. The topoisomerase I inhibitor agent camptothecin is known as the most effective anticancer agent around the world (with respect to agents which are used in clinical side). There are different forms of camptothecins: topotecan and irinotecan, and they are approved by FDA in the treatment of ovarian and lung cancer and colon cancer. There are, however, several limitations of these drugs. These limitations include spontaneous inactivation in the blood; because of rapid reversal of the drug, they need much more time for infusions; and because some types of cancer cells which express more

**FIGURE 1.3**

The DNA topoisomerase II are one of the major target for cancer treatments. The chemical molecules take role at least one step of the topoisomerase activity steps and they are called topoisomerase inhibitor or topoisomerase poisons. The agents are able to stabilize DNA + isomerase enzyme complex are called topoisomerase poisons. But the agents that take role at different stage of the topoisomerase activity are called catalytic inhibitors. In the figure; the ATPase domains of topoisomerase II are shown in light blue, the core domain in dark blue, and the active site which is tyrosine residue are shown in red color. Aclarubicin and suramin drugs act as inhibitors and they impede the binding between DNA and enzyme complex. Merbarone, ICR-187 act as poisons and they stabilize the bound between DNA and topoisomerase. Novobiocin is a kind of drug whose mode of action is based on inhibition of ATP binding.

Source: Reprinted with permission from Elsevier.

membrane transports respect to other cancer cells can show resistance to these derivatives drugs (Pommier, 2006).

Unlike the type I form, topoisomerase II enzymes are found in multimeric form which are called a and b domains. These enzymes bind to the double strand of the DNA, forming double strand breaks in the DNA to pass through between two strands during replication steps. When the topoisomerase II inhibitors take a role in the cell, they form double strand breaks and cells verged themselves into apoptosis mechanism. The epipodophyllotoxins, which contain etoposide, and teniposide are the most well-known topoisomerase II inhibitors (Lind, 2011).

1.2.4.7 The unexpectedly good results from the proteasome inhibitor drugs in cancer treatment

Through the discovery of the first proteasome inhibitor, bortezomib (velcade, PS-341), proteasome inhibitors have taken a leading role in the treatment of cancer, especially in the treatment of multiple myeloma. Proteasome inhibitors have been

investigated nearly for 20 years alongside cancer therapy. As mentioned, bortezomib is known as the first proteasome inhibitor drug, which has a history since 1994 from lab-bench to 2003, and its approval by FDA (Teicher and Tomaszewski, 2015). Now, second and third generations drugs take place in the drug markets. Before detailing information about these drugs and their drug studies, it would be good idea to give summary information about proteasome.

What is the proteasome? And why it is so important in MM treatment?

Proteasome, is a multimeric protease complex and takes a vulnerable role in cellular protein regulation; this regulation has vital importance in the activation/deactivation of important paths (Palombella et al., 1994). Well controlled protein recycling is very important for cell viability and the pKa value of protein must stay in a neutral position. As indicated many times in this chapter, the concentration of proteins must be under strict continuous control, since they are the main players of genomic expression and are therefore lead the work of the cell. Their concentration is taken under control by degradation mechanisms and this degradation process is performed by the ubiquitin-proteasome system (“Garland Science—Book: The Biology of Cancer + 2,” n.d.). Those proteins which have multiple domains are degraded by proteasomes (Varga et al., 2014). The balanced function of the proteasome is important for cell viability since it takes a role in cell daily life. Due to the half time and evolution rules of the cell, proteins have a half-life and regarding to this system proteins completed their life and function; they are polyubiquitylated. Through the complexity of the ubiquitylation, ubiquitylated proteins are transported to proteasomes and their degradation is performed. As is known, malignant cell activity is much higher than normal cells and they can increase their activity by higher expression of required proteins. A higher number of protein means higher proteasome activity. After realizing the vital importance of the proteasome for malignant cells due to their genetic instability and rapid proliferation, researchers focused on inhibition of proteasome activity and the explained or suspected action mode of proteasome inhibitors are summarized in Fig. 1.4.

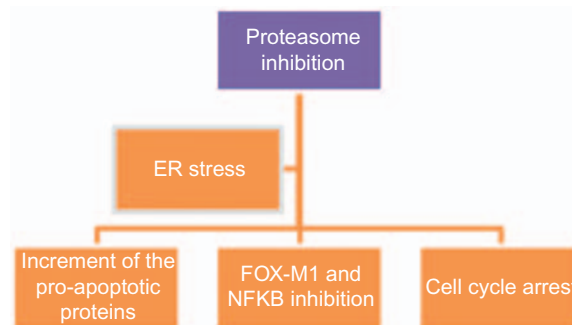


FIGURE 1.4

Mode of action of proteasome inhibitor drugs. The known actions are: increment of pro-apoptotic proteins, downregulation of oncogenic pathways, and cell cycle arrest.

Even in its 20-year short history, proteasome inhibitor drugs yield unexpectedly good results in cancer treatment, therefore they have a premium role in the drug markets and studies are still going on ([Crawford et al., 2011](#)).

1.3 TARGETED THERAPIES AND IMMUNOTHERAPY

One of the most promising treatment strategy in cancer treatment. Targeted therapies can also be called a subgroup of immunotherapy and can be defined as the type of therapies that directly and solely affect cancer cells. Unlike traditional therapies, targeted therapies are expected to not to have harmful effects on normal body cells. As discussed earlier, for example, in chemotherapy, drugs block all of the proliferating cells indiscriminately, whether they are malignant or not. It is expected that these therapy agents also target cell cycle, oncogenic paths, metastatic cells like chemotherapeutic drugs, but specifically kill the malignant cells not other normal cells ([Joo et al., 2013](#)). The complex nature of the biology of mammalian organisms remind us that we need to continue this research. Normally, immune systems have a high erection capacity and are very effective against foreign cells such as bacteria, viruses, and abnormal cells. This feature of the immune system has been used for medical purposes for a long time. The first targeted therapy was performed in the 1940s ([Lee, 2012](#)). The major aim of these targeted therapy studies are based on educating the immune system cells and factors to be able to recognize as foreign and kill cancer cells specifically. The immune system is normally programmed to recognize and eliminate foreign cells, while unperturbed by the body's own cells, blocking malignant cells in daily life. We also know that cancer cells are the body's own cells, they are just a differentiated form of the normal tissue cells. Even with these differentiation steps, cancer cells are also native to body and, in most cases, immune system cells are not enough to realize their differentiated form and label them as foreign. Therefore, the main question here is: which changes are required to change immune cells for focusing them to cancer cells?

In this chapter, we will try to explain the functionality of immune cells in cancer treatment, ongoing targeted therapy, immunotherapy studies, and FDA-approved targeted therapy drugs. To be able understand this topic, we have included summary knowledge on how immune cells perform the selective identification steps. Understanding the immune systems mechanism would be helpful to overcome the main obstacles of these therapy studies.

Before detailing this part, we intend to highlight the irreplaceable role of biomarker studies in targeted and personalized medicine therapy. For biomarker discovery, the Cancer Genome Atlas is of vital importance to identifying novel targets and cancer-specific expressions. At the end of this project, it is assumed that it would be possible to be able to overcome present limitations. Characterization of tumor heterogeneity is open to developing new and specific

treatment strategies. These methods are also important for cancer therapy and for the cell-based therapy applications in medicine. This topic is too complex to explain in just one part, so we aim to focus just the main points of immunotherapy and targeted therapy.

1.3.1 WHY IMMUNOTHERAPY IS THE MAGICIAN IN CANCER THERAPY?

The immune system is the defense mechanism of the organism and protects the organism against different kinds of foreign factors to block disease formation. To be able to succeed in its function, the system must be able to recognize and label the cause of disease factors as foreign. These factors are pathogens, viruses, fungi, etc. The important point of this labeling is based on the ability of the immune system to distinguish self and nonself cells. At this point, immune system cells are also specialized to distinguish unnatural form of the cell from their native stage. Most species' immune systems are mainly divided into two groups; innate immunity and adaptive immunity. Mode of actions of the two types of immune mechanism are humoral immunity and cell-mediated immunity, respectively (Beck and Habicht, 1996).

As just discussed, the immune system serves two types of attack and they discriminate their targets via the recognition of specific surface proteins which are served by these agents and these surface molecules, which can be called antigens. Via unique protein–protein interactions, immune systems became capable of discriminating self and nonself and label the foreign agents (Berek et al., 1991; Liu et al., 1996; Restifo and Gattinoni, 2013).

The immune system cells contain a high number of different types of cells to be able to manage their complex function. Each group and subgroup of cells have different and interwoven functionality cells. The most common and well known group of the cells are T lymphocytes, macrophages, B-lymphocytes, dendritic cells, and natural killer cells. We do not plan to discuss the function of these cells in detail. Even if they all have different function, they are member of an infrangible group. Macrophages are the first activation part of the immune system and generally act as an “eater” of the foreign factors, to be able to warn and alert other immune system cells. Dendritic cells are also found as first activated ones and they are the main antigen-presenter cells. B cells are the factory of the immunoglobulins/antibodies. These protein structure molecules are one of the main players of immunotherapy. These molecules label the cell and start a path cascade to provide the eating of the foreign factor by macrophage. T lymphocytes are the “headliner” of the immune system since they are the killers. They are activated by antigen-presenting cells. Antigen presentation is performed by HLAs for human or MHCs for other organism. As mentioned, for the activation of immune system cells, they must be triggered by the specific antigens. During this antigen presentation, there are a huge number of proteins and they are all potential target

for cancer treatment. In this chapter, it is not an option to be able to explain whole immune system but, to sum up the general mechanism: when a mammalian cell is a target, infected or malignant cell expresses antigens (these antigens are the biomarkers of the malignant cells) on their surface. After that, the antigens which are represent via HLA/MHC-1 or antigenic protein, are opsonized by antibody molecules or digested by macrophages. At the end of these interactions, specific T cells come and kill the target cells (Andersen et al., 2006; Castelli et al., 2000; Boon and van der Bruggen, 1996; O'Byrne and Dalglish, 2001).

During the evolution of cancer, malignant cells use different mechanisms, such as low availability (which is actually a protection mechanism to block auto-immune disease), blocking the expression of the HLA expression (Cho et al., 2000; Drake et al., 2006); now they can escape from the immune system. A low number of tumor-specific immune cells, such as T cell $CD4^+CD25^+FoxP3^+$ (regulatory T cells), myeloid-derived suppressor cells, tumor-associated macrophages is found and it is one of the application to increase the number of these cells and also it is important to regulate the number of the intratumoral secretion of inhibitory cytokines such as transforming growth factor- β , tumor necrosis factor (TNF- α), and interleukin-10, the expression of negative accessory molecules for T-cell activation within the tumor microenvironment (B7-H1, B7-H4), and various phenotypic alterations that result in immune escape by the downregulation of tumor antigen expression, MHC molecules, and other molecules essential for antigen processing (Chen et al., 2013).

In this chapter, it is commonly mentioned that tradition therapy applications are just such as killing the fly by using hatchet. While killing the malignant cells, patient also loose her/him own cells, too. And also these methods are not totally kill the cancer cells, they always have potential to live up and metastasize over and over. But to direct the body's own defense mechanism to kill these transformed cells or usage of well targeted strategies can be more promising on getting permanent and less adverse results.

1.3.2 WELL KNOWN IMMUNOTHERAPEUTIC AGENTS AND THEIR ADVANTAGES/DISADVANTAGES

Cancer treatment is still one of the uncomplete studies from around the world. In spite of the favorable effect of the small chemicals, hormone therapies, radio-therapies, and surgery applications; cancer is still one of the most harmful diseases around the world and the treatment strategies have adverse side effect on patient lifestyle. One of the most promising treatments is targeted therapy and immunotherapy. Recently, monoclonal antibodies are hot topics in this area but in addition to that cell based therapies also have good ratio in patient survival (Kim et al., 2004; Mendelsohn, 1997; Weiner and Link, 2004).

There are several antibody-based drugs in the sector which are approved by FDA. Their structures show some varieties: some of them are found in chimeric

form, some of them are murine-sourced, while some are found in humanized form. These drugs mainly target cancer-specific biomarkers. If the cell has these markers, they are opsonized by the monoclonal antibodies and this monoclonal labeling ends up with killing of the cells. Here again we see the importance of the biomarker studies. Rituximab (Rituxan), Tositumomab (Bexxar), Ibritumomab (Zevalin), Gemtuzumab (Myolotarg)*, Alemtuzumab (Campath), Trastuzumab (Herceptin), Cetuximab (Erbix), Panitumumab (Vectibix), Bevacizumab (Avastin) drugs are those monoclonal antibodies that are approved by FDA to be able to use in different cancer treatments (Okur and Brenner, 2010).

Immunotherapy applications are divided into the three main groups in this chapter: cellular therapy, adoptive cell transfer therapy, and genetic modifications of the immune cells.

1.3.2.1 Cellular immunity

Cellular immunotherapy can be explained as the kind of therapy which is based on usage of cell-based vaccines. The source of these vaccine cells is derived from tumor cells or antigen presenting cells, which are direct to express tumor-specific antigens or the adoptive transfer of the effector T cells. These applications are still under investigation for future treatments. The main aim of these applications is to govern the immune cells to a specific target, which is the tumor tissue, for the elimination of the cancer cells. Recent key topics in immunotherapy applications are verged to adoptive T cells therapy, production of immunomodulatory agent, activating the specific and durable immune response to cancer cells.

Vaccine strategy has been used for long time to prevent disease. In this treatment type, vaccines are used both to prevent and treat the disease formation. In this therapy, there are different kinds of vaccines: DNA vaccines, protein/peptide-based vaccines, and whole cell vaccines. In Table 1.4, we summarized a short list of vaccines that are used or promise to use vaccine types.

As mentioned before, tumor cells have special immune escape mechanisms and to overcome these mechanisms is also one of the key topics in immunotherapy studies. Fig. 1.5 summarizes the immunotherapeutic applications. To increase immunogenicity of the tumor cells, these cells take out and they are genetically modified. After normalized immunogenicity levels, APC cells detect tumor cells and they recruit cytotoxic T cells. In addition, expanding the tumor specific T cells (e.g., CD40L or IL2) is one of the promising ways of cancer treatment. At this stage, the transferred cells can be self or nonself and both of them have discriminate advantage/disadvantages. Self transplantation has advantages in not requiring use of immune suppression drugs because of matched HLA types. However, the nonself transplanted cells are much more standardized and therefore, in some cases, they can be more advantageous for patient; both approaches have been used in treatment (Foster and Song, 2014).

In this part, we wish to focus on the role of protein/peptide vaccines in immunotherapy. In this type of vaccination, peptides are used the same as traditional

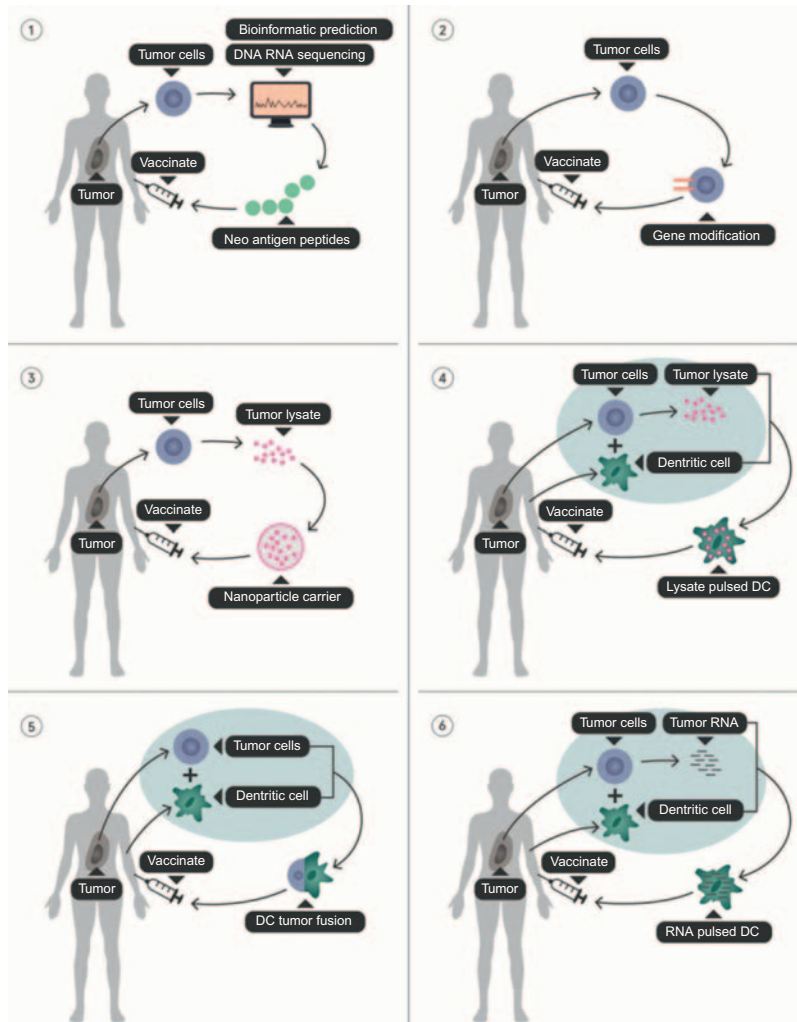
Table 1.4 Different Vaccination Strategies Used in Cancer Therapy and Their Production (Okur and Brenner, 2010)

Vaccination Strategy	Method of Production
Peptide/protein	Manufacturing peptides from class I/II MHC epitopes of tumor target antigens or protein subunit containing multiple epitopes
Subunit vaccines	Cloning genes encoding tumor target antigens into a plasmid and delivering it to host by different methods (injection, electroporation, gene gun)
DNA vaccines	
Whole-cell vaccines	– Irradiated unmodified autologous tumor cells – Autologous/allogeneic tumor cells engineered to express costimulatory molecules, cytokines, chemokines
Dendritic cell vaccines	– Derivation of immature DCs from CD34 + hematopoietic stem cells or peripheral blood monocytes – Loading them with tumor antigens (peptides/whole protein, DNA, mRNA), whole necrotic or apoptotic tumor cells, tumor cell lysates, or transducing with viral vectors or fusing with tumor cells – Ex vivo maturation with cytokine cocktails (+) TLR agonists

vaccine strategies. Tumor cell—specific peptides, which are in a cocktail of adjuvants, are encountered with immune system cells. The content of adjuvant is, of course, very important; it must have specific agents which are able to activate monocytes and macrophages via their toll- like receptors. After this stimulation, concerned cells label this agent as foreign and generate required signals for the activation and maturation of immune cells that function as antigen-presenting cells. At this point, design of the peptides are important. They must be designed as capable of presenting with HLA (or MHC I) on the antigen-presenting cells, since they must interact with CD8 + cytotoxic T cells to be able to start an immune response (Xiao et al., 2015). In addition, to be able to improve effectiveness of immunogenicity, these peptides can be designed so they are recognized by CD4 + T helper cells, since it is known that helper T cell—triggered immunity lasts longer than CD8 + cytotoxic T cells (DuPage and Bluestone, 2016; Kuo et al., 2016; Okur and Brenner, 2010; Schoenberger et al., 1998). Furthermore, single protein usage in the activation of immunity has several advantages, since they have more chance to be able to load a wider range of HLA types. The main obstacle of peptide-based vaccination is the fast loss of epitope activity of these peptides, therefore immune responses become shorter (Okur and Brenner, 2010).

1.3.2.2 Adoptive cell therapies by using lymphocytes

Passive administration of the tumor cell—specific T cells is called adoptive T cell therapy. This therapy application is very effective, since these cells are capable of

**FIGURE 1.5**

Different applications of immunotherapy. (1) To minimize dead of normal cell, firstly malignant specific antigenic parts must be identified. These newly identified peptides can be used as neoantigens for vaccination. (2) Whole tumor cell can be transplanted with adjuvant. Because of specialized adjuvant cocktail, immunogenicity can be increased. (3). Whole tumor lysate can also be injected by nano-encapsulation. Nano-encapsulation can trigger an immune response. (4) Tumor lysate can be encountered with dendritic cell which is one of the best antigen presenting cell. Via these presented antigens, T cell will initiate the immune response. (5) In a specific niche, tumor cells interact with dendritic cells and dendritic cells can process the tumor specific antigens to present T cells to start

(Continued)

transporting through the body and an educated T cell is well focused to its antigen-specific tumor tissue. However, this kind of therapy is not specific for only T cell, it is suitable for other lymphocytes, which are capable of tumor infiltration. For the success of this therapy in clinical use, we need to learn much more about immune systems; their recognition mechanism, labeling mechanism, tolerance and infiltration mechanisms, etc. There are different kinds of applications in adoptive cell treatment strategy, which are summarized in Table 1.5. To sum up, when immune regulation has totally succeeded, there can be expected results in the treatment of the cancer.

Even if there are a lot of limitations for now, adoptive lymphocyte therapy will have a bright future, especially if it is used coupled with drug therapies. In addition, antibody and ALT application combinations will be the main cure of cancer in the future.

1.3.2.3 Genetic engineering in T cell

Genetic modification or gene therapy is one of the most promising application in the area of the cancer treatment, but the complex nature of the biology continues

Table 1.5 Different Application Types of Adoptive Transfer of the Lymphocytes (Okur and Brenner, 2010)

Name of the Application	Advantage of the Application	Limitation of the Application
Not antigen specific actions <i>Donor lymphocyte infusion and natural killer cell infusion</i>	HLA unrestricted manner usage which is useful for the tumor cell that evolved immune escape mechanism	Adverse effect in the case of infection and GVHD
Tumor infiltrating cells	Isolation of the tumor infiltrating cells and induce expansion of these cells with different applications	Tumor-induced energy effect and nonspecific expansions can be observed
Generating antigen-specific T cell and transfer of them	High expansion rate of tumor specific T cell and continuous reach of antigen presenting cell	Unresolved complex structure of the virus infection which is used for tumor expansion mechanism

◀ immune response. (6) Tumor RNA can also be used to start an immune response via dendritic cells.

Source: Reprinted with permission from Elsevier (Ophir, E., Bobisse, S., Coukos, G., Harari, A., Kandalaft, L. E., 2016. Personalized approaches to active immunotherapy in cancer. *Biochim. Biophys. Acta BBA - Rev. Cancer, The role of the immune system in cancer: from mechanisms to clinical applications* 1865, 72–82).

to block this. There are a lot of big projects, such as the cancer Genome Atlas Project, Human Genome Project, Human Proteome Project, etc., which aim to solve this big biology problem. The genetic modification of immune cells are also one of the most promising ways for cancer treatment. The nature of immunity is based on the production of billions of antigen receptors. Due to tolerance mechanisms by clonal deletion or the energy mechanism, these antigen recognition receptors are eliminated. In most cases, tumor-associated antigen carrier cells are eliminated in this stage because of low affinity of TCRs. To overcome this limitation, production of transgenic receptor-expressing cells can be a good option. Another option for this situation is the production of chimeric antigen receptors (CAR). The CAR T-cells are designed to recognize antibodies and have a well designed signal cascade, which will be activated when a cell encounters its antigen, activating the killing mechanism. Recently, CAR T cell strategies gave promising results, especially in B cell malignancies (Davila and Sadelain, 2016). In spite of the current successful results, there are still several challenges, such as unexpected cytokine release, neurologic toxicity, nonspecialized killing of the tumor tissue, hypogammaglobulinemia, and anaphylaxis (Bonifant et al., 2016; Brudno and Kochenderfer, 2016).

To be able to overcome these limitations, several applications are still ongoing. For example, in B cell malignancy treatment to prevent uncontrolled cytokine release, an inducible mutation system was tried. In this system, the CD19CAR vector was fused with Tet-on/off vector to control expression of CD19CAR only in the presence of doxycycline.

This study showed us that controlling the expression of a CAR can be solution for uncontrolled cytokine release situations (Sakemura et al., 2016).

The recent trend in cancer therapy is the use of adoptive transfer systems and monoclonal antibody based drugs. Immune modulator drugs are still under investigation, but there are already successful results. Effectiveness of the results are demonstrated in B cell malignancies and there are promising results in Phase I solid tumor studies (Almåsbaek et al., 2016; Frigault and Maus, 2016; Ladjemi et al., 2010; Mendelsohn, 1997). However, these marked results are not available for everyone, because of their high cost. This is one the main challenges of these therapies, but it is also hoped that, as with human genome sequencing costs, CAR T cell or other immunotherapy applications will be cheaper in the near future.

CARs are genetically engineered receptors that combine the specific binding domains from a tumor targeting antibody with T cell signaling domains to allow specifically targeted antibody redirected T cell activation. Despite current successes in hematological cancers, we are only in the beginning of exploring the powerful potential of CAR-redirectioned T cells in the control and elimination of resistant, metastatic, or recurrent nonhematological cancers. This review discusses the application of the CAR T cell therapy, its challenges, and strategies for successful clinical and commercial translation.

1.3.2.4 Antibody-drug conjugates

Over the past half century, cancer management has improved significantly. One part of cancer treatment studies contain antibody-drug conjugates (ADC). In this structure, there are two forms: organic (monoclonal antibody) and nonorganic (chemotherapeutic drug). By using special linker molecules, this couple are connected each other and they have the potential to kill cancer cell specifically. This “killing” is performed by chemotherapeutic drug while specificity is performed by monoclonal antibody. Adcetris and Kadcyla are the first ADCs that are approved by FDA, but by 2016 there are nearly 60 ADCs in both drug market and clinical trials. In this part we will shortly aim to explain technologies in the conjugation and linker chemistry and clinical success of ADCs; the general aspects are shown in Fig. 1.6. Being able make useful ADCs, conjugation is one of the main steps in design, so researchers focused on the conjugation strategies, and because it is not preferable to break the pharmacokinetics (PK)/pharmacodynamics balance of the molecule. This balance is important for drug effectiveness in the organism.

Despite its huge potential, to succeed in cancer treatment, it is required to make further studies to be able to understand the immunological and biochemical nature of this couple. To achieve better immunological activity, target antigen selection has vital importance. In addition to that, for the pharmacochemical properties of the ADCs, detailed investigations are required in linker chemistry. Linker chemistry is important for the impact of the drug, since it effects PK features, important for absorption, distribution, metabolism, and excretion of the drugs. Additionally, for the optimal epitope interaction, linker chemistry and conjugation applications also play an important role (Gao et al., 2016) (Table 1.6).

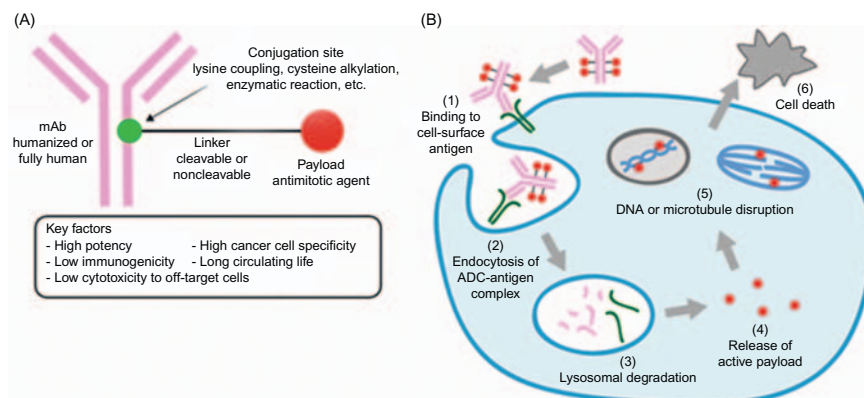


FIGURE 1.6

Main structure of antibody and mode of action antibody drug conjugates.

Source: Reprinted with permission from Elsevier (Tsuchikama, K., An, Z., 2016. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. *Protein Cell* 1–14).

Table 1.6 Advantages and Disadvantages of the Conjugation and Linker Chemistries Described

	Strategy	DAR ^a	Advantages	Disadvantages
Chemical conjugation	Lysine coupling	0–7	Simple process Used in FDA-approved and clinically tested ADCs	Distributed DAR Heterogeneous mixtures of products Potential reduction of antigen binding
	Cysteine coupling	0,2,4,6,8	Simple process Used in FDA-approved and clinically tested ADCs	Heterogeneous mixtures of products Increased clearance rate with high DAR
	THIOMAB	2	Defined DAR	Requires genetic engineering
	Cysteine rebridging	4	Homogeneity Defined DAR	Potential disulfide scrambling
	Nonnatural amino acid	2	Homogeneity High structural stability Defined DAR	Requires special techniques and biological agents
	Sortase	3–4	Homogeneity Tightly-controlled DAR No adverse effect on antibody binding	Potential immunogenicity Requires incorporation of LPETG motif on the heavy chain
(Chemo) enzymatic conjugation	Microbial transglutaminase	2	Defined DARs	Requires removal of <i>N</i> -glycan on N297
	Glycan engineering (GlycoConnect)	2	Homogeneity Defined DARs	Requires multiple steps (i.e., <i>N</i> -glycan trimming, glycosylation, and conjugation)

(Continued)

Table 1.6 Advantages and Disadvantages of the Conjugation and Linker Chemistries Described *Continued*

	Strategy	DAR ^a	Advantages	Disadvantages
Cleavable linker	Hydrazone		pH-responsive cleavage	Premature cleavage during circulation
	Val-Cit ^b -PABC ^c ,		Stability during circulation	Hydrophobicity
	Val-Ala-PABC ^c		Traceless release of payload	
	Disulfide		Intracellular release of payload	Potential premature cleavage during circulation
	Pyrophosphate diester		Stability during circulation Hydrophilicity Traceless release of payload	Unknown mechanism of lysosomal cleavage
Noncleavable linker	Stable linker without cleavage mechanism		Stability during circulation	An amino acid residue attached on the released payload

This table is reprinted with permission by Springer (Tsuchikama, K., An, Z., 2016. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. Protein Cell 1–14).

1.4 SMART ROLE OF THE NANOTECHNOLOGY IN CANCER TREATMENT

Before discussing the nanomedical treatment of cancer, it would be better to give some brief information about nanotechnology. Nanotechnology is the science of nano-sized materials and use of these nano-sized elements for different purposes. Even if the potential of nanoparticles were discovered in 1959 by theoretical physicist Richard Feynman, researchers have just discovered the way of controlling them only in past 10 years, in the science of medicine (“‘There’s Plenty of Room at the Bottom’ (Richard Feynman, Pasadena, 29 December 1959)—Metamodern,” [n.d.](#)). In cancer treatment and diagnosis, nano-particles have vital importance and a bright future. In this part, we try to summarize selected studies which are being undertaken in nanomedicine. The main focus of all nanomedicine studies is to establish a way of creating the functional nanoparticle, direct it to the correct tissue/cell, reprogramming its duty in the organism. They are expected to act as a nanorobot which is functional in living body.

There are many efforts to enlighten the ways of using nanoparticles in cancer treatment research. Most companies and researchers work together to be able overcome limitations of therapeutic application of nanoparticles. To be able understand the potential of nanotechnology, to give number of studies would be good example. A PubMed report shows us there were 73,000 articles between 2010 and May 2013, and interest in this technology is growing sharply (Bertrand et al., 2014). Most studies focus on several fundamental concepts, which are: increasing the enhanced permeability and retention effect of the drug, developing target specific drugs, binding mechanism of drug to tumor cell, or immunomodulatory effect of nanoparticles.

1.4.1 BRIEF LIST OF STUDIES FOR USAGE OF NANOTECHNOLOGY IN CANCER TREATMENT

All efforts in cancer nanotherapy aim to produce more efficient drugs. Especially with Doxil and Abraxane, these are more likely to be realized. There are plenty of nanodrugs which are commercialized or in phase stages. In addition to these developments, the theoretical knowledge is growing by each study. Even if it is not possible to explain all science behind the nanotechnology studies, we will briefly outline it.

One of the treatment strategies still under development but give promising results is targeted nanotherapy. This strategy is called CytImmune and they published their Phase I study result recently (Koonce et al., 2015). In this study, TNF- α protein is bound with a gold nanoparticle and thiol-derivatized polyethylene glycol. In this way, they hid the antitumor protein from the immune system and, therefore, the particles can freely transport to the tumor site and give better results, according to the traditional delivery of TNF.

As mentioned above, one of the challenges in immunotherapy is the low duration time of the transplanted cell in the tumor tissue. To overcome this problem, researchers apply the feature of nanoparticles. By conjugation of adjuvant drug-loaded nanoparticles to the transplanted cell, they achieve block pseudoautocrine stimulation to transplanted cells. Therefore, they achieve better ratios in the killing of malignant cells (Stephan et al., 2010).

Another strategy is based on the use of accumulation and ability of nanoparticles. What is the meaning of accumulation property? The nature of nanoparticles allow them to enter any kind of cell easily. This nature is dictated by size and biochemical features of the particles. Because of this easy entry mechanism, nanoparticles can be accumulated in any part of the cell in active or passive state. The meaning of active/passive state is same as able to be triggered property. After a required time is passed, the accumulated drugs can be easily triggered by different types of applications. One of them can be radiowave, other can be heat, or photosensitivity. There are several examples of this kind of treatment

application and they all result in the following way: with the help of conjugated or solo nanoparticles, it became possible to be able to overcome proliferation and metastasizes of the cancer (Sano et al., 2013). Auroshell is one of the example of this kind of drugs. This nanoparticle-containing tumor cell became an infrared light adsorber. By the implementation of heat to the body, the tumor cell absorbs all light and, because of an excess amount of heat, malignant cells die (Alphandéry et al., 2015).

One of the limitations of cancer therapy is the secretion of cancer drugs from malignant cells. Since malignant cells are capable of pumping cancer chemotherapeutic drug out of the cell, the effectiveness of the cancer drug would decrease. To overcome this limitation, nanotechnology give us a solution, which are called nanodiamonds. Nanodiamonds are diamonds which has a size smaller than 1 micrometer. These nanoparticles can be used as delivery vehicles since their chemical formula prevents the pumping out mechanism of the malignant cell. Therefore, the effect of the drugs can be enhanced (Ryu et al., 2016; Shimoni et al., 2016).

As mentioned previously, gene therapy is also one of the promising ways for cancer treatment and treatment of other diseases that are caused by genetic abnormalities. At this point, delivery mechanisms generally consist of using virus-like particles. This encounters another problem; being unable to control this virus genome and unpredictable results of this tool. Nanotechnological applications can provide another solution: nanotrain. Nanotrain is the conjugation of nano-sized particles with nanotechnological applications. DNA can also be a member of this nanotrain. Ongoing research gives hopeful results; researchers have used nano-trains to conjugate DNA (special sequences) particles and therefore it would be possible to deliver directly DNA particles to tumor tissues. Delivered DNA particles control the malignant cell expression to produce apoptosis signals. Through this application, researchers detect a high ratio in the killing of malignant cells (Zhu et al., 2013).

Nanoparticles are also can be used in the bio-mimicking process, which also means that it is possible to produce a nanoparticle resembling an organic molecule. It would be better to explain this strategy by an example. High density lipoproteins (HDL) are a kind of protein and they carry cholesterol. Research shows us that lymphoma malignant cells tend to use HDL as a substrate to overcome its high metabolic rate. By the application of nanotechnology strategies, researchers developed a particle which totally resemble natural HDL (mimic the HDL) but it contains a gold nanoparticle in the core. This special formula can bind selectively to lymphoma cells but this binding is not transient. The permanent binding of this synthetic molecule prevents the nutrition uptake of the malignant cell and, as a result, the malignant cell would die because of starvation (Foit and Thaxton, 2016; McMahon et al., 2015).

To sum up the advantage of nanotherapeutic application, we can list the purpose of using nanoparticles in cancer treatment. They can be used directly

Table 1.7 Summary of the Nano-Medicine Limitations (Dawidczyk et al., 2014)

Limitation	Projected Solution
Tumor size/heterogeneity dependent activity	Developing the well imaging techniques would give better values about tumor features
Does stabilization problems and inconsistent physicochemical properties	Further high-throughput studies give better option in reporting of dose-dependent factors and biochemical features of the drug. Need time to accumulate theoretical knowledge on controlling of nano-drugs

as drug, drug delivery vehicle, and conjugating agent of different drugs. Despite great advantages of nanotechnology, there are several limitations that are summarized in Table 1.7, although advantages have a higher number, in comparison.

Of particular applications, nanotechnology applications have high importance in cancer treatment and give solutions to limitations of traditional therapies. Once the uniformity of the tumor diagnosis and transient physicochemical properties are overcome, combination of nanotechnology with other traditional and targeted therapy can be solution of cancer-caused death.

1.5 CONCLUSION

Cancer is known as abnormal cell growth but the physiology of the cancer is complicated, therefore management is also sophisticated. With recent fossils we know that cancer is a kind of disease observed in 2 million years ago. According to National Cancer Institute, 2016 1,685,210 instances were newly diagnosed in United States and 595,690 people died. Even if there are 3000 biotechnology firms and thousands of researcher focusing to find a cure for this disease, there are still no certain results. The main results for this situation is tumor heterogeneity and complexity of the biology. By recent development in the high throughput biomedical area, which is still under ongoing development, it can be possible to solve this problem. However, in our knowledge, as it is not reasonable to get a certain therapy for the cancer. Therapeutic agents are under development, including low molecular weight drugs, proteins, monoclonal antibodies, and gene therapy strategies. In this chapter, we have concentrated on a small number of recently developed therapies that illustrate the science of the cancer studies and how molecular diagnosis will increasingly play a part in the development and clinical introduction of novel therapies. These therapeutic strategies hold great promise and we hope to see certain results of these studies.

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FURTHER READING

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Nanotechnology-based drug delivery systems: Challenges and opportunities

2

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CHAPTER OUTLINE

2.1 Introduction	40
2.1.1 What is Nanotechnology?	40
2.1.2 Nanotechnology Research Fields.....	40
2.1.3 Drug Delivery to Targets	41
2.2 Fundamentals of Nano-Targeted Drug Delivery	44
2.2.1 Shape and Size	45
2.2.2 Realistic concerns with nanodrug delivery.....	53
2.3 Evaluation of Nanotechnology for Industrial Applications	54
2.3.1 Research Pipeline.....	55
2.3.2 Clinical Trials	58
2.3.3 Developmental Questions	58
2.3.4 Assessment of Marketed Products and Technologies	61
2.3.5 Techniques for Further Development of Nanodrug Delivery	63
2.3.6 Nanotoxicology	64
2.4 Conclusion	68
References	68

2.1 INTRODUCTION

2.1.1 WHAT IS NANOTECHNOLOGY?

Norio Taniguchi, an engineer at Tokyo University, was the first person to use the word “nanotechnology”, in the year 1974. It was almost two decades before the term was rediscovered and now we have a commonly used definition, which describes nanotechnology as the production of materials and systems with a dimension or manufacturing limit less than 100 nanometers (nm). However the size of the 100 nm is not a rigid boundary, it varies according to the application. For example, in medicine, any size less than the micrometer is considered as nanometric; with the help of this dimension, distribution of drugs in the body or the interactions between implant and tissue can be controlled. A significant interest in nanotechnology has spread to all branches of science over a last decade (Bath & Tuberfield, 2007; Porter & Youtie, 2009). The intrinsic nanoscale properties of various compounds have resulted from the arrangement of their atoms and electrons within a nanometer range. Prior to the concept of nanotechnology, there were practices and process which used compounds like carbon black, silica, zinc oxide, titanium dioxide, etc. hence, it was prevalent in its crude form and now it is a full-fledged discipline (Maskos & Stauber, 2011; Min et al., 2008; Shin et al., 2015). The products such as drugs, cosmetics, nutraceuticals, and therapeutic agents that are developed currently through nanotechnology may not have a quantum behavior at all times but they can intermingle with the biological molecules in an exceptional manner, in comparison to their larger counterparts. The nanosystems have an advantage over other types of delivery systems; the former can be targeted directly to the sites of interest in the body. One application of nanotechnology is the nanomedicine.

2.1.2 NANOTECHNOLOGY RESEARCH FIELDS

Nanomedicine is the application of nanotechnology, which serves an important role in the diagnosis, monitoring, prevention, and treatment of diseases. The possible applications are: Nanoscale drugs and targeted drug delivery, antimicrobial medical dressings and textiles, in vivo imaging methods, bone and dental prostheses, and coatings for in vivo implants to support substances for the regeneration of tissues. With the help of nanotechnology, biomarkers can also be better identified and understood, which will lead to the diagnosis of the disease stage, blood glucose metering and insulin delivery using nanotechnology, 3-dimensional (3D) nanomaterials for the immobilization of stem cells at the site of injury, image-guided implantation of neuro stimulators, etc. Further development in this area enable patient-specific, targeted, and regenerative medicine technology. Some active research areas of this field are given in Table 2.1.

However, with new technologies emerge new responsibilities. For example, it is necessary to assess the impact of the production, use, and disposal of nanoproducts. There is a growing health and environmental concern in the society about the ill effects associated with the usage of nano products. For example, inhalation of carbon

Table 2.1 Active Research Areas in Nanomedicine

Health Field	Application	Example
Diagnosis	In vitro tests with sensors for determination of molecules that react with particular disease (Biomarkers).	Detection of hereditary diseases by DNA-coated gold nanoparticles in combination with a biosensor chip, to read the protein values.
Diagnosis	In vivo measurements of biomarkers by imaging techniques, using nanoparticles as contrast media.	Visualization of small tumors by magnetic resonance tomography (MRT) using magnetic iron oxide nanoparticles which are attached to supports.
Diagnosis	Diagnostic test: “one-chip lab”(“Lab-on-a-chip”).	Monitoring of potassium levels in the blood from home.
Therapy	Passive drug release.	Therapeutic drugs are made using hollow nanostructures to the tumor (e.g., with liposomes).
Therapy	Active drug release.	The injection of nanocapsules using molecular antennas. The capsules release their contents when they are in contact with certain disease structures.
Therapy	Thermotherapy	Nanoparticles accumulate in the blood vessels of tumors and are activated via physical components such as sound, light or magnetic waves. The heat is produced by the particles and destroy the tumor cells.
Tissue regeneration	Regeneration of tissue with help of nanoscale biomaterial.	Matrix contains peptides, nanofibers, and other nano-based materials in which cells can proliferate and tissue formation can occur.
Tissue regeneration	Regeneration of tissue cell therapy.	Cells are first formed outside the body and then used to support self healing.

black and titanium dioxide cause reduction in sperm count, carcinogenic effect, proinflammatory, and increased risk of oxidative stress-mediated diseases.

2.1.3 DRUG DELIVERY TO TARGETS

Drug delivery to the target is the process of drug accumulation at the target site and this method is not dependent on the route or method of administration of drug (Torchilin, 2000). The term nanoparticles refers to particles having a size of one to several hundred nanometers (European Science Foundation, 2005). On decreasing

the size of nanoparticles, their macroscopic physical properties will also change. According to the laws of Quantum mechanics, below the size of 50 nm, the particle changes its color, transparency, hardness, along with magnetic and electrical properties. For example, quantum dots change their color, depending on their size. It has been observed that surface area increases on increasing the total mass of the particle. As a result, physical parameters, such as melting and boiling point, change. Nanoparticles easily cross biological barriers to get inside the body and thereby have an advantage over conventional drug delivery systems (Fig. 2.1).

For an efficient drug delivery to the target, there are four requirements needed, which includes, retaining of drug carrying vehicles in the system, evading the immune system, reaching the target, and releasing the drug (Mills & Needham, 1999). The drugs which are administered through intravenous route require efficient loading into a delivery vehicle. This could lead to their accumulation and residence at the target site through circulation, retention of their specific characteristics, which could lead them to the target and effective release of the drug at the site in time. Different types of drug delivery systems are required, depending upon the type of route and the target site selected.

Drug targeting is classified into two categories: Passive and active. Passive targeting entails the preparation of a complex that includes the drug and the carrier in a way that it can avoid its elimination from the body and reach the target through circulation and be taken up by its target. In this type of targeting, the drug accumulates at the diseased site which is commonly known as enhanced permeation and retention effect. Whereas in active targeting, the drug–carrier complex is attached with a specific marker, such as antibody, receptor, etc., that can efficiently direct it to a specific target. It is based on the specific interactions between the drug delivery vehicle and the target cells or tissues (Bee & Park, 2011). Through these targeting mechanisms, the drug can accumulate efficiently and effectively at the target sites.

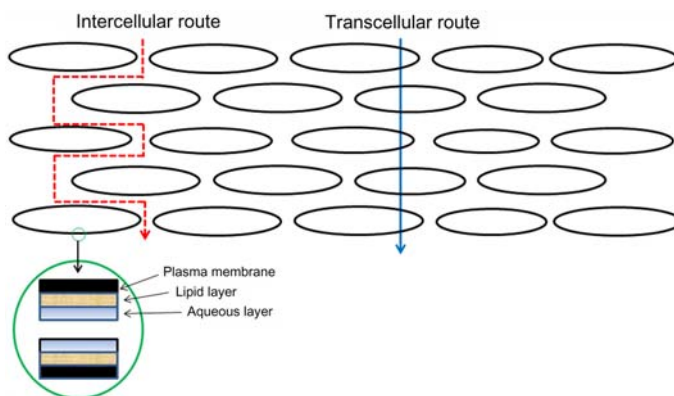


FIGURE 2.1

Represents the various barriers and ease of movement of nanoparticles drug carrier.

There are two areas by which drug delivery can be targeted. These areas include systemic and intracellular targeting. In systemic targeting, the drug–carrier complex reaches the target through circulation and extravasations through the vessel walls. It can also be categorized into two types: ligand–receptor-mediated and locally activated drug delivery. In the former, the drug–carrier complex is linked with the ligand or a receptor, which leads to its targeting to the specific site; in the latter, the complex is self-triggered by a specific signal released at the target site or by externally activating the release of drug from its vehicle.

Self-triggered targeting is the passive targeting in which internalization by the target site is dependent upon particular factors such as enzymes or differences in pH at the specific site, inside the body and on the outside. It is dependent on physical entities such as light, ultrasonic waves, temperature, and electromagnetic field (Lentacker et al., 2008; Lin et al., 2010; Rapoport, 2004).

For successful drug targeting, the direct delivery of a drug–carrier complex to the target is very important, such as in intracellular targeting. To release a drug, the carriers or vehicles must enter the cytoplasm of the cell and release the drug inside at an optimal rate for its therapeutic effect. As a result, to develop the drug delivery systems for target delivery, it is important to understand the role and mechanisms of intracellular localization and trafficking. To study the mechanism of drug delivery by intracellular trafficking, various studies have developed several means and tools (Nam et al., 2009; Raagel et al., 2009; Richardson et al., 2009; Sasaki and Kinjo, 2010; Sauer et al., 2009;). The knowledge of these intracellular trafficking mechanisms will help in the designing of drug–carriers and help in effective targeting the specific site in the cytoplasm of the cell.

The most important biological barriers include the epithelia of skin, intestinal tract, and lungs. Epithelia can be single or multi-layered. Multilayer epithelia, e.g., mouth and nasal mucous membranes or cornea of the eye, consist of several cell layers, usually of the plate or column-shaped. Single-layered epithelia are found in intestine and in the alveolar region of the lung. Small molecules that are sufficiently lipid- and water soluble can passively pass through the cells (transcellular transport) (European Science Foundation, 2005). Hydrophilic substances can be found between adjacent epithelial cells (paracellular transport) (Hillyer & Albrecht, 2001). Paracellular transport is carried out by the apical region of the cells’ “tight junctions”. The tight junctions are cell–cell connections that occur between the membranes. Adjacent cells are formed lead to enhancement of permeability of the cell cluster to relatively small molecules. Furthermore, it is possible to use molecules (e.g., peptides with up to three amino acids) with the help of special carrier systems. An active transportation, under adenosine triphosphate (ATP) consumption, can be via ATP binding cassette (ABC) transporter (Jani et al., 1990). Large molecules enter through endocytosis in cells, and in the membrane, vesicles are transported through the epithelial barrier. Molecules transport across the epithelial barrier through the efflux system. Nanoparticles attached to the apical membrane of epithelial cells localize and transport through the cell (Dong & Feng, 2005). The best-studied efflux system is P-glycoprotein. This is a

170 kDa heavy glycoprotein, which belongs to the family of ABC transporter proteins.

The oral bioavailability of substances plays a major role in the drug delivery of medicines, due to the fact that oral medication is easy to perform and has high patient acceptance. Orally applied substances are absorbed by the mucous membranes of the small intestine. Many drugs are subject to enzymatic reactions in the gastrointestinal tract and therefore do not show better bioavailability.

To overcome this barrier, drug is encapsulated in nanoparticles which increase the transport rate across the barrier. Biodegradable polymers are an important class of polymers used in the manufacturing of the drug-encapsulated nanoparticles, e.g., poly(lactic-co-glycolic acid) (PLGA), Xanthan gum and Guar gum (Sharma *et al.*, 2016). The transport properties of the particles depend on many factors, such as particle size, surface properties, or dwell time in the gastrointestinal tract. Hillyer and Albrecht examined the extent to which nanoparticles of gold in the sizes of 4, 10, 28 and 58 nm orally administered via water to mice are distributed in the body of the animals. After 12 h, the animals were sacrificed and individual organs were inspected for the presence of the nanoparticles. It was clearly recognizable that the particles were distributed according to the size (e.g., many gold particles of the size 4 nm were found in blood, particles of size 28 nm were found in kidneys, intestines, and stomach) (Hillyer & Albrecht, 2001). Surface-modified nanoparticles are used to further enhance the transport of nanoparticles through cells. The “coating” particles with bioadhesive materials, such as polyethylene glycol (PEG), polyvinyl alcohol (PVA) or vitamin E- α -tocopheryl PEG succinate, can improve the adhesion to cells or affect the ABC transporters mediating the efflux system. This increases the dwell time of nanoparticles inside the cells.

Targeting the colon using lectins and neo-glycoconjugates is another interesting approach known as “glycotargeting”. It can be achieved in two ways: Firstly, oligosaccharides or neo-glycoconjugates may be part of the active transport system that is present from the cell surface-binding proteins (the lectins) and then by endocytosis. Conversely, the active substance transport system includes certain lectins that interact with the sugar molecules on the cell surfaces.

2.2 FUNDAMENTALS OF NANO-TARGETED DRUG DELIVERY

The background of research involving nanotechnology is changing faster; whether it is the size and composition of nanoparticles or the driving technology (Wagner *et al.*, 2006). This section discusses the basic ground rules that are to be considered and required for understanding the material and its interaction with biological molecules.

To reach the target, the drug carrier should be internalized by the target cell through various barriers and their uptake is managed by several aspects, including

the particle size, shape, charge and interactions between them and cell membranes (Lu et al., 2009; Trewyn et al., 2008; Vivero-Escoto et al., 2010).

2.2.1 SHAPE AND SIZE

The shape and size of the drug vehicle is an important criterion for targeting and delivering it to the specific site with controlled behavior of transport, especially in the small blood vessels and congested tumor vessel network. Shape and size dictates the behavior of cells and their response to particles for internalization. (Goldsmith, 1986; Illum et al., 1982; Lamprecht et al., 2001; Moghimi et al., 2001; Patil et al., 2001; Stolnik et al., 1995; Tabata & Ikada, 1990). Shape and size also alters the surface-to-volume ratio of particles, which influences the plasma kinetic, metabolism, and elimination of drug vehicles (Dunne et al., 2000; Panyam et al., 2003). The carrier after entering the circulation must cross the vessel wall, reach the target and act specifically (Jain & Stylianopoulos, 2010). The size of the nanoparticle affects its biodistribution; its concentration in the blood, diffusion through vessels and its clearance from the circulation (Vieseh et al., 2011). The small size permits the entrance of biomacromolecules to the vehicle arrangements of that dimension in drug delivery.

Over the last 50–60 years, a large number of nanoparticle-based vehicles were synthesized chemically mostly using grass to top approaches. Some of the grass to top approaches include liposomal nanocarriers, solid polymeric nanoparticles, micellar formulations, and polymer-drug conjugates (Duncan, 2003; Jeong et al., 2007; Pasut & Veronese, 2007; Samad et al., 2007; Torchilin, 2005). These approaches have demonstrated very encouraging results, which provided significant impetus use nanoparticles as drug carriers. Very recently, a number of research groups have shown keen interest in top-to grass approaches which make use of fabrication processes that are based on nano- and micro-electromechanical systems, generating nano and micro drug carriers of uniform characteristics with desired size and shape (Ainslie & Desai, 2008; Gates et al., 2005). These top to grass approaches confer advantages over grass to top approaches in terms of easy control over shape and size, which are difficult to maintain in grass to top approaches.

2.2.1.1 Shape and size concerns

Amongst nanotherapeutics, the size and shape has an important part in each and every case. The nano-sized drug components, nanocrystals, are developed because of good and advanced solvation rate, and the loaded nanotherapeutics are prepared for their good plasma kinetics and other advantages. Liu et al. (2016) have demonstrated that particle size is an important parameter influencing the phagocytosis of drug-loaded poly(lactic-co-glycolic) acid microspheres (PLGA-MS) by the target cells (Liu et al., 2016). Some studies have investigated the size concerns of nanoparticles and shown that nanoparticles having diameter greater than 5 μm get caught in the capillary bed of organs such as liver, while the nanodrug

carriers having size range in between 1 μm and 5 μm concentrated at the target site of liver, where Kupffer cells engulf them (Illum et al., 1982; Tabata & Ikada, 1990). Some have shown that the nanodrug carriers with a size less than 1 μm and greater than 200 nm percolated easily in the spleen, but the particles having size less than 100 nm are trapped in the pores of endothelial lining of the blood vessels (Illum et al., 1982; Stolnik et al., 1995; Tabata & Ikada, 1990). Polystyrene nanoparticles of less than 200 nm can easily escape from the macrophage system recognition and thereby improve the half-life of nanoparticles in the blood circulation and can reach the target easily (Kulkarni & Feng, 2013).

The forces that resist the movement of particles in the blood circulation are called hemodynamic forces. When the diameter of the spherical particles is increased, these hemodynamic forces also increase and no margination dynamics is seen in the circulation, until some external forces such as electrostatic interactions or van der Waals forces exist in between the particle and the endothelial cells (Goldman et al., 1967; Liu et al., 2012). The hemodynamic forces also cause the unbalancing, rolling, or tumbling of nonspherical nanodrug carriers, because of the random forces created by blood circulation. For example, the cuboidal nanoparticles face greater force by the blood flow, due to the plane that is facing the flow and separation of the flow of blood at the particle's corners; because of this, the particles face great pressure to the flow in comparison to spherical nanoparticles. Some studies have showcased the effect of the shape of particles in their flow in the blood circulation, i.e., ellipsoidal particles of 3.5 μm wander sideways to the capillary wall, due to their unbalancing in the flow, when compared to spherical particles that flow in the center. Some studies have reported that the filamentous polymer micelles that assembled on their own and having high width-to-height ratio, i.e., diameter of 22–60 nm and 2–8 μm length, persist in blood flow for a longer time than the spherical particles (Geng et al., 2007; Nishiyama, 2007).

2.2.1.2 Performance based on size and shape

Adjusting the critical distance of the bonds holding the atoms, nanomaterials can be prepared hard or soft. A small-sized polycrystalline material can become hard because of the lack of displacement of bonds and others become softer, due to the low amount of bonds between the atoms. A particle consists of two sorts of energies: surface forces and body forces. All the molecules of particles are involved in body forces, while molecules at the surface exert the surface forces. With the decrease in size, the surface forces increase and affect the properties of material. Van der Waals interactions are responsible for the attraction among the particles and hence are accountable for body forces (Hodges, 2007; Min et al., 2008). The decrease in size of crystalline substances results in large surface area, which is compensated by changes in crystallography, i.e., lattice contraction, lattice deformation, defects, rearrangements, and morphological changes (Auffan et al., 2009). These changes lessen the extent of crystallinity and thereby increase the solubility

of the material, which is the most important decisive factor for drug delivery to the target site.

The shape and size aspects of nanodrug carriers affect the dynamics of the nanoparticles in the blood vessels towards their wall (Decuzzi et al., 2005; Ferrari, 2005). Some studies have shown the role of theoretical modeling in signifying the importance of size of the nanoparticles in their interaction with the blood capillaries of tumor during their transport (Decuzzi & Ferrari, 2006; Decuzzi et al., 2006, 2009). Some recent studies have also suggested the important role of the shape of nanoparticles in their behavior in vivo (Champion et al., 2007a,b; Geng et al., 2007). Similarly, other size- and shape-related factors, such as size-to-shape ratio or geometry of its edge also have an influence on the transport features, interaction with the cell, and the pharmacokinetics of the drug carrier (Champion et al., 2007a; Nishiyama, 2007).

2.2.1.3 Matter

For the delivery of active material or drug by the carrier, the material of the carrier has a role to play in preparation of nanotherapeutics. The chemical nature of the linker and the polymer used in the polymer–drug conjugates conveys different properties to the drug on the whole. The nanoparticles of proteins, such as serum albumins, facilitate the penetration of nanoparticles across the wall of the blood vessel. Assessment of immunotoxicity is required if proteins from other animals or mammals other than human origin are to be employed in delivery of the drug. The two important issues, biodegradability and biocompatibility, are based on the type of material used in nanotherapeutics. The material used for the development of nanoparticles or nanotherapeutics should be biocompatible and inert, because if the material is not biocompatible or inert, then it would produce some untoward effects or other side effects by reacting with other body tissues. Biodegradability is principally influenced by the structure of the material used. The biodegradable matter that is used for the manufacturing of nanotherapeutics should permit the prolonged release of drug at the target site (Kohane & Langer, 2010). Some of the polymers undergoing hydrolytic degradation are the derivatives of carboxylic acid, such as polyesters, polyanhydrides, polyphosphoesters, polyphosphazenes, polyamides, etc. Some studies have examined the role of PLGA and polylactide (PLA) nanoparticles for their prolonged release of drug (Parveen et al., 2012). Hydrolysis is influenced by the types of degradable bonds.

2.2.1.4 Charge

The Brownian motion affects the intrinsic stability and instability of the colloidal particles. Interfacial forces have an important role in maintaining the thermal stability of the suspensions and emulsions. Derjaguin, Landau, Vervy and Overbeek (DLVO) theory explained that the stability of these colloids is mainly due to the steadiness between the crucial physicochemical properties of van der Waals interactions and ionic repulsion. The charge on the surface of particle is required for the separation of diffused particles and for stability over a prolonged period of

time for storage. Nearly every particle get the charges of dissimilar magnitude on the surface in the dispersed system. The theory of DLVO is broadly applied to envisage the suspensions' stability, believing the interaction of particles takes place by van der Waals interactions and ionic repulsion forces. The sum of the repulsion probability and the attractive potential is the total potential energy of the particle, or particle interaction. The attractive energy is reliant on two factors: The size of the particle and the distance between the particles; whereas the repulsion energy is dependent upon various factors such as the size of the particle, distance between the particles, concentration of the ions, pH of the system, zeta potential, and medium dielectric constant. With the increase in the concentration of the ions in the medium, the reduction in the width of the double layer of the charges occurs, which causes the aggregation of the particles. Electrical energy at the shear plane is zeta potential, which is extensively used in predicting the constancy of the emulsion or suspension. The surfactants are used in imparting the charge stability to the surface of the particles (Devadasu et al., 2013).

2.2.1.5 Importance of surface characteristics of nanomaterial

The surface properties of the nanoparticles have a significant impact upon their prolonged stability and their plasma kinetics, as well as their interaction with the biological matter. This has an effect on attaching the ligands and loading the drugs in the nanoparticle that direct it to the target site.

For the preparation of nanoparticles by milling, the determination of surface characteristics facilitates identifying a correct stabilizer (Parsons et al., 1992). It is likely to obtain the surface Gibb's free energy, from the contact angle measurements. If there is similarity in the surface Gibb's free energy of the stabilizer to the drug's surface Gibb's free energy, then with a few small variations in the dimensions of the nanoparticles, the preparation of the drug can be done with that stabilizer (Lee et al., 2008). Surface characteristics have an important role in maintaining the stability of the particle and their interaction with the biological molecules. By changing the surface charges of the nanoparticles through replacing one functional group with another, the uptake of particles by the cells increases (He et al., 2010; Hirm et al., 2011). For examining the role of nanoparticle coating on their penetration through the mucus, it is necessary to engineer the surface of the nanoparticles. A few studies have shown that engineering a nanoparticle with neutral charge surface effectively improves their penetration through the mucus (Lai et al., 2009; Tang et al., 2009).

2.2.1.6 Agglomeration consequence

Nanoparticles' large surface area makes them thermally unsteady. Consequently, these particles have a tendency to agglomerate in a steady system, having fewer surfaces Gibb's free energy. In the nanosuspensions, agglomeration undergoes particle settlement, growth of the crystals, and variations in the dose of the drug (Wu et al., 2011). During preparation of the nanosuspensions, addition of

preservatives prevents their agglomeration/aggregation by delivering ionic and steric stability to the surface. For studying the cellular uptake of these nanoparticles by the living systems, the agglomeration, aggregation, and settlement of these particles should be considered and it could simultaneously occur in physiological media (Limbach et al., 2005). Agglomeration is the process by which the particles are bound loosely to each other by noncovalent interactions (Hackley & Ferraris, 2001). The total agglomerate surface area is equal to the sum of the surface areas of each entity of all particles that are participating. Aggregation, dissimilar to agglomeration, is that in which particles are brought together in close contact by the chemical or metallic bonds, which often results in the slightly smaller surface area in comparison to the surface areas of the individual particles that are participating. Agglomerates may be reversible, while aggregates, generally, are irreversible. Agglomeration alone can considerably modify the performance of the nanodrug-carrier complex. The results of agglomeration or aggregation of nanotherapeutics are related significantly to their toxic effects during their use. As some of the nanotherapeutics can agglomerate very rapidly in biological and culture media, it is very important to study the phenomenon of agglomeration of nanotherapeutics (Murdock et al., 2008). The concentration of the nanoparticles and their size are the deciding factors in their rate of agglomeration. Using the open flocs in the dispersion medium and mixing with steric or electrostatic stabilizers with the nanoparticles, the agglomeration of these particles can be avoided (Tam et al., 2010; Van Eerdenbrugh et al., 2008).

2.2.1.7 Approaches

There are two techniques by which nano systems can be studied or approached at micro and macro-scales because the nano systems lies between these scales. These two techniques are, “bottom-up” and “top-down”. The former technique deals with the nano systems at molecular or micro level and the latter approach deals with them at macro-level. For obtaining the particles of active pharmaceutical ingredient at nano level, the top-down approach is the principal approach compared to the other bottom-up approach. The large particles of active pharmaceutical ingredients are reduced mechanically by wet milling and homogenization at high pressures to a nano scale in the top-down approach. Presently, this approach is the most used technique to produce nanoparticles of drugs in industries at large scales. While in bottom-up techniques, the nanoparticles of drugs are produced easily, knowing the solubility of the drug in the solvents that are pharmaceutically acceptable.

One of the simple methods is to solubilize the drug in a water-based solvent and water is then added to it. This method is the hydrosol process (Gassmann et al., 1994). When the organic solution is added to the water-based solution, it changes the property of the solvent and precipitation takes place in the solution. Stabilizers are also added to the solution of nanoparticles to stop their growth in size. There are also other methods of bottom-up technique for drug nanoparticles preparation which include precipitation of the particles into aqueous solvent

through evaporation, cryopreservation through spraying into the liquid, and rapid ultra cryopreservation (Chan & Kwok, 2011; Chen et al., 2002; Hu et al., 2003; Rogers et al., 2002).

The defective surface of the produced nanoparticles is the major problem in top-down approaches. The surface-to-volume ratio of nanoparticles is very large, therefore the defects in the exterior of the particles can significantly alter the function of the nanoparticles. Whereas the nanoparticles produced by the bottom-up approaches are fabricated by effective and well-organized control of the molecules, atoms and macromolecules' arrangement (Varadan et al., 2008). The nanoparticles that are manufactured by bottom-up techniques have low Gibb's free energy which represents their equilibrium state. These nanoparticles have few surface defects and these are homogeneous with the short-range and long-range ordering. To prevent damage and defects in the nanoparticles during their production from polymers, bottom-up approach is employed, because this involves precipitation under high pressure and solubilization.

2.2.1.8 Specificity of nanodrug carriers

The benefit of using nanodrugs is that they can reach the specific site through active or passive means and conversely they minimize the toxic effects of drugs and increase their efficacy and efficiency.

Because of the small size of the liposomal formulations, they have an advantage of enhanced permeation and retention effect and thereby can accumulate at the tumor sites selectively and control the release of drug while traveling in the blood circulation and due to this effect of liposomal preparations; they have less toxicity in the body. The nanodrugs and nano delivery vehicles accumulate at the target site due to the enhanced permeation and retention effect is the method of passive targeting. Polymer–drug complex or polymer nanotherapeutics can also be targeted to the specific tumor sites by passive targeting. Various drugs cannot cross the blood brain barrier and consequently are not able to act as a brain disease therapeutic, but by making nanoformulations and doing some surface modifications, they are able to cross this barrier, localize and show its therapeutic effect, specifically at the diseased site (Clemons et al., 2002). Nanotherapeutics can also be considered for targeting diseased sites through lymphatics (Moghimi, 2003). To localize the nanoformulations extensively at the target organ or tissue, and to increase its absorption or permeation through the blood vessel walls to the target site, the nanodrug or nanotherapeutics are attached with a specific ligand, receptor or antibody. This type of drug targeting is active targeting.

In vivo, the response of a drug rarely reached the saturation response, which is a response that can be accomplished by the drug usage. Due to the toxicity to the other tissues, it is limited and is always dose-dependent. For clinical practice, the drugs have two concentration limits of plasma, which are dependent upon their response in vivo. One is the dose that is needed for achieving an assessable response, called minimum effective dose; the second

one is a dose that shows side effects and toxicity and is called the maximum tolerable dose.

For the formulation of nanodrug, the substance used in its manufacturing, size, and shape of the nanoparticle and the properties of the surface primarily decides its specificity towards the specific target site. Substances used in the nanoformulations can be responsive towards pH and temperature. They can also have other properties, such as magnetic and photo-thermal characteristics. Collectively, these properties can improve the effectiveness of the nanodelivery vehicles. For their prolonged bioavailability and targeting through passive means, the size is the most important factor. The properties of nanodrug carrier systems can also be improved by their shape, particularly by increasing its uptake by the cells to arrive at the specific cytoplasmic or nuclear targets. The surface characteristics of the nanodrug carrier can specify its particular target, so that it can accumulate at the target site specifically. All these characteristics collectively increase the specificity and affinity of the nanodrug delivery systems towards the target site. Besides internal properties of nanoparticles, external factors such as electromagnetic radiation and a magnetic field also contribute in targeting the nanotherapeutics to the specific site.

2.2.1.9 Delivery to the target of nanotherapeutics

Biological therapeutics have taken a major part of the drug market. Insulin, blood clotting factor VIII, and thyroid hormone are the first protein therapeutics that were approved for human use in the 1920s. There was an increase in the development of biological therapeutics with the introduction of molecular biology and recombinant DNA technology in 1960s. Appropriate delivery systems or carriers are required for the proficient clinical use of the biological therapeutics, as they have large size and unstable nature. There are already various biological therapeutics present in the market, which are manufactured by using the nanotechnology approaches. The most important are the polymer–protein conjugates that are involved in delivery of the biological therapeutic; many types of proteins have been already delivered successfully by using these complexes. Biological therapeutics comprise one quarter of the drugs that are in development pipelines. The first generation of biological therapeutic patents are close to expiring, and because of this there is a search for similar therapeutics raised by the pharmaceutical industry (Knezevic & Griffiths, 2011).

It is essential to retain the integrity, function, and composition of the biological therapeutic throughout its formulation. The functioning of a peptide is dependent on the side chain functional group of amino acids and also on the noncovalently bound three-dimensional structure of the proteins. Accordingly, it is not easy to encapsulate the proteins or peptides in microspheres (van der Walle and Olejnik, 2011; van der Walle et al., 2009). Some oligonucleotides also have therapeutic effects. For example, short interfering RNAs (siRNAs) have a therapeutic effect in cancers, hereditary or gene disorders, and viral diseases.

However, these RNAs also have some disadvantages, such as they cleared rapidly from the circulation, are more negatively charged, and have high molecular mass, which makes them less available at the target site. Due to these reasons, these require a carrier that can deliver them specifically to the target site, preventing other cells from its toxicity.

2.2.1.10 Pharmacokinetics with *in vitro*–*in vivo* correlation

Due to a discrepancy in shape, size, surface characteristics, and composition of materials used in production, nanoparticle-based therapeutics have different pharmacokinetic profiles, keeping in view the role of encapsulated drug in the nanoparticle. There have been considerable discrepancies observed in the behavior of nanoformulations *in vivo* when the nanodrug–carrier complex was administered via different routes. This can be characterized by the prolonged release of drug from the nanoparticles, their modified distribution to tissues, and specificity towards the target site. The primitive or conventional drug delivery carriers or systems cause the release of drug at a specific site in the body for a particular interval of time, at which the drug is absorbed; except the drug is given by the intravenous route. Whereas the nanotherapeutics specifically release the therapeutics at the target region only until their life span ends in the blood circulation.

The absorption, distribution, metabolism and excretion (ADME), are the most important components of pharmacokinetics study of any drug or therapeutic. These parameters involve the drug distribution in the tissues, its half-life in the blood circulation, and the time taken for its clearance from the body tissues. To study the different pharmacokinetic parameters of the therapeutics or drug molecule, there are different compartmental models (Li et al., 2010; Nestorov, 2003; Prokop & Davidson, 2008). The most important model that is appropriate for studying the pharmacokinetics of large number of drug molecules is physiologically based pharmacokinetic (PBPK) modeling. However, these pharmacokinetic models are not suitable for studying the distribution of nanoparticle therapeutics because the nanoparticles have sustained absorption stages, which causes their extended release in the cell or tissue compartments, resulting in their accumulation in tissues in particular quantities and different mechanisms of excretion. For a drug, its low concentration in the blood circulation corresponds to the reduced toxicity of that drug, but nanodrug delivery vehicles contradict this fact, because they are safe at high concentrations, and due to this their serum pharmacokinetics cannot be explained (Heinemann et al., 1997). To assess the pharmacokinetics of nanotherapeutics, there is a need to ascertain some *in vitro* techniques that can easily and quickly evaluate their stability, quality, function, and performance. *In vivo*–*in vitro* correlation defines the building of a connection between the properties attributed *in vitro* and the same drug response shown *in vivo*. For oral

formulation development and the quality control of nano-based therapeutics, it is necessary to assess the *in vitro*–*in vivo* correlation of the rates of dissolution and absorption of these therapeutics. These correlation studies could also be provided as a substitute for biological equivalent studies, for making formulations that can save money and time in the process of drug development. Most of the successful *in vitro*–*in vivo* correlations are linear. Non linear *in vitro*–*in vivo* correlations, also known as *in vitro*–*in vivo* relationships, are also very useful in drug development (Polli et al., 1996).

2.2.2 REALISTIC CONCERNS WITH NANODRUG DELIVERY

In medical industries, nanotechnology develops the means by which drugs are administered in the body and thereby revolutionizes healthcare. Administering the medicines by various unconventional routes, e.g., insulin delivery through oral route, inhalation, intradermal injection, etc., is difficult and all of these are not even possible. However, this has not made any considerable difference and benefit to the patient. Utilization of nanotechnology in the industries is done by refining the hydrophobic compounds/drugs to increase their solubility, by decreasing their size and thereby their surface area-to-volume ratio. However, this is confined to a few selected compounds and, conversely, increasing their solubility and ADME properties would also increase the toxicity of many compounds. Consequently, polymers and lipids are being used to encapsulate the hydrophobic drugs in the nanoparticles that emphasize improving the ADME properties or bioavailability of the drug and lessening the toxicity of that compound. This has caused the production of first generation nanoparticles.

There are some studies indicate that nanotechnology does not yield all desirable efficacies. On the other perspective, these generalize that nano is toxic, which give the origin to “nanotoxicology”. There are some essential steps for obtaining the drug-loaded nanoparticles using preformed polymers. These are mixing of two solvents, decreasing the size of drug/polymer, dissipating the organic solvents and lyophilization of drug-carrier complex. The formulation of a drug is a complex procedure that depends on many factors. Such as in conventional drug delivery each ingredient has its specific role to play. The selection of these ingredients depends on drug dose, route and physicochemical properties of the ingredients. This results into a complex process. Conversely, the recent delivery methods including nano-based carriers are being synthesized with more prominence on the chemical nature of the delivery vehicle rather than much limited knowledge of the medicine or drug and the treatment of the disease. Therefore, it is necessary to know about the methods that can improve the delivery of the drug to the target through various delivery systems.

2.3 EVALUATION OF NANOTECHNOLOGY FOR INDUSTRIAL APPLICATIONS

In recent years, the swift advances in nanotechnology and nanoscience have opened up various scenarios for several industrial and consumer areas that can be considered as a new industrial revolution.

Nanotechnology, in the context of food-related applications, offers several advantages to consumers (Chaudhry & Castle, 2011). Some of the food-related applications of nanotechnology includes:

1. Processed nano-structured or nano-textured food products: This application includes the production of food having better taste, with less and stable fats and emulsifiers. Some examples may include mayonnaise, ice-cream, etc. This type of processing also kills microbial pathogens. The limitation of this type of application is that these food nano-structures are prone to be assimilated in the gastrointestinal tract and therefore unable to take materials to the bloodstream.
2. Delivery of nutrients and supplements as liposomes or biopolymer-based nano-encapsulated substances: This type of application includes the masking of taste of various food ingredients (Chaudhry & Castle, 2011; Khosravi-Rashidi & Darani, 2011) such as fish oils, processing and improving certain ingredients, increased physiological availability of healthy nourishment and supplements, improved antimicrobial effect and alternative health advantage. The increased availability of supplements and other additives in living systems can alter the delivery of substances to the tissues and in turn alters its pharmacokinetics.
3. Organic nano-sized additives for food applications: Due to its organic nature, no additional fats or emulsifiers are needed to enhance the taste. Because of the large surface area and organic nature, they are required in lesser quantities and have higher absorption and improved bioavailability.
4. Inorganic nano-sized additives: In addition to the benefits provided like those of organic nano-sized additives, they also have increased antimicrobial activity. However, this type of additive may contain insoluble, indigestible, and biopersistent nanoparticles.
5. Plastic polymers containing nanomaterials: This improves the packaging and temperature/moisture stability of food stuff and it also prevents microbial growth (Chaudhry & Castle, 2011; Khosravi-Rashidi & Darani, 2011). However, this can pose potential consumer exposure to nanoparticles and their ingestion.
6. Nanofiltration: This includes water purification and elimination of unwanted constituents in food stuff, with the use of porous silica and regenerated cellulose membranes. Agglomerated colloidal silica is used to clarify beers and wines, but these nanoparticles may end up in filtered products and pose health risks.

7. Nano-sized agrochemicals: These are used in the production of fertilizers, pesticides, biocides, etc., for better efficacy; their increased delivery in the field with less use of solvents. This can pose health risks to people working with hazardous substances.
8. Food labeling nanosensors: This type of application involved in food packaging can monitor food conditions during transportation and storage. This kind of packaging will release preservatives only when needed.

All these applications lowered the use of fats, emulsifiers, preservatives, etc. in food products and led to improved taste and flavor of food (Chaudhary & Castle, 2011).

Nanotechnology has various advantages, but this exciting technological progress may also be associated with risks. The small size of engineered nanomaterials can have major toxicological consequences. They could enter the human cells (Piccinno et al., 2012). There is a concern about the risks posed by engineered nanomaterials that may contaminate the environment and consequently the population. The information about the toxicity and biokinetics of nanoproducts is necessary for production of nanomedicines. Toxicology of nano-engineered drugs is necessary for evaluation of human beings and atmospheric exposure, pharmacokinetics, detection of effective hazards, and persistence or accumulation of nanomaterials in cells and subcellular structures to demonstrate a hazard assessment (Oberdorster, 2009).

2.3.1 RESEARCH PIPELINE

This section describes the potential nanotherapeutics currently in progress. The development part involves the use of polymers, lipids, and delivery of drugs that is done by inorganic materials.

2.3.1.1 Polymeric nanoparticles and nanocapsules

For the production of nanotherapeutics, polymeric nanoparticles are a largely used group that delivers the molecules to their target. They are biodegradable and prepared by processes such as solvent displacement, emulsion-diffusion, dialysis, salting out and super critical fluid technology (Bhardwaj et al., 2005; Heurtas et al., 2011; Rao & Geckeler, 2011). These techniques are not validated and accuracy in achieving the perfect characteristics of the particle is doubtful, as the nanoparticles obtained by preparing these methods often have wide size distribution ranges and some of the nanoparticles may be lost during the lyophilization. This process of concentrating nanoparticles is time-consuming and bulky (Devadasu et al., 2013). Polymer nanoparticles can also be obtained through polymerization of monomers.

These methods produce spherical-shaped nanoparticles. But now, significance of other shapes is also increasing. The methods used for manufacturing of nanoparticles include the photolithography, nonwetting template molding,

self-assembly, elongation of globular particles, etc. These techniques were used for nanoparticle widespread manufacturing and obtaining accuracy in shape (Champion et al., 2007b). A stabilizer is used in preparation of these nanoparticles. These stabilizers encapsulate the nanoparticles and also provide an electrostatic and steric pacification to block agglomeration.

Drugs are loaded in the nanoparticles by either entrapment or adsorption, and drugs are released from these nanoparticles through destruction of polymer and removal from the surface, where it is absorbed and dispersed (Bhardwaj et al., 2005). Generally, anticancer agents are encapsulated in nanoparticles to make its delivery to the target organ or cell. This type of drug delivery system leads to life expectancy of the patient. Preclinical studies have shown promising effects, but clinical studies are yet to be performed for these nanodrug delivery systems (Vauthier & Bouchemal, 2009).

Nanocapsules display the vesicle-like structure having a core-shell. Drugs are loaded in the core and shell is formed of solid material (Mora-Huertas et al., 2010). Nanocapsules can be either hydrophilic or lipophilic and act as a drug reservoir.

2.3.1.2 Metal and inorganic nanoparticles

Several metal and inorganic element-based nanomaterials such as gold, silver, silica, and carbon have been researched for medical resolution. Some nanoparticles are widely used as nanomedicine, for example, gold nanoparticles are used due to their distinctive optics. However, they have some side-effects, such as hyperthermia shown in preclinical studies and clinical studies are underway (Kennedy et al., 2011). Magnetic materials like iron oxide can be used for preparation of nanoparticles that generally entrap or adsorb the drug. They should be nontoxic, biocompatible, nonimmunogenic, and hydrophilic. The advantages of using magnetic material for preparing nanoparticles are facile diagnosis, attractive manipulation and transfer of energy. Body tissues are nonmagnetic, therefore it is very easy to detect the magnetic nanoparticles by creating a magnetic field and dragging the nanoparticles to the site of interest (Varadan et al., 2008). Its application is to use them in anticancer therapy to destroy cancer cells at the tumor site by inducing hyperthermia. Size optimization and surface modification of these nanoparticles are necessary to target the specific organ or tissue. The material used for preparation should be nontoxic and biocompatible.

2.3.1.3 Self-nanoemulsifying drug delivery systems (SNEDDS)

SNEDDS are formed by the mixture of oil, surfactant, and co-solvent with solubilized drug. This mixture forms the microemulsion by its dilution with aqueous medium, causing the formation of droplets in the nanoscale range. These are called nanoemulsions (Shah et al., 2010). This type of drug delivery system was launched to advance hydrophobic drugs oral delivery.

2.3.1.4 Solid lipid nanoparticles and nanostructured lipid carriers (SLN and NLC)

Solid lipid nanoparticles are used to transport inefficient hydrophobic drugs as they are composed of solid lipids, glycerides, waxes, fatty acids, and fixed by surface active agents. They have major advantages over various such as routes of administration. Their suitability in optimizing drug loading and releasing profiles, stability, low toxicity, protection of loaded labile drugs, and target specificity are some main features of SLNs (Fangueiro et al., 2013; Souto et al., 2013).

Nanostructured lipid carriers are second-generation lipid nanoparticles, composed of biocompatible solid lipid and liquid lipid. Biocompatibility and controlled drug release are some advantages of NLCs (Obeidat et al., 2010; Zhuang et al., 2010).

2.3.1.5 Protein vaults

Protein vaults are composed of ribonucleoprotein complexes in the sub-100 nm range, expressed almost ubiquitously in eukaryotes and are highly conserved. These vaults can form a barrel-shaped nanocapsule, making a hollow interior. These protein cages, such as virus capsids and ferritin, can serve as a template for drug delivery vehicles (Buehler et al., 2011; Matsumoto et al., 2013). Protein vaults are used as drug carriers and manufactured by recombinant technology (Buehler et al., 2014). They can be made to sheath and deliver the drug at target location (Kickhoefer et al., 2009; Yu et al., 2008). These vault nanoparticles can be used to deliver vaccines (Champion et al., 2009).

2.3.1.6 Dendrimers

Dendrimers are macromolecules with highly branched 3-D structures, with three components: core, interior, and shell (Mintzer & Grinstaff, 2011). They have a size in the nano-scale range and consequently can be used to encapsulate the drug molecules and function as a drug carrier. Due to their unique surface functionalities, they can be suited as a nano-scaffold for the targeted delivery of drugs (Svenson & Tomalia, 2012). One of the most important benefits of dendrimers is that they can be cleared through renal route because of their small size (Gillies & Frechet, 2005). One limitation of using them is their toxicity. They can shear the lipid bilayer membrane from cells and wrap it around itself (Roco, 2005).

2.3.1.7 Polymeric micelles and polymerosomes

Polymeric micelles were first introduced by Kataoka's group in the early 1990s, for the delivery of anticancer drug, doxorubicin (Kedar et al., 2010). Core and shell are the two main parts of polymeric micelles; the core is water repellent, encapsulates the lipophilic drugs, controls their stability and release of drug, while the shell is hydrophilic and involved in in vivo pharmacokinetic behavior of micelles (Gong et al., 2012; Kedar et al., 2010). Due to both the properties of hydrophobicity and hydrophilicity, the polymeric micelles are amphiphilic. They

assemble to form micelles within the 5–100 nm size range. The selection of polymers for the formation of polymeric micelles is based on their hydrophobicity and hydrophilicity (Oerlemans et al., 2010). The hydrophilic portion of the delivery vehicle generally consists of Poly(ethylene glycol), poly (ethylene oxide), poly (vinyl pyrrolidone), etc. These blocks can be arranged in diblock and triblock polymers (Kedar et al., 2010; Miyata et al., 2011; Oerlemans et al., 2010). The polymers used for the formation of hydrophobic core are polyamino acids such as polyglutamate and polyaspartate, polyesters such as poly (glycolic acid), poly (lactic acid), etc. (Kedar et al., 2010; Oerlemans et al., 2010). Micellar stability persists even below the critical micelle concentration (CMC) (Oerlemans et al., 2010).

Polymersomes are also nano-range amphiphilic vesicles made up of diblock copolymers. They are composed of a bilayer membrane making the both outer shell and inner core hydrophilic to encapsulate the hydrophilic drugs, therapeutic molecules such as DNA and RNA, enzymes, peptides, etc. (Lee & Feijen, 2012; Letchford & Burt, 2007; Shum et al., 2011). They are more stable and have prolonged circulation times, and they can be targeted to the specific organ or tissue by incorporating targeting moieties in the bilayer membrane of polymersomes (Lee & Feijen, 2012). They can be made responsive to pH, temperature, ionic strength, etc. by using various block copolymers that can escape the lysosomal enzymatic digestion (Lee & Feijen, 2012; Lomas et al., 2007).

2.3.2 CLINICAL TRIALS

Adagen (Sigma-Tau Pharmaceuticals, Inc., MD), a PEG-based bovine adenosine deaminase was the first approved nanotherapeutic in the 1990s. Currently, 40 nanomedicines have been approved worldwide and more than 200 nanomedicines are under different phases of clinical trials (Devadasu et al., 2013; Schutz et al., 2013). Recent studies have shown that 20 to 30% of Phase I drugs have been profitably passed from Phase II to Phase III and 40 to 50 % of drugs are actually marketed. This is because of their toxicity problems. About 17 nanotherapeutics are in Phase III clinical trials and about 100 are in Phase I or II of clinical trials (Schutz, 2013). The nanomedicines under clinical trials are shown in Table 2.2.

2.3.3 DEVELOPMENTAL QUESTIONS

This section describes how the emerging concepts, funding, human resources, and recognition help in driving the development of nanotechnology (Roco, 2008). With the success of marketed nanotherapeutics, industrial development of nanomedicine is just getting started, but there is an ambiguity in implying the new strategies for development of nanomedicine (Devadasu et al., 2013). Nanosizing therapeutics is more important, compared to their preparation. During development of product, the suitable selection of raw materials and method play a vital role (Sahana et al., 2008). For reducing the interfacial tension and stabilization of

Table 2.2 Nanotherapeutics in Different Phases of Clinical Trials

S. No.	Drug Name	Therapeutic	Nanomaterial	Company	Clinical Trial Phases	References
1.	ABI-009, ABI-010, ABI-011	Rapamycin, 17-AAG, Thiocolchicine dimer	Albumin nanoparticles	Celgene	Phase I	Desai (2007-2008)
2.	ANX-514	Docetaxel	Liposome	Adventrx	Phase I	Curran & McCormack (2008)
3.	CALAA-01	siRNA targeting the M2 subunit of ribonucleotide reductase	Polymer nanoparticles	Calando Pharmaceuticals	Phase I	Eifler & Thaxton (2011)
4.	Docetaxel-PM™	Docetaxel	Polymer micelle	Samyang Co.	Phase I	Simon (2006)
5.	MAG-CPT	Camptothecin	Polymer-drug conjugate	Pharmacia & Upjohn Inc.	Phase I	Heidel & Davis (2011)
6.	ABI-008	Docetaxel	Albumin nanoparticles	Celgene	Phase II	Desai (2007, 2008)
7.	AMG 223	Phosphate-binding polymer	Polymer-polymeric drug	Amgen	Phase II	Zamboni (2008)
8.	AP5280	Carboplatine palatinatate	Polymer-drug conjugate	Access Pharmaceutical	Phase II	Rademaker et al. (2004)
9.	Aurimmune	TNF, recombinant human TNF- α	Gold nanoparticles	CytImmune Sciences	Phase II	Stern et al. (2010)
10.	ADI-PEG 20	Arginine deiminase	Polymer-protein conjugate	Polaris Group-Phoenix pharmacologics	Phase III	Shen & Shen (2006)
11.	Clariscan™	MRI diagnostic	Superparamagnetic iron oxide nanoparticle (SPION)	GE Healthcare	Phase III	Vogel et al. (2005)

(Continued)

Table 2.2 Nanotherapeutics in Different Phases of Clinical Trials *Continued*

S. No.	Drug Name	Therapeutic	Nanomaterial	Company	Clinical Trial Phases	References
12.	Lipoplatin	Cisplatin	Liposome	Regulon	Phase III	Stathopoulos & Boulikas (2012)
13.	PEG-IFN	IFN- β -1a	Polymer-protein conjugate	Biogen	Phase III	Zamboni (2008)
14.	Taxoprexin	Paclitaxel	Polymer-drug conjugate	Luitpold Pharmaceuticals	Phase III	Zamboni (2008)
15.	SH L 643 A	Gadolinium	Dendrimer	Bayer Schering Pharma AG	Phase III	Herborn et al. (2003)
16.	Trastuzumab	Mertansine (DM1)	Antibody-drug conjugate	Genentech	Phase III	Mathew & Perez (2011)

the system, the selection of solvent has a considerable effect on surfactant selection because compatibility of solvent and surfactant with the drug and the delivery system is important (Mittal et al., 2007; Polli et al., 1996). Characterization of the nanotherapeutics size is an important requirement to call it a nanotherapeutic. An accepted technique called dynamic light scattering (DLS) accurately measures the hydrodynamic diameter of the nanoparticles. It is important to know the viscosity of the medium and temperature, as they influence the particle size measurement. Transmission electron microscopy and energy dispersive X-ray, coupled with scanning electron microscopy reveals the distribution of nanoparticles inside the tissues. Atomic force microscopy (AFM) displays the three-dimensional surface outline of the nanoparticles, and AFM and electron microscopy also determines the size. Zeta potential suggests the stability of the nanoparticles in the solution (Muller et al., 2001).

Some analytical methods are used for the characterization of nanotherapeutics with complex multiple functions (Adams, 2011). Matrix assisted laser desorption/ionization-Time of flight (MALDI-TOF) is used in the characterization of dendrimers.

Efforts are being made to come up with different regulatory methodologies to evaluate the nanoparticles' safety as there is an interference with the in vitro assays used to determine their safety. Due to their optical and catalytical properties, they have unreliable reactivity in in vitro assays. Nanotechnology characterization laboratory (NCL), USA has set up a goal to develop the tests that accurately assess the safety and efficacy of nanoparticles (McNeil, 2009).

For systemic administration of nanoparticles, they need to be tested for complement activation through polyacrylamide gel electrophoresis, Western blotting and immunohistochemistry (Dobrovolskaia et al., 2008; Harboe et al., 2011). Doxil and Ambisone, the marketed liposomal preparations activate the complement system, leading to hypersensitivity reactions (Chanan-Khan et al., 2003; Szebeni et al., 2012). In effective vaccine delivery by subcutaneous and intradermal route, complement activation is required (Dobrovolskaia et al., 2008; Reddy et al., 2007). Nanotechnology involves all areas of science and demands huge investments.

2.3.4 ASSESSMENT OF MARKETING PRODUCTS AND TECHNOLOGIES

Various nanotherapeutics made up of polymer, liposomes, vaccines, micelles, nano-sized therapeutics, and protein-based nanoparticles have been approved and already marketed. Some of the marketed nanotherapeutics are shown in Table 2.3.

There are various types of nanotherapeutics present in the market and pipeline (Devadasu et al., 2013) are as follows:

1. Dendrimers: branched nanoparticles;
2. Liposomes: vesicles formed of lipids to encapsulate drugs;
3. Micelles: spherical monolayered arrangement of amphiphilic molecules;
4. Nanocrystals: Drug crystals;

Table 2.3 Approved Nanotherapeutics Available in Market

S. No.	Product	Nanomaterial	Company	Approval Year	Therapeutic Use	References
1.	Adagen [®]	Polymer-protein conjugate	Sigma-Tau Pharmaceuticals	1990	Immunodeficiency	Vicent et al. (2009)
2.	Taxol [®]	Polymer micelle	Bristol Myers Squibb	1992	Chemotherapy	Heidel & Davis (2011)
3.	Endorem [®]	SPION	Amag Pharmaceuticals	1996	MRI diagnostic	Jung (1995)
4.	Inflexal [®] V	Virosome	Crucell	1997	Vaccine	Herzog et al. (2009)
5.	Depocyt [®]	Liposome	Sigma Pharmaceuticals	1999	Chemotherapy	Glantz et al. (1999)
6.	Rapamune [®]	Nanocrystal	Pfizer	1999	Antibiotic	Junghanns & Muller (2008)
7.	Welchol [®]	Polymer-polymeric drug	Daiichi Sankyo Co. Ltd	2000	Antidiabetic	Goldfine et al. (2010)
8.	Depodur [®]	Liposome	EKR Therapeutics	2004	Analgesic	Howell (2001)
9.	Micera [®]	Polymer-protein conjugate	Hoffman-LaRoche	2007	Anemia	Curran & McCormack (2008)
10.	Cimzla [®]	Polymer-protein conjugate	Nektar	2008	Rheumatoid arthritis	Mease (2011)
11.	Feraheme [®]	SPION	Amag Pharmaceuticals	2009	Anemia	Singh et al. (2008)
12.	Marqibo [®]	Liposome	Thalon Therapeutics	2012	Acute lymphoblastic Leukemia (ALL)	Rodriguez et al. (2009)

5. Polymer–drug conjugates: Drug bound to polymer/peptide/antibody by covalent interactions;
6. Protein-based nanoparticles: Drug bound to proteins.

2.3.5 TECHNIQUES FOR FURTHER DEVELOPMENT OF NANODRUG DELIVERY

Nanotechnology brings many improvements in treatment and diagnosis. It plays an important role in analytics and diagnostics, the production of active pharmaceutical ingredients, local transport of active substances, and production of biocompatible materials and surfaces. At present, nanotechnology-based products are used in packaging, nutrition, color, and additives. In future, nanotechnology is going to play important role in the field of bioactive substances. Nanotechnology is playing an important role in cosmetics, e.g., in sunscreen lotions with improved properties (Singh & Sharma, 2016). One of the important aspects in which the nanotechnology will play an important role is in sensors used in analytical experiments to obtain reliable measurements, for example on microtiter plates. In the field of the medicinal products, nanotechnological process are used to formulate medications, and in nanomedicine therapy; e.g., in the formulation of abraxane (chemotherapeutic agent), in which aluminum nanoparticles are used as drug delivery systems (Ernsting et al., 2012). Catalytic applications of nanomaterial are also a big step for future scientific research, in terms of green technology and sustainable environment (Sharma et al., 2015). Using harmonized standards and giving access to manufacturers, distributors, patients, and physicians to nanotechnological advances are also an important aspect of this field. The use of nanotechnology in emergency pharmaceutical products opens up new possibilities for decontamination, wound-healing, and radioprotector formulations (Pathak et al., 2016; Rana et al., 2014).

Nanoscale-based sensors will be used in the field of the analysis, identification, and destruction of the bacterial pathogens (Verma et al., 2016). Furthermore, nanotechnology-based powders of active pharmaceutical ingredients will be used for better drug delivery and improved bioavailability. In the field of diagnostics, nanotechnology play an important role; various chemotherapeutic agents are been replaced by nanotherapeutics, i.e., chemotherapy agents with pioneering nanoparticle reformulation. Changes in the landscape of medicines by the addition of new approaches in nanotechnology-related drug delivery are starting from diagnosis and drug development, in the case of patent and intellectual property rights. Drug delivery is the source which reproduces considerable outcomes. There are several examples of site directed drug delivery in the form of dendrimers, liposomes, nanoshells etc available which has demonstrated their potential as suitable effective drug carriers. The development of productive feasible nanomedicine by-products that can effectively improve a patient's quality of life and lessen the costs of health care which is controlled by the durable patent system. It

is important to draft the guidelines, regulations and protocols for the production of the nanotechnology-based pharmaceutical products.

Nanomaterials contrast fundamentally from different materials because of the accompanying two noteworthy central components: the expanded surface region and quantum impacts. These elements can improve properties, for example, reactivity, quality, and in vivo conduct. Nanotechnology and nanoscience are broadly observed as having an extraordinary potential to convey advantages to numerous zones of research and applications. The use of nanotechnology in the field of human services has gone under significant consideration. There are numerous medications today that take a great deal of time and are also extremely costly. In utilizing nanotechnology, much less expensive medication can be produced. There is another perspective for utilizing nanotechnology: ordinarily, drugs work through the whole body before they achieve the influenced region. Utilizing nanotechnology, the medication can be focused to an exact area which would make the medication a great deal more powerful and decrease the odds of conceivable symptoms.

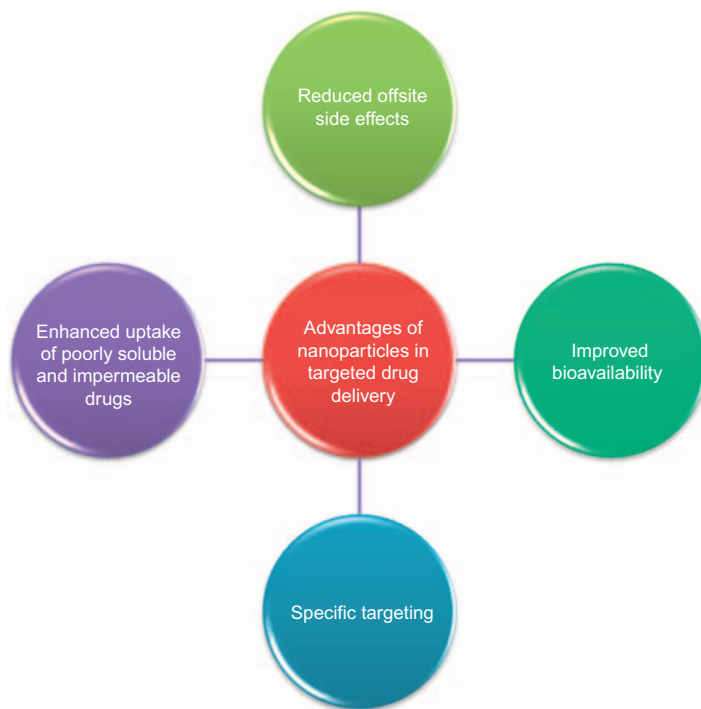
A perfect medication conveyance framework has two components: the capacity to target and to control the medication discharge. Focusing on these two components will guarantee a high proficiency of the medication and lessen the reactions, particularly when managing drugs that are intended to act against malignant cells. The lessening or aversion of symptoms can likewise be accomplished by controlled discharge. Nanoparticle drug delivery systems (NPDDS) achieve superior entry for the particles inside the body, as their size permits conveyance by means of intravenous infusion or different courses. Another important use of the NPDDS is in the production of the antibodies based specific proteins against the malignant cells.

Nanoparticles can be used in targeted drug delivery at the site of disease to improve biodistribution (Fig. 2.2).

2.3.6 NANOTOXICOLOGY

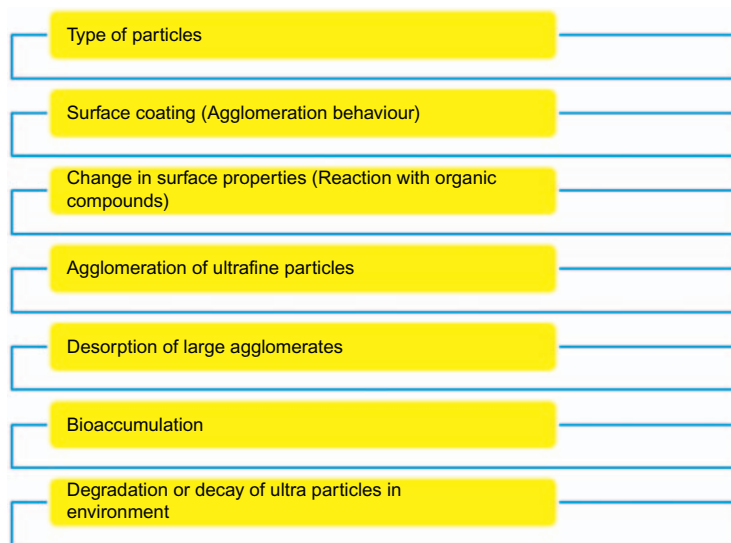
Nanoscale particles show properties which are significantly different from analogous bulk phases, which swiftly established a nanotechnology field. Due to this datum, the environmental nanotoxicology remains very much dependent on mass-based dosemetrics. The duration and amount of human exposure to nanomaterials are hardly available. For evaluation, dosemetrics must be considered all together to see if nanoscale particles produce toxicological effects in contrast of bulk phases. Some studies claim that the nanoparticles are harmless for medical purpose (Alkilany & Murphy, 2010).

Hull et al. has stated the importance of characterization information for correct assessment of toxicity of engineered nanomaterials. In the same work, 25 representatives studies were collected over a period of one year. It was observed that most of studies showed toxicity in terms of mass rather than mass dosimetry basis. eventhough 20% of these studies consist of sufficient characterization information to obtain reasonable mass dosimetric base estimation of toxicity of engineered nanomaterials. Conversion of mass concentration needs information about

**FIGURE 2.2**

Advantage of nanoparticle drug carriers in improving the drug delivery.

the size, shape, and crystallinity of the particle and these are reported as 60%, 48% and 20%, respectively (Hull et al., 2012). Toxicological response was measured by the characterization, which was performed in the experimental media. Only 8% of the studies reported that information, and no one reported the presence of the fraction of soluble ions. Production of nanomaterials at industry level contributes to the number of particles in the outside air, with respect to other sources of ultrafine dust. Exposure to nanoparticles through the air affects all organisms in the environment. In the preparation and use of synthetic nanoparticles, these can be released into the air and directly inhaled. This is the predominant exposure path. Accordingly, inhalation exposure is a major risk path for the nanotoxicology to humans. So far, assessment data of this exposure are not available, either for ultra-fine dust particles or for synthetic nanomaterials. The release of synthetic nano-objects from nanotechnological products is also not studied. However, it is expected that, in the near future, individual data on the exposure of the environmental components, such as air and water, will be seen. There are multiple factors that have individual consequences in the environmental media (Fig. 2.3) (Krug, 2005).

**FIGURE 2.3**

Representation of factors that have influence in environment media.

There is lack of reliable data on the concentrations of ultrafine objects in environmental media. It is essential to detect ultrafine particles in the air in the environment and its toxicological effect on the human body. There are various research approaches to rule out the possible exposure of the nanoparticles to human beings. Here is an example: Karlsruhe Institute of Technology (KIT, formerly a research center) developed an exposure apparatus in which cell cultures are exposed to a particle-laden test atmosphere (Krug et al., 2007; Paur et al., 2008). The test atmosphere simulates the tempered and moistened human lungs on which the particles in the test aerosol are being measured. On the basis of the cell culture parameters, such as oxidative stress, inflammatory mediators, and cell death. Possible absorption of the nanoparticles through food is studied by Bund. It has been observed in research worldwide that no binding safety standards and tests for nano products exist (Bund, 2008). This fact is also supported by a study from the Swiss Center for Technology Assessment (TA-Swiss) (Möller et al., 2009), although pharmaceutical packaging material can be watched and monitored. In a recent report, the National Institute for Health and environment of the Netherlands accessed and compiles the exposure of nanomaterials in consumer products (RIVM, 2009). It is reported that sunscreens, adhesives, and care and cleaning products have serious consequences if used in nano form. With regard to the application of nanotechnological products on 24 March 2009, the European Parliament adopted a new cosmetic regulation (EU, 2009). In it for the first time, the use of nanomaterials is introduced. The labeling of nanoparticles is mandatory, and a procedure for assessing risks even before admission is required.

Manufacturers must inform the EU authority about the product with nanoparticles in the market. Exposure to nanomaterials can occur over environmental (air, water, soil) as well as on pharmaceutical products. During the manufacturing of synthetic nanomaterials, they are released into the air and directly inhaled. Inhalation of the nanoparticles is primary exposure path causing the toxicity. So far there are no standardized measuring methods for concentration determination, and there is also a lack of reliable data on the concentration of nano objects in environmental media. For the use of nanoproducts in the pharmaceutical sector there are no safety standards available worldwide. Recently EU introduced the new regulations for labeling and risk assessment of nanomaterials in cosmetics.

There are also several factors that induce cellular toxicity by the substance used in its fabrication and also by various unclear mechanisms (Favi et al., 2015). The size has an important role in generating cytotoxicity. For this, the particle has to cross the epithelial layer of skin (Bellmann et al., 2015; Lefebvre et al., 2015). By inhalation, it has to reach the parenchyma of cell and convert into liquid phase with the fluids of respiratory tract (Gliga et al., 2014; Qiao et al., 2015). Silver nanoparticles with different sizes also produce cytotoxicity when inhaled (Braakhuis et al., 2015). For evaluating the cytotoxicity, pharmacokinetics must be considered. Many research groups have investigated the distribution of nanoparticles in vivo and concluded that the smaller sized nanoparticles cannot be removed through the kidneys and accumulated in the organs such as liver and spleen until the mononuclear engulfing system comes into action (Albanese et al., 2012; Varna et al., 2012).

With respect to the nanoparticles' size, its interaction with the cell and thereby its uptake and effectiveness are also important factors in determining the cellular toxicity (Kou et al., 2013; Zhao et al., 2011). Several studies have examined various cellular uptake pathways for the internalization of nanoparticles. Polyvinyl-coated silver nanoparticles, polystyrene nanoparticles, silica-coated iron nanoparticles, gold nanoparticles, and gold nanoparticles conjugated with bonds in peptide are internalized through clathrin-mediated endocytosis (Bohmer & Jordan, 2015; Greulich et al., 2011; Jiang et al., 2010; Klingberg et al., 2015; Suresh et al., 2014). Gold nanoparticles are also internalized by cells through caveolae-mediated phagocytosis and other nanoparticles, such as silica nanoparticles, silver nanoparticles and polyethyleneglycol–polycaprolactone nanoparticles conjugated with IL-13 peptide, are internalized through pinocytosis (Gao et al., 2014; Meng et al., 2011; Trefry & Wooley, 2013; Wang et al., 2010). Due to their small size, the nanoparticles have large surface area that is responsible for low melting point and high reactivity with cells, which leads to inflammation of the target tissue, causing cytotoxicity (Suttiponparnit et al., 2010). The rate of aggregate formation of nanoparticles depending upon its surface characteristics also determines the cytotoxicity (Raboli et al., 2011). Biocompatibility is also an important factor in determining the nanoparticle uptake (Verma & Stellacci, 2010; Wang et al., 2012). To evaluate the toxicity of nanoparticles, they should be tested by various methods on ex vivo conditions that mimic the cell environment before proceeding to in vivo studies (Albanese et al., 2013; Esch et al., 2011; Valencia et al., 2012).

Changes in the surface charges of the nanoparticles also disturbed the uptake of these particles by the cell, which causes high level of toxicity in the cell (Asati et al., 2010; Frohlich, 2012; Schaeublin et al., 2011).

In nanotoxicology, the morphology of the particles is also involved. A study has reported the toxic effect of carbon nanotubes on lungs, causing their inflammation (Firme & Bandaru, 2010). The agglomeration factor of nanoparticles can also induce lung cells' inflammation in humans and consequently be responsible for lung cancer (Bantz et al., 2014). For the safety of nanoparticles in living systems, the physicochemical properties and toxicity of the nanoparticles should be determined at the cellular, tissue, organ, and organism level.

2.4 CONCLUSION

Nanotechnology is an upcoming technology which has been investigated for different applications, ranging from material science to life science. Although various nanoparticle-based products are available in the market, limited knowledge is available in literature regarding toxicology and biodistribution of nanoparticles. Therefore, this chapter has highlighted various factors that influence applications, pharmacokinetics and nanotoxicology of nanoparticles.

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Nanotechnology applications in drug controlled release

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CHAPTER OUTLINE

3.1 Introduction	82
3.1.1 Nanotechnology in Drug Delivery.....	84
3.1.2 Targets for Drug Delivery	86
3.1.3 Advantages of Nanoparticles.....	87
3.1.4 Drug Release From Nanoparticles	88
3.2 Nanotechnology-Based Drug Delivery Systems	89
3.2.1 Fullerenes.....	89
3.2.2 Solid Lipid Nanoparticles	91
3.2.3 Routes of Administration of Solid Lipid Nanoparticles	93
3.2.4 Liposomes	94
3.2.5 Microemulsions.....	95
3.2.6 Nanoemulsions	96
3.2.7 Dendrimers	97
3.2.8 Carbon Nanomaterials.....	98
3.2.9 Magnetic Nanoparticles	98
3.3 Nanoparticles Preparation Methods.....	99
3.3.1 Solvent Evaporation Method	99
3.3.2 Polymerization Method	100
3.3.3 Coacervation or Ionic Gelation Method	100
3.3.4 Spontaneous Emulsification or Solvent Diffusion Method	101
3.3.5 Double Emulsion and Evaporation Method	101
3.3.6 Salting Out Method	101
3.3.7 Precipitation Method	102
3.3.8 Miniemulsion	102
3.3.9 Microemulsion	103

3.3.10 Interfacial Polymerization.....	104
3.3.11 Controlled/Living Radical Polymerization	105
3.3.12 Desolvation	105
3.3.13 Supercritical Fluid Technology.....	106
3.3.14 Spray-Drying	106
3.4 Limitations/Toxicology of Nanomedicines	106
3.5 Future Trends for Nanomaterials in Drug Delivery.....	107
References	108

3.1 INTRODUCTION

Nanotechnology is the manipulation of matter, involving physical and chemical transformations, in order to produce materials at the nanoscale. The word “nano,” which derives from Latin, signifies “dwarf” and mathematically is the billionth part of a meter. Nanotechnology comprises the study, design, creation, synthesis, characterization, manipulation and application of materials, apparatus and systems through the control of material structure at the nanoscale, and the exploitation of phenomena and properties of matter at atomic or molecular scale. When manipulating matter at nanoscale, the material properties change. For example, quantum effects have dominance, the surface area increases, and thermal, electrical, and magnetic property of the material varies. Nanotechnology is emerging as a field in medicine that is expected to elicit significant therapeutic benefits to improve drugs bioavailability.

Drug delivery research is clearly moving from the micro to the nanoscale. An ideal drug-delivery system must be able to target and control the drug release. Targeting will ensure high efficiency of the drug and reduce the side effects, especially when dealing with drugs that are presumed to kill cancer cells but can also kill healthy cells when delivered to them. The reduction or prevention of side effects can also be achieved by controlled release. The formulation of nanosized systems for the delivery of drugs is very attractive to scientists for different reasons, of which one of the most important is that there is an increase in the ratio between the number of atoms or molecules on the surface and the total number of atoms or molecules. Accordingly, the surface area increases, helping to bind, adsorb or carry compounds such as drugs, probes, and proteins.

Moreover, the drug particles themselves can be designed to form nanoscale-sized materials (Hadjipanayis et al., 2010). Nanosized devices can eventually go much further, and access inaccessible areas, such as inflamed tissues or cancer cells, because they have increased permeability and retention effect and can damage lymphatic drainage. Accordingly, they can be used for genes and proteins administration through the oral route of administration (De Jong and Borm, 2008).

Nanoparticles (NPs) are solid colloidal particles, ranging from 1 to 1000 nm in size, consisting of various macromolecules in which the therapeutic agents can be adsorbed, entrapped, or covalently attached. The distinct advantages offered by solid NPs in drug development can be ascribed to their physical stability as well as the possibility to modify the formulating material in order to achieve controlled release characteristics. The ability to formulate NPs to achieve sustained release offers an opportunity to extend the life cycle of drugs whose patent is about to expire, developing formulations with lower dosing frequencies.

Nanoparticles have various morphologies, including fullerenes, solid lipid nanoparticles (SLNs), liposomes, nanostructured lipid carriers, nanoshells, quantum dots, superparamagnetic nanoparticles, dendrimers, etc. Some examples of nanoparticles that have been evaluated as drug delivery systems are shown in Fig. 3.1.

Drug delivery is intended to achieve a sustained (or slow) and/or controlled drug release, accordingly improving safety, efficacy, and/or patient comfort (Varshosaz, 2007). Ideally, Nanoparticle drug delivery systems (NDDSs) should reach the necessary organ or tissue to accumulate selectively therein and, at the same time, penetrate target cells to release the bioactive agent (Maeda et al., 2000). NDDSs have outstanding advantages, such as their ability to penetrate

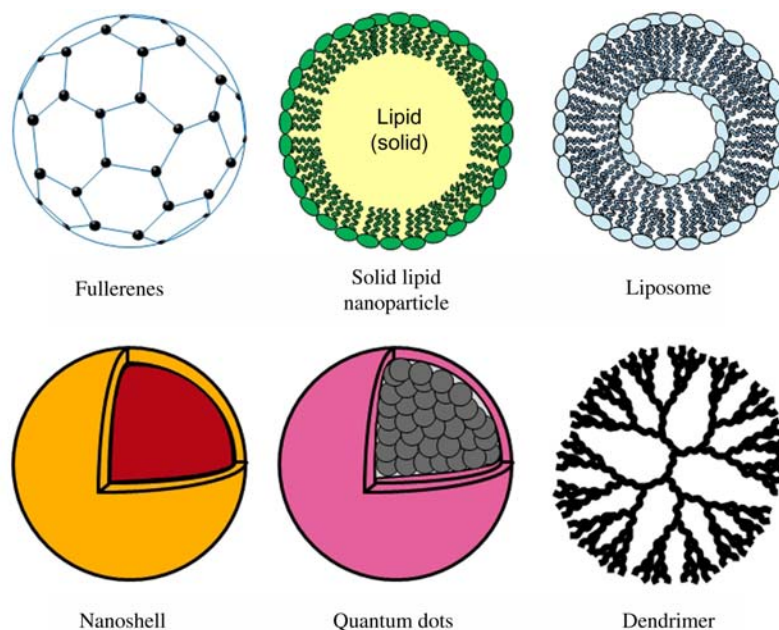


FIGURE 3.1

Nanoparticulate drug delivery systems.

cells and tissue, reaching target organs such as spleen, lung, spinal cord, liver, and lymph. They can also improve the usefulness of drugs, reducing toxic side effects. As their volume is ultra small, they can be transported through the thinner capillaries, avoiding the phagocytosis and, accordingly, they have an extended blood flow. Another advantage of NDDSs is that they can be used for control drug release because the material properties are sensitive to temperature, pH, and biodegradability. These systems are also promising because they can improve the stability of therapeutic agents against chemical/enzymatic degradation (Moghim *et al.*, 2001). All the advantages presented by NDDSs lie in their nanosize (Vinogradov *et al.*, 2002). For all these reasons, NDDS are highly promising in increasing the efficiency and efficacy of therapy, minimizing side effects at the same time.

Over the years, NDDSs have demonstrated enormous potential in medical, pharmaceutical and biological applications (Illum, 2007). So far, NPs have been widely used to deliver different active agents, such as drugs, proteins, polypeptides, genes, nucleic acids, and vaccines, among others. The drugs or biomolecules can be trapped into the NP interior structure or can be absorbed onto the exterior surface.

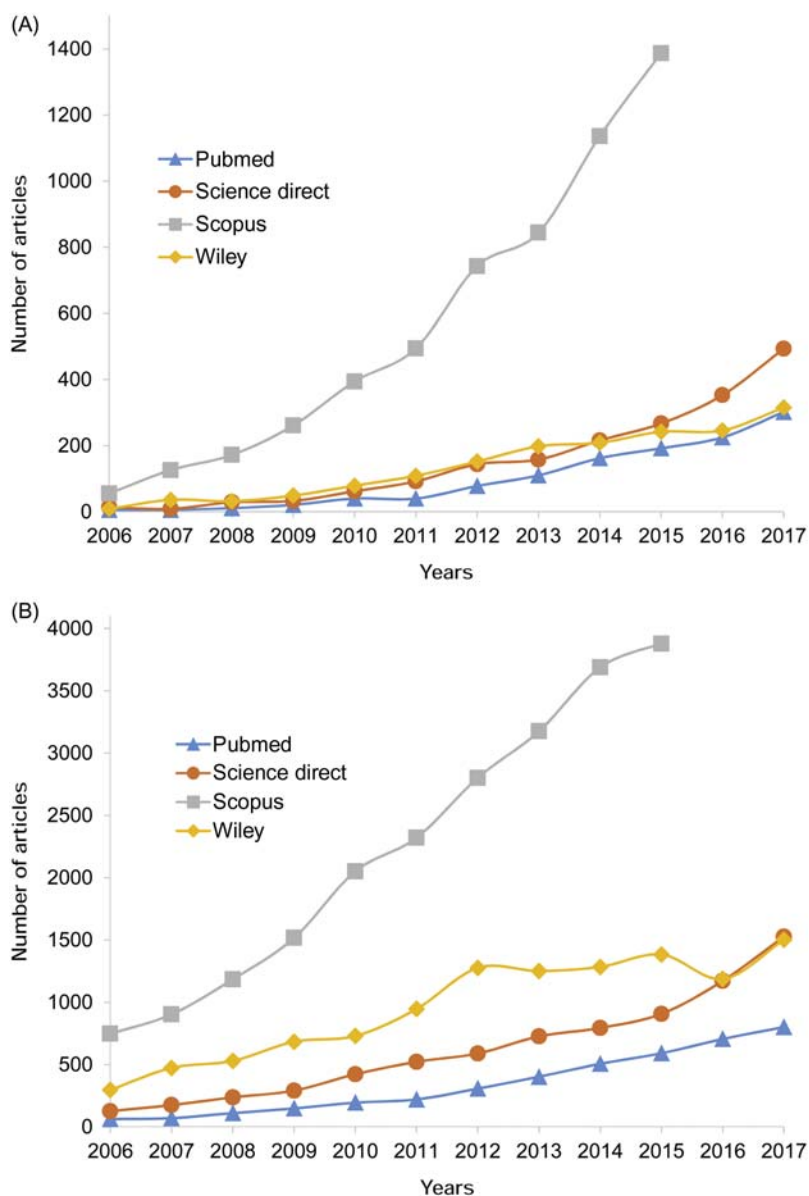
Nanomaterials can be used for the design and development of new drug delivery systems contributing to extended drug patents, as well as to reformulate existing drugs to improve their effectiveness, safety, patient-compliance, and moreover decreasing the cost of health care (Couvreur, 2013).

The increasing scientific interest in NPs can be clearly seen in Fig. 3.2, which shows the rising number of published articles using different keywords such as “nanocarrier and drug delivery” (Fig. 3.2A) or “nanoparticle and drug delivery” (Fig. 3.2B) in different databases (Scopus) in the period from 2006 to 2017. While a lesser number of articles were published in Sciencedirect, PubMed and Wiley in the same period.

The effectiveness of NPs in drug delivery can be attributed to many factors, such as physical and biological stability, good tolerability of the components, simplicity of the manufacturing process, possibility for easy scale-up of the manufacturing process, and amenability to freeze-drying and sterilization. The manufacturing process excludes organic solvents or potentially toxic ingredients. All the formulation components should be commercially available, safe, affordable, nontoxic and biodegradable. NP should be stable with respect to size, surface morphology, size distribution and other important physical and chemical properties.

3.1.1 NANOTECHNOLOGY IN DRUG DELIVERY

In the area of controlled release of active substances like drugs, a strong interest in nanoparticles as carriers of active ingredients has arisen. Some of the active components are extremely hydrophobic, without cellular permeability, and susceptible to metabolic degradation, limiting their use. These kind of agents can be

**FIGURE 3.2**

(A) Articles published in different databases using as keywords "nanocarrier and drug delivery" in the period from 2006 to 2017. (B) Articles published in different databases using as keywords "nanoparticle and drug delivery" in the period from 2006 to 2017.

transported without inconvenience through physiological media using NPs. Particle size is an important parameter because it governs the efficiency of the delivery system. Using nanotechnology for drug delivery, improved pharmacokinetic and pharmacodynamics properties of an active agent can be achieved (Shubhika, 2013).

Drugs bind to nanoparticles by adsorption, absorption, and “entrapment” or covalent bonding. They are released by desorption, diffusion, polymer degradation, physical or chemical polymer transformation (e.g., lower critical solution temperature, protonation/deprotonation), or a combination of these mechanisms. One advantage of NPs is their high dissolution rate, leading to increased bioavailability and lower drug doses, thereby reducing the toxicity (Pathak et al., 2007).

The main objective is to achieve a high control degree of their properties: excellent stability for circulation in blood, new functionalities for subsequent bioconjugation/biovectorization, narrow nanoparticle size distributions, biocompatibility and/or biodegradability, and sustained drug delivery. Normally, the release profiles follow a constant decreasing curve over a certain period.

Nanoparticles that release their content by external triggering open up new possibilities for therapeutic strategies. The external trigger could be light, heat, pH changes, ultrasound, etc., creating the possibility that it can be released according to demand.

3.1.2 TARGETS FOR DRUG DELIVERY

One of the main challenges in drug delivery is that, once having reached the target tissue, the drugs must have the ability to selectively kill diseased cells without affecting healthy ones (Elgindy et al., 2012). Nanoparticles seem to have the potential to increase the intracellular concentration of drugs and reduce toxicity; accordingly, they could be effective as drug carrier systems (Sudhakar, 2009).

Cellular and Intracellular Targets: Intracellular targeting is the supply of active agents to specific compartments or organelles within the cell. The mechanisms by which an active agent can reach the target cell are: simple diffusion or the use of cellular machinery (Shang et al., 2014). Endocytosis is the main route by which an active agent enters the target cell (Saboktakin et al., 2011; Oh and Park, 2014). Cytoplasmic delivery is another way of intracellular drug delivery. Steroid receptors are present in the cytoplasm, so delivering the drug nearby or in the receptor sites can achieve an improved therapeutic effect, reducing in addition, collateral effects (Tsigelny and Simberg, 2012). Another mechanism is nuclear targeting, used to deliver active agents specifically in the cell nucleus (Huang et al., 2010). To prevent oxidative damage, administration of mitochondrial drugs is important for the delivery of antioxidants to mitochondria (Rajendran et al., 2010).

Brain Targeting: NP can also be used for getting drugs into the brain. The blood–brain barrier (BBB) is a highly permeable selective barrier that separates the circulating blood from the brain extracellular fluid in the central nervous system (CNS). The BBB is formed by brain endothelial cells, which are connected

by tight junctions, and it does not allow many drugs to enter the brain. The administration of nanodrugs is useful to treat certain CNS disorders, due to some NPs being able cross the BBB. NP transport through the BBB occurs through paracellular or transcellular pathways, among others (Saraiva et al., 2016).

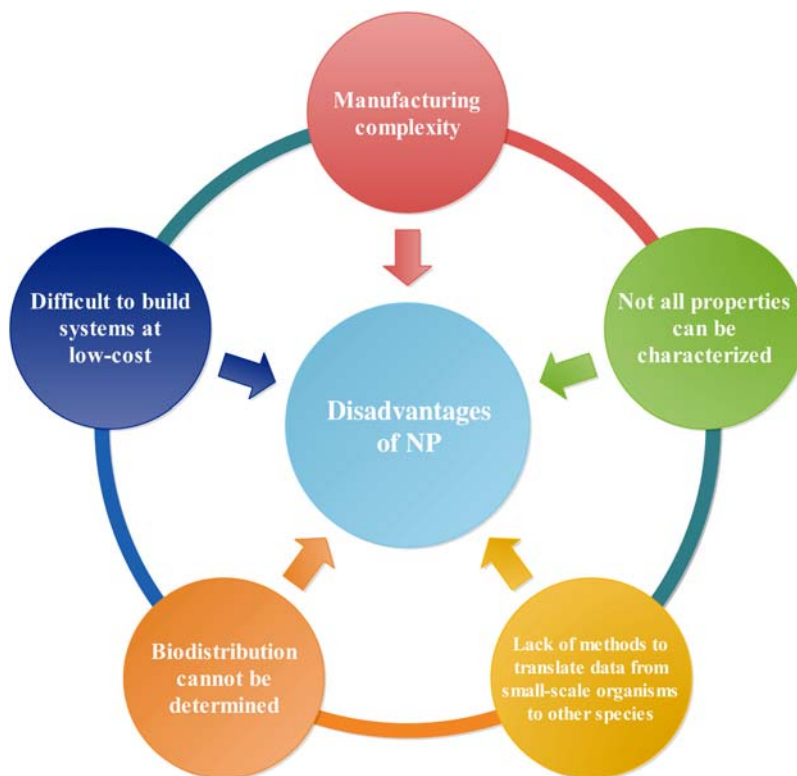
3.1.3 ADVANTAGES OF NANOPARTICLES

The advantages of NPs are summarized in Fig. 3.3 (Shinde et al., 2012; Bhokare et al., 2015). Another advantage of NPs in drug delivery is that it is easy to scale-up production. The fact that drugs can be incorporated into systems without any chemical reaction is important for preserving the drug activity. Finally, the systems can be administered for different routes, such as oral, intraocular, parenteral and nasal.



FIGURE 3.3

Some of the main advantages of nanoparticles.

**FIGURE 3.4**

Main disadvantages of nanoparticles.

Besides the advantages of NP, some disadvantages have been observed and are summarized in [Fig. 3.4](#) ([Shinde et al., 2012](#); [Bhokare et al., 2015](#)).

3.1.4 DRUG RELEASE FROM NANOPARTICLES

Drugs contained in nanoparticles, coated by polymers, are released by diffusion or erosion mechanisms through the polymeric membrane. This membrane acts as a barrier to drug release; therefore, the solubility and diffusibility of the drug in the membrane are decisive factors for the release process. Two important factors in developing a successful nanoparticulate system are: drug release and polymer biodegradation. Drug release rate depends on several factors, among which are: (1) drug solubility, (2) absorbed drug desorption process, (3) drug diffusion through the NP matrix, (4) erosion/degradation of the nanoparticle matrix, and (5) combination of erosion/diffusion process. Therefore, matrix materials solubility,

diffusion, and biodegradation are the governing factors of the release process (Mohanraj and Chen, 2006; Mudshinge et al., 2011).

Drugs can be delivered from NP to tissue sites by one of the three following general physicochemical mechanisms:

1. hydrating the polymer nanoparticles, causing their swelling, and subsequent drug release by diffusion;
2. enzymatic reaction resulting in polymer rupture, cleavage or degradation, allowing the entrapped drug from the inner core to be released;
3. dissociation of the drug from the polymer and its desorption/release from the swollen nanoparticles (Nagavarma et al., 2012).

3.2 NANOTECHNOLOGY-BASED DRUG DELIVERY SYSTEMS

Micro- and nanoparticles in biomedicine, and especially in drug delivery, have many advantages when compared to conventional systems: enhanced delivery, high performance characteristics of the product, lower drug load in the system, longer bioactivity due to the particle protects the drug from environmental effects in biological media, and more effective treatment with lower side effects. In addition, research is focused on the design of more effective delivery systems using an already known drug, which is more economical than trying to find of a new bioactive molecule.

Currently, some of the goals of NDDS researchers are focused on the selection and combination of carrier materials to reach suitable drug release rate and the modification of NP surface to enhance their targeting ability. Studies are also centered on the optimization of NP preparation for the possibility of an industrial scale production. Efforts are also focused on investigation of in vivo dynamic processes to elucidate the interaction of nanoparticles with blood, target tissues, and organs.

The effectiveness of NPs in drug delivery can be attributed to many factors, such as physical and biological stability, good component tolerability, simplicity of the manufacturing process and the possibility of easy scale-up, and amenability to freeze-drying and sterilization.

3.2.1 FULLERENES

Carbon has different morphological forms: diamond, graphite, coal, and fullerene. This last one has a hollow structure with sphere, ellipsoid, or tubular shapes. A combination of their spherical shape, small size, and hollow interior provides important therapeutic opportunities (Nasir, 2010). Spherical fullerenes are known as buckyballs, while cylindrical ones are called buckytubes or nanotubes. The buckyballs composed of less than 300 carbon atoms are known as endohedral fullerenes. This group includes the most common fullerene, the buckminsterfullerene (C₆₀).

The name of buckminsterfullerene was named in honor of Richard Buckminster Fuller, an architect who created a dome in 1967 with the carbon cluster shape. C₆₀ is a truncated icosahedron containing 60 carbon atoms with pentagons formed by C₅–C₅ single bonds, and hexagons constituted by C₅–C₆ double bonds (Kroto et al., 1991). Fullerene structures are characterized by sp² carbons symmetric cages, which belong to either 5- or 6-member rings on the cage surface. Different sizes of cages (C₆₀, C₇₀, and so on) are composed by different numbers and ratios of these rings (Anilkumar et al., 2011). These ball-like structured compounds have very good electronic properties, high affinity toward electrons, and are able to accept multiple electrons.

Friedman et al. and Schinazi et al. discovered that the hydrophobic cleft of the human immunodeficiency virus (HIV)-1 protease can host a C₆₀ molecule without problems (Friedman et al., 1993). This was the first evidence that fullerenes could be of pharmaceutical importance due to interactions with biological targets, highlighting their great potential for medicinal applications. They have been used in various applications in biological systems, taking advantage of their excellent radical scavenging ability (Bosi et al., 2003; Anilkumar et al., 2011).

Fullerenes have poor solubility in commonly used organic solvents, and much lower in aqueous solutions. Carbon disulfide, *o*-dichlorobenzene, toluene, and xylene are good solvents for fullerenes (Ruoff et al., 1993). The most used methodologies to improve water affinity are:

1. encapsulation or microencapsulation in a specifically designed carrier;
2. suspension by cosolvents;
3. introduction of solubilizing agents by chemical functionalization (Chawla et al., 2010).

Fullerenes have neuroprotective activity because they can react with oxygen radical species (reactive oxygen species, ROS). The fullereneols have antioxidant activity and reduce apoptosis in cultured neurons; they are also able to cross the BBB (Kumar and Kumbhat, 2016). Carboxyfullerenes are inhibitors of lipid peroxidation in nerve tissue and they have demonstrated to be powerful neuroprotectors against cytotoxic, apoptotic, and metabolic injuries in cortical cell cultures (Jain, 2011; Chawla et al., 2010). Fullerenes also have antioxidant activity; they inactivate circulating free radicals and can therefore stop the progression of diseases caused by excess free radical production (Anilkumar et al., 2011).

There is evidence that fullerenes inhibit human immunodeficiency virus or HIV protease and the HIV transcriptase. This inhibition prevents the virus replicating and infecting more cells. Fullerenes are advantageous because the HIV virus can mutate quickly (Anilkumar et al., 2011). Moreover, the inhibitory activity of fullerenes against some enzymes as papain, trypsin, thrombin, etc. has been demonstrated (Tokuyama et al., 1993). However, the mechanism by which this activity is exerted remains unknown.

Furthermore, it has been reported that fullerenes have antimicrobial activity against some microorganisms, such as *Candida albicans*, *Bacillus subtilis*,

Escherichia coli and *Mycobacterium avium* (Da Ros et al., 1996; Mashino et al., 1999; Dizaj et al., 2015; Patel et al., 2013). Some derivatives of fullerenes inhibit gram-positive bacteria but not gram-negative ones. These derivatives can cause the death of gram-positive bacteria because they disrupt the structure of the cell wall, while the cell wall of gram-negative bacteria have an outer membrane that prevents the passage of fullerenes (Da Ros et al., 2001). The transference of the growth factor TGF- β is the main cause of apoptosis. Antioxidant treatment is one way to stop or at least decrease the damage; in apoptosis, ROS species are released. Huang et al. have proved that fullerene derivatives can prevent apoptosis, neutralizing the TGF- β -induced ROS (Huang et al., 1998).

Fullerenes also have anticancer activity. It has been shown that metallofullerenes can be used to treat cancer tumors by delivering radioactive atoms directly into the diseased tissue (Chawla et al., 2010). Endohedralmetallofullerenes have shown potential to be applied in diagnostics (Wilson, 1999). Metallofullerenes are nontoxic and stay in the body for approximately one hour, allowing imaging of the circulatory system (Naga Anusha and Siddiqui, 2011).

3.2.2 SOLID LIPID NANOPARTICLES

SLNs are submicron colloidal carriers (50 nm–1 μ m), developed at the beginning of the 1990s. They are alternative carrier systems for drug controlled release of polymeric nanoparticles, emulsions, and liposomes (Abhilash, 2010). SLNs are spherical with a solid lipid core matrix, stabilized by surfactants, where the lipophilic molecules can be solubilized. The melting point of these particles must be greater than the body temperature, so that they remain in solid phase after being supplied. The main lipids investigated with high melting points are triacylglycerols (triglycerides), steroids, acylglycerols, fatty acids, waxes, and their combinations. Regarding surfactants, the most studied ones are biological membrane lipids, such as lecithin; biocompatible nonionic, such as ethylene oxide/propylene oxide copolymers; sorbitan esters; fatty acid ethoxylates; bile salts such as sodium taurocholate, and combinations of them (Sharma et al., 2011).

SLNs are prepared from one kind of solid lipid or mixtures of different lipids. The solid matrix has two functions: providing chemical stability to the encapsulated drugs, and controlling their release.

SLNs have a wider application range than polymeric nanoparticles (dermal, oral, pulmonary, and intravenous applications), due to the fact that they are prepared with physiologically well tolerated and generally recognized as safe excipients, minimizing their cytotoxicity. Second generation lipid nanoparticles (NLC) were developed to improve the ability of SLNs for drug delivery. In NLCs, the solid lipids are mixed with fluid lipids so that the final particle is solid at room temperature (Bahari and Hamishehkar, 2016).

In the last decade, SLNs have acquired considerable interest as new particulate drug delivery systems. SLNs allow incorporation of high amounts of lipophilic and hydrophilic drugs within the lipid matrix (Gupta and Jain, 2010). The main

SLN advantages are that they can control drug release and drug targeting, they increase drug stability and allow high drug loads; it is possible to incorporate both lipophilic and hydrophilic drugs, the carrier is not bio toxic, there are no problems with large-scale production, they allow sterilization, and have good tolerability (Varshosaz et al., 2010).

Solid lipid nanoparticulate carrier system performance depends on its ability to entrap the loaded drug and release it appropriately when required. Drug entrapment refers to the location of the drug incorporated into the solid lipid matrix. This results in a strong alteration in SLN properties, such as particle size and particle size distribution, charge characteristics and thermotropic properties, lipid orientation modification, among others. Many factors affect drug entrapment in SLNs, such as crystallinity and polymorphism of the lipid formulation, drug solubility and miscibility in the lipid melt, physicochemical orientation of the lipid matrix, among others. Before SLN formulation, solubility of the drug in the lipid melt must be deeply studied. High drug solubility in the solid lipid means high drug-loading capacity, and therefore high drug release from the SLNs.

Drug release and drug-loading capacity are affected by lipid crystallinity and orientation. A high degree of imperfections in the lipid allows high drug entrapment. If a weakly bound or entrapped drug exists on the large surface of SLNs, an initial rapid release or burst effect takes place (Fig. 3.5). Consequently, a more controlled and slower drug release occurs due to either diffusion of drug through

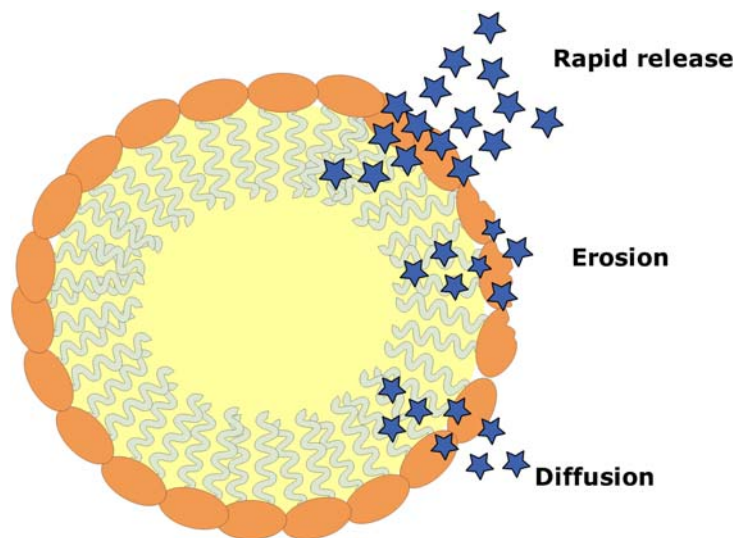


FIGURE 3.5

Drug release from a solid lipid nanoparticle.

the lipid matrix system of the SLNs or erosion/degradation of the lipid matrix system. Drug diffusion-dependent release from SLNs could follow zero-order kinetics, commonly found in SLNs formulated with biodegradable lipid core; or first order kinetics, when formulated with no biodegradable lipid core ([Attama and Umeyor, 2015](#)).

3.2.3 ROUTES OF ADMINISTRATION OF SOLID LIPID NANOPARTICLES

There are different routes of administration for SLNs. They can be orally administered as dispersions, SLN-based tablets, pellets or capsules, as well as lyophilized unit dose powders, which must be reconstituted ([Thakkar et al., 2016](#); [Nirbhavane et al., 2016](#); [Wu et al., 2016](#)). The routes of particle uptake after oral application are mainly transcellular (via the M cells in the Peyer's patches or enterocytes) but they can also be paracellular (diffusion between the cells) ([Attama and Umeyor, 2015](#)).

Another possibility to administer SLN is the parenteral way. This is the most common and effective way to administer drugs with low bioavailability and very narrow therapeutic index ([Sachin and Vishal Gupta, 2013](#)). The possibility of thromboembolism development must be considered when administered via this route.

SLN can also be administered by the rectal way. This route of administration is of vital importance and often used for pediatric patients under special clinical conditions, such as seizures. When the rectal route of administration is used, plasma levels and therapeutic efficacy of drug-loaded SLN of the same dose were higher than when oral or intramuscular routes were used ([Din et al., 2015](#); [Mohamed et al., 2013](#)).

Another possible route of administration of SLN that is painless, noninvasive, and well tolerated, is the nasal route, which favors fast absorption and rapid onset of drug action. These avoid both degradation of labile drugs in the GIT and insufficient transport across epithelial cell layers. The nasal cavity also has reduced enzymatic activity, high availability of immunoactive sites, and moderately permeable epithelium ([Muntimadugu et al., 2016](#)).

SLNs can also be administered topically, ensuring immediate availability of products. Due to the nonirritant and nontoxic lipid core, it could be used for drug administration on damaged or inflamed skin. The substances used for the preparation of dermal SLNs are the ones used in conventional dermal formulations ([Bharadwaj et al., 2016](#); [Kaur et al., 2016](#)).

The ocular route can also be used. Eye drops for drug administration to the ocular tear film have high patient acceptance and compliance level because they are easy to use and require no specialized skills or instrumentation ([Kumar and Sinha, 2016](#); [Sharma et al., 2016](#)).

3.2.4 LIPOSOMES

The discovery of phospholipids spontaneously forming spherical, self-closed bubbles known as liposomes, upon dispersion in water, ushered in a new era in drug delivery technology.

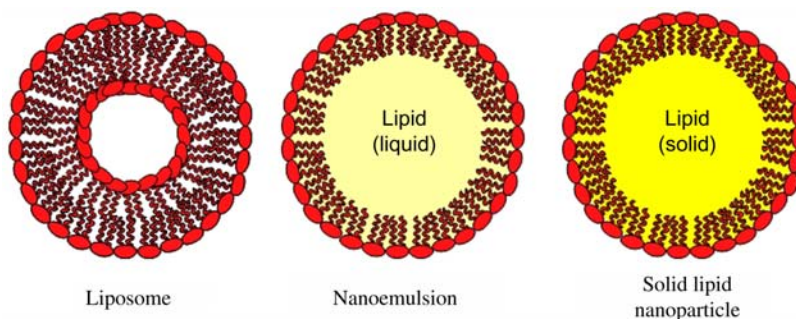
Liposomes, first described by [Bangham et al. \(1965\)](#), are a kind of colloidal system formed by the self-assembly of amphiphilic lipids, which self-associate to enclose an aqueous compartment surrounded by one or more concentric lipid bilayers. The assembly into closed bilayered structures is mainly driven by the hydrophobic effect, which organizes amphiphilic molecules so as to minimize entropically unfavorable interactions between hydrophobic acylchains and the surrounding aqueous medium ([Tanford, 1980](#)). This effect is further established by various intermolecular forces such as electrostatic interactions, hydrogen bonding, as well as van der Waals and dispersion forces ([Lasic, 1993](#)).

Liposomes depend on the preparation method, surface charge, composition, and size. They can have one or multiple bilayers. In the first case, they are named unilamellar vesicles, which can be small or large, based on their sizes ([Mozafari and Sahin, 2005](#)). In the latter, they are called multilamellar vesicles. Liposomes are commonly used as model cells or carriers for various bioactive agents including drugs, vaccines, cosmetics and nutraceuticals. The availability of a large array of vesicular systems differing in composition and applications has led to the effective delivery of bioactive molecules, either by passive targeting through enhanced permeation and retention phenomena, or by active binding to specific target cells and subsequent delivery of cargo.

Coupled with the increased blood circulation time through the development of stealth technology, various liposomal formulations have already been approved for clinical use, while others are in various stages of clinical trials. Drugs associated with liposomes have markedly altered pharmacokinetic properties, compared to free drugs in solution. Liposomes have the unique feature of compartmentalizing and solubilizing both hydrophilic and hydrophobic materials by nature. This ability, together with biocompatibility and biodegradability, make liposomes very attractive as drug delivery vehicles.

Conventional liposomes have nonspecific reactivity. Stimuli-responsive liposomes are reactive liposomes that can change their structure under the action of an external stimulus. In this way, a number of different stimuli have been explored to trigger the release of the encapsulated cargo from liposomes, including temperature, pH ([Simoes et al., 2004](#)), light ([Troutman et al., 2009](#)), redox potential ([Ong et al., 2008](#)), near-infrared ([Volodkin et al., 2009](#)), and ultrasound (US) ([Schroeder et al., 2009](#)); each stimulus with its specific advantage, depending on the application ([Sawant and Torchilin, 2010](#)).

Recent advances in nanotherapy have led to engineering liposomes to emerge as battlehorses in nanomedicine, providing better therapeutic control of diseases through improved therapeutic or diagnostic agents in diseased tissues. This has paved the way for the development of second generation liposomes, with greater

**FIGURE 3.6**

Liposome, nanoemulsion and solid lipid nanoparticles scheme.

therapeutic benefit and could lead to a paradigm shift from conventional drug delivery technologies.

The scheme in Fig. 3.6 shows the difference among liposome, nanoemulsion, and SLN.

3.2.5 MICROEMULSIONS

Microemulsions are isotropic, thermodynamically stable systems composed of oil, water, and surfactant. Thermodynamic stability rather than size, is the defining hallmark of a microemulsion, although the droplet sizes are still below 100 nm (and in many cases even smaller) (Majuru and Oyewumi, 2009). Nonetheless, what is critical about microemulsions is that they contain two phases, consisting of two immiscible liquids that are mixed together and stabilized with the aid of a surfactant, with or without a cosurfactant.

The difference between microemulsions and emulsions is that the later are opaque mixtures of two immiscible liquids, thermodynamically unstable and usually require the application of high torque mechanical mixing or homogenization to produce dispersed droplets in the range of 0.2–25 μm . Both types can be made as water-in-oil (w/o) or oil-in-water (o/w) (Majuru and Oyewumi, 2009).

The choice of the dispersed and continuous phases for microemulsions formulations is based on the hydrophilicity of the model drug. Additionally, surfactants that have hydrophilic-lipophilic balances (HLBs) of 3–6 tend to promote w/o microemulsions formation, while those with HLB values of 8–10 tend form o/w microemulsions.

The choice of microemulsion components affects its stability. It has been reported (Majuru and Oyewumi, 2009) that the formation and stability of microemulsions are dependent on the interfacial tension between the dispersed and continuous phases. Microemulsion instability can lead to Oswald ripening, leading to dissolution of the small droplets with a resultant increase in the size of the

large droplets; therefore, stabilization against Oswald ripening is critical because the resultant change in droplets sizes could lead to loss of physical stability of the dosage form.

Microemulsions have been proposed as drug delivery systems to enhance the absorption of drug across biological membranes (Majuru and Oyewumi, 2009). Some of microemulsions advantages include: (1) increased solubility and stability of drugs, and (2) ease and economical scale-up. Some of the disadvantages are: (1) premature leakage/release of incorporated drug, (2) phase inversion, (3) many of the effective surfactants and/or cosurfactants do not have a pharmaceutically acceptable toxicity profile, and (4) microemulsion systems often require development of complex systems that may be time consuming.

3.2.6 NANOEMULSIONS

Nanoemulsions are isotropic dispersed systems of two nonmiscible liquids, normally consisting of an oily system dispersed in an aqueous system, or an aqueous system dispersed in an oily system, forming droplets or oily phases of nanometric sizes. Nanoemulsions often have a higher loading capacity for lipophilic active ingredients than microemulsions, which can be an advantage in some applications.

Nanoemulsions are thermodynamically unstable systems, in contrast to microemulsions, because some nanoemulsions need significant energy to be formed. Three methods are most often used to prepare nanoemulsions: high-pressure homogenization, microfluidization, and phase-inversion temperature method.

Nanoemulsions are susceptible to Oswald ripening and, as a consequence, susceptible to creaming, flocculation, and other physical instability problems associated with emulsions. Despite this, they can be stable (metastable) for long periods, due to their extremely small sizes, if adequate surfactants are used. Oswald ripening can be retarded by adding sufficient quantities of a ripening inhibitor to the oil phase prior to nanoemulsion formation (Lim et al., 2011; Julian McClements et al., 2012).

Hydrophobic and hydrophilic drugs can be formulated in nanoemulsions. Like microemulsions, nanoemulsions can be fabricated in a concentrated form that is diluted with an aqueous solution containing other functional ingredients to form the final liquid product. Nanoemulsions therefore have characteristics that make them suitable for application as delivery systems in clear liquid formulations. Nanoemulsions are nontoxic and nonirritant systems that can be used for skin or mucous membranes, parenteral and nonparenteral administration in general, and have been utilized in the cosmetic field.

Transdermal delivery using nanoemulsions has decreased due to stability problems inherent to this dosage form. Some examples of drugs using nanoemulsions for transdermal drug delivery are gamma tocopherol, caffeine, plasmid DNA, aspirin, methyl salicylate, insulin, and nimesulide (Sonneville-Aubrun et al., 2004).

3.2.7 DENDRIMERS

These hyperbranched molecules were discovered in the 1980s by Tomalia and coworkers, and they called them dendrimers (Tomalia et al., 1985). The term “dendrimer” originates from the Greek word *dendron*, meaning “tree,” because their architecture closely resembles the branches (and roots) of a flourishing tree. They are polymeric macromolecules, highly branched, varying from 2.5 to 10 nm in diameter.

From a chemistry perspective, the first library of synthetic dendrimers, or “cascade molecules,” as they were termed by their creator Fritz Vögtle, was reported in the late 1970s (Buhleier et al., 1978). Dendrimers are generally symmetric around the core and adopt spheroidal three-dimensional morphology in water, when they are extended. It is possible to identify in their structure a central core with at least two identical chemical functionalities. Starting from these groups, other molecule repeating units can be originated with at least one branching junction. Chains and branching repetitions result in a series of crowded, radially concentric layers (Albertazzi et al., 2012). Dendrimer shapes range from spheres and flattened spheroids (disks) to amoeba-like structures, especially when surface charges exist, giving macromolecules a starfish-like shape.

Dendrimers can be synthesized from both synthetic and natural monomers, e.g., monosaccharides, amino acids, or nucleotides. Two kinds of dendrimers are commonly used for biomedical applications: polyamidoamines and polypropyleneimines (Dhanikula et al., 2009). Due to their well-defined structure, stability, monodispersity of size, and surface functionalization capability, dendrimers are attractive as drug carrier candidates (Abhilash, 2010).

To generate asymmetric dendrimers, dendrons of different generations (G1–G4) are coupled to a linear core, creating a branching dendrimer with nonuniform orthogonal architecture. Asymmetry allows versatility in relation to molecular weights and structures, giving precise control over the number of available functional groups on each dendron for the attachment of drugs and other therapeutic moieties (Medina and El-Sayed, 2009).

Studies of biomedical application of dendrimers are becoming more and more attractive, especially in the field of nonviral gene vector and NDDSs (Ihre et al., 2002).

Photodynamic therapy of cancer involves the systemic administration of photosensitizers to solid tumor tissues and local illumination with light of a specific wavelength, leading to photochemical destruction of cancer cells via generation of singlet oxygen or superoxide from molecular oxygen. Suitable carriers and photosensitizers should have simple but effective strategies to be highly selective, photodynamic efficient, and have less side effects. Zhang et al. (2003) reported the successful use of dendrimer polymeric micelles to deliver photosensitizers. Dendrimers also have several unique characteristics that make them good nanoparticle platforms for antimicrobial drug delivery (Medina and El-Sayed, 2009).

3.2.8 CARBON NANOMATERIALS

Two types of carbon nanocarriers are used for drug delivery systems: nanotubes (CNTs) and nanohorns.

CNTs have a singular architecture, consisting of single or multigraphite layers (SWCNTs: single walled carbon nanotubes, MWCNTs: multiwalled carbon nanotubes) with a large surface area and excellent thermal and electronic conductivities (Amenta and Aschberger, 2015). Chemical modification of their surface allows an improvement of their biocompatibility (Bianco et al., 2011); e.g., by covalent attachment of amphiphilic diblock copolymers (Di Crescenzo et al., 2011), polyamidoamine dendrimers (Caminade and Majoral, 2010) or PEG layers (Bottini et al., 2011) on CNT surface or by dispersing within a hyaluronic acid matrix (Filip et al., 2011). The mechanical strength of SWCNTs has been used to improve the properties of other carriers, such as polymeric or nonpolymeric composites (Shin et al., 2011).

The immobilization of drug in the carbon nanocarriers can be achieved by three different ways. One of them includes the anchoring of the drug to functionalized CNTs (Sahoo et al., 2010). In the second one, the drug can be chemically absorbed on the surface or in the spaces between the nanotube (Pan and Xing, 2008). The third immobilization method involves the encapsulation of the active agent in the CNT (Bessrouer et al., 2012), presenting the advantage over the other two in protecting the drug from being degraded during its transport to the cells and releasing it only under certain conditions. In all cases, the control of drug release can be performed electrically or chemically.

Nanohorns are formed only by single-walled nanotubes and have similar properties to nanotubes. They can be obtained by simple methods with high purity and low cost, since metal catalysts are not necessary in their manufacture process. They are generally synthesized by laser ablation of pure graphite and form radial aggregates, reaching a high production rate and yield (Zhu and Xu, 2010). The drugs can be immobilized either by adsorbing them on the nanohorns (Nakamura et al., 2011) or by nanoprecipitating the active agents with the nanohorns (Ajima et al., 2005).

3.2.9 MAGNETIC NANOPARTICLES

In recent years, numerous investigations have been carried out on magnetic nanoparticles (MNPs) as very promising carriers for targeted drug delivery, because they have unique attributes, such as their singular physical properties and ability to function at molecular and cellular level of biological interactions. However, some difficulties are encountered at the time of applying magnetic nanoparticles, generally associated with inappropriate features of magnetic nanoparticles or inadequate magnet system. Magnetic nanoparticles, e.g., tend to aggregate into larger clusters, causing the losing of some properties related to their small size and making physical handling difficult. Furthermore, magnetic force may not allow the accumulation of magnetic drugs solely at the target site, because it

could be insufficient to overcome the force of blood flow (Neuberger et al., 2005). For all of these reasons, many factors must be considered when designing magnetic drug delivery systems, such as drug loading capacity, strength of magnetic field, size and magnetic properties of particles, accessibility of target tissue, and rate of blood flow.

The immobilization of drugs in MNPs can be carried out by electrostatic interactions (Bhattarai et al., 2008), covalent binding (Ulbrich et al., 2016), encapsulation process (Zhang and Misra, 2007), and adsorption (Yallapu et al., 2011). MNPs can be divided, depending on their magnetic properties, into pure metals (such as manganese, nickel, iron, and cobalt), their oxides, and alloys (Assa et al., 2016). However, when it comes to biomedical applications, the choice of magnetic material is drastically reduced. This is due to the lack of knowledge of the negative effects that most of these nanomaterials have on the human body. Iron oxide nanoparticles are the only type of MNPs approved for clinical use by Food and Drug Administration (Wilczewska et al., 2012).

3.3 NANOPARTICLES PREPARATION METHODS

NP can be prepared from different materials, such as polysaccharides, proteins, and synthetic biodegradable polymers. The selection of the polymer depends on the nanoparticle sizes needed, the properties and solubility of the drug to be encapsulated, surface characteristics, biodegradability and biocompatibility, and drug release profile from the nanoparticles (Bhokare et al., 2015).

There are different methods for NP preparation. The choice of the method depends on the raw material and the properties of the drug. The raw material is selected depending on the biocompatibility, the route of administration, the drug release profile, and biomedical applications of NP (Maravajhala et al., 2012).

3.3.1 SOLVENT EVAPORATION METHOD

Polymer solutions are prepared using volatile solvents and then the emulsions are formulated. Dichloromethane and chloroform were the most used solvents in the past, but they have been replaced with ethyl acetate, due to its better toxicological profile (Rao and Geckeler, 2011; Allouche, 2013). The polymer/drug solution mixture is then emulsified in an aqueous solution, containing an emulsifying agent or surfactant, to form an oil-in-water (o/w) emulsion. The organic solvent is evaporated after stable emulsion formation, reducing the pressure or by continuous stirring. High-speed homogenization or ultrasound can be used to reduce particle sizes (Bhokare et al., 2015).

Poly(lactide-co-glycolide) (PLGA) (Xie et al., 2016), polylactide (PLA) (Rachmawati et al., 2016), or poly(-caprolactone) (PCL) (Devrim et al., 2016) are reported in literature as polymers used to prepare nanoparticles with the solvent evaporation method.

3.3.2 POLYMERIZATION METHOD

In the polymerization method, monomers are polymerized in aqueous solution to form nanoparticles. The drug can be dissolved in the polymerization medium or adsorbed onto the nanoparticles after the polymerization is completed (Bhokare et al., 2015). The method can be classified as conventional or surfactant-free emulsion polymerization, depending on whether a surfactant is used. The ingredients that form the conventional system are: water, low water solubility monomer, water soluble initiator, and surfactant. In surfactant-free emulsion polymerization, the reagents used are deionized water, a water soluble initiator (i.e., KPS, potassium persulfate) and monomers (vinyl or acryl monomers are the most common) (Rao and Geckeler, 2011). To remove the stabilizers and surfactants, the nanoparticle suspension is purified by ultracentrifugation and the particles are resuspended in an isotonic surfactant-free medium.

Surfactants and stabilizer concentration is fundamental in nanocapsule formation and in their particle size (Bhokare et al., 2015). This technique has been used to prepare polybutylcyanoacrylate or poly(alkylcyanoacrylate) nanoparticles (Zhao et al., 2014).

3.3.3 COACERVATION OR IONIC GELATION METHOD

Most research is focused on chitosan, gelatin, and sodium alginate polymers (biodegradable hydrophilic polymers) for nanoparticles preparation. Calvo and coworkers developed an ionic gelation method to prepare hydrophilic chitosan nanoparticles that involves two aqueous phases. One of them contains chitosan and the diblock copolymer of ethylene oxide or propylene oxide (PEO-PPO), and the other one is polyanion sodium tripolyphosphate. Chitosan's positively charged amino group interacts with negatively charged tripolyphosphate, forming nanometer-sized coacervates. Coacervates are formed due to the electrostatic interaction between the two aqueous phases, whereas the ionic gelation involves the transition from liquid to gel, due to ionic interaction at room temperature (Calvo et al., 1997). This technique has been used to prepare cefixime nanoparticles using chitosan, and *Basella alba* leaf mucilage as matrix formers (Harika et al., 2016).

3.3.4 SPONTANEOUS EMULSIFICATION OR SOLVENT DIFFUSION METHOD

This is a modified version of the solvent evaporation method. It requires a polymer solvent partially soluble in water. The oil phase is formed by a water miscible solvent with a small amount of a water-immiscible organic solvent. A spontaneous diffusion of solvents takes place, creating interfacial turbulence and leading to small particle formation. The particle size depends on the water

miscible solvent concentration: as it increases, the particle size decreases. This method, as well as solvent evaporation method, can be used for both hydrophilic or hydrophobic drugs. For hydrophilic drugs, multiple w/o/w emulsion needs to be formed, and the drug should be dissolved in the inner aqueous phase (Bhokare et al., 2015).

Polymer solvents such as ethyl acetate (Lokhande et al., 2013) and surfactants such as polyvinyl alcohol (Pandav and Naik, 2014) are often used for stabilizing and modifying the emulsion droplet sizes. The most used and most suitable polymers are the same as mentioned in the solvent evaporation method: PLA (Ravikumara et al., 2013), PLGA (Fu et al., 2015), and PCL (Katakam et al., 2015), but gelatin or chitosan nanoparticles have also been synthesized by this technique (Wilson et al., 2010).

3.3.5 DOUBLE EMULSION AND EVAPORATION METHOD

Emulsion and evaporation methods have the problem of poor entrapment of hydrophilic drugs. Therefore, the double emulsion technique is employed to overcome this inconvenience. It includes vigorous stirring of an aqueous drug solution and the organic polymer solution, to form w/o emulsions. This emulsion is poured into another aqueous phase with continuous stirring, to form the w/o/w emulsion. The solvent is then evaporated, nanoparticles are isolated by high speed centrifugation and thoroughly washed before lyophilization.

The NP characteristics are affected by the amount of hydrophilic drug incorporated, the stabilizer and polymer concentration, and the volume of aqueous phase (Bhokare et al., 2015). This method has been used to prepare insulin-loaded poly(ϵ -caprolactone) nanoparticles (De Araújo et al., 2013).

3.3.6 SALTING OUT METHOD

The salting out procedure can be considered a modification of the emulsification/solvent diffusion method. It is based on the separation of a water miscible solvent from aqueous solution via a salting out effect. Polymer and drug are dissolved in a common solvent, such as acetone, the solution is then emulsified into an aqueous gel that contains the salting out agent and a colloidal stabilizer. The salting out agents are electrolytes, such as magnesium chloride, calcium chloride, and magnesium acetate; or nonelectrolytes such as sucrose (Balakrishanan and Rajan, 2016). The colloidal stabilizer could be polyvinylpyrrolidone or hydroxyethylcellulose. To enhance the acetone diffusion into the aqueous phase, this oil/water emulsion is diluted with water or aqueous solution, inducing nanosphere formation. Cross-flow filtration is used to eliminate solvent and salting out agents (Nagavarma et al., 2012).

3.3.7 PRECIPITATION METHOD

Solvent displacement implies precipitation of a preformed polymer from an organic solution, and the organic solvent diffusion in the aqueous medium that also has surfactant. A semipolar water-miscible solvent is used to dissolve the polymer and drug, together with a lipophilic surfactant. The solution is then mixed with an aqueous solution containing stabilizer. Nanoparticles are formed instantaneously due to the fast solvent diffusion, and the solvent is removed by evaporation at low pressure (Bhokare et al., 2015).

The polymer could be synthetic, semisynthetic or natural. Those most used are biodegradable polyesters, especially PCL, PLA, and PLGA (Alshamsan, 2014). Eudragit can also be used, as well as polyalkylcyanoacrylate. Although synthetic polymers have higher purity and better reproducibility than natural polymers, allylic starch and dextran ester are natural polymers also used (Rao and Geckeler, 2011). Copolymers of PEG are used in order to decrease nanoparticle recognition by the reticular endothelial system.

The polymer organic solvent could be ethanol, acetone, hexane, methylene chloride, or dioxane, which is miscible in water and easy to remove by evaporation. Accordingly, acetone is the most frequently employed solvent for this method (Dalpiaz et al., 2009). Sometimes, solvent mixtures are used, such as acetone/water solutions, consisting mainly of acetone, and blends of acetone with ethanol and methanol. Conversely, the nonsolvent phase could be formed with a single nonsolvent or a mixture of nonsolvents, with one or more natural or synthetic surfactants (Allouche, 2013).

3.3.8 MINIEMULSION

This method differs from conventional polymerization and surfactant-free emulsion polymerization, in that the nanoemulsions are generated through high-energy methods and a low molecular mass compound is generally used as costabilizer, prior to initiating polymerization (Allouche, 2013). Usually, the formulation consists of water, costabilizer, monomer mixture, initiator, and surfactant (Rao and Geckeler, 2011). The nanoemulsion droplets generally consist of a pure monomer phase stabilized by an adsorbed suitable surfactant (Nagavarma et al., 2012).

One of the widely used polymerization mechanism is the radical polymerization, which starts in the emulsion droplets by the initiator incorporation in the continuous phase. The droplet nucleation mechanism is the one assumed in the literature, suggesting that the radicals are generated in each droplet of monomer, taken as an individual reaction site (Asua, 2002).

Therefore, the number and size of particles do not change during the polymerization process. The initiator choice and its solubility obviously have a great influence on the final properties of the particles, especially in their size.

Even if the reverse polymerization of the nanoemulsion is possible using hydrophilic ingredients, the hydrophobic character of the dispersed monomer

phase requires, in most cases, an oil-soluble initiator, which is more suitable for obtaining well defined nanoparticles. Although radical polymerization is frequently selected for NP generation in miniemulsion polymerization, nonradical polymerization methods are less aggressive for drug encapsulation applications and can also be used. These may be: polyaddition (Crespy and Landfester, 2010), anionic polymerization (Crespy and Landfester, 2010) or metal catalyzed reactions (Tomov et al., 2000). For example, the preparation of polyurethane latex nanospheres through reaction between diisocyanate and activated diol in nanoe-mulsion droplets is found in the literature (Anton et al., 2008). The main factors leading to successful synthesis are the low water solubility of the reactants and slower polymerization kinetics than emulsification.

3.3.9 MICROEMULSION

Microemulsion is a nanoparticle synthesis technique in which two immiscible fluids such as water in oil (w/o), oil in water (o/w) or water in supercritical carbon dioxide (w/sc CO₂) are thermodynamically stabilized with the aid of a surfactant. The microemulsion is named o/w if water is the bulk fluid and oil is in less quantity, with a small surfactant amount. In the w/o system the liquids proportions are reversed (Bhokare et al., 2015).

The most relevant difference between microemulsion and miniemulsion polymerization processes, is based on the produced dispersed phase kinetic character in the emulsified system. Contrary to miniemulsion, microemulsion is a spontaneous and thermodynamically stable system prepared with a high load of surfactant and characterized by almost zero interfacial tension at the oil/water interface (Nagavarma et al., 2012). Usually, generation of nanoparticles through microemulsion polymerization creates smaller particles (typically less than 80 nm) than the ones obtained by miniemulsion polymerization processes. A water-soluble initiator is introduced into the aqueous phase to initiate the polymerization in only a few swollen micelles of the monomer-containing microemulsion. As time goes by, the osmotic and elastic influence of the polymer chains that are being formed, destabilizes the microemulsions creating an increased particle size, empty micelle formation, and leading to a secondary nucleation (Allouche, 2013).

The highlighted factors influencing the final properties of the particles, according to reported literature, are: reaction temperature, initiator type and concentration, and surfactant and monomer concentration. Polyvinyl acetate (Gómez-Cisneros et al., 2005), polyaniline (Yufeng et al., 2016), polyacrylamide (Feng et al., 2000), and polypyrrole (Sun et al., 2012) nanoparticles are found in literature having been prepared with this technique. Microemulsion polymerization has great potential in several applications, but has some disadvantages related to its dilute formulation and its high surfactant amount, which greatly limit the commercial use of this technique.

3.3.10 INTERFACIAL POLYMERIZATION

Interfacial polymerization is characterized by the monomer polycondensation at the interface of the droplets, which generates mainly nanocapsules instead of nanospheres. Several strategies have been developed, depending on the selected formulation and the choice of the monomers introduced in the continuous and/or dispersed phase (Rao and Geckeler, 2011).

One way is to introduce the monomer into the continuous phase of the emulsified system, which can then react with the droplets of the emulsion to form nanocapsules. Because most of the monomers used are hydrophobic in character, the resulting emulsions are generally w/o type. Accordingly, critical factors influencing the success of this process are the good monomer solubility in the external phase and its sufficient reactivity toward the aqueous phase (Allouche, 2013).

In the case of the polymerization of alkylcyanoacrylates such as isobutylcyanoacrylate, the ions of the hydroxyl are those that catalyze the reaction and nanocapsules can be generated (Hillaireau et al., 2006). Due to its high hydrolyzable properties, lipophilic diisocyanate can also be used, properties that induce the isocyanate function conversion to amine (Anton et al., 2009). The amine function can then react with another monomer molecule and polymerization at the droplet interface can occur.

In the second strategy, the monomer is introduced directly into the droplet phase and the polymerization takes place by reacting with the continuous phase or by adding an initiator in the outer phase. The initiator may also be added in the dispersed phase and the polymerization begins when the temperature is altered. In this case, the gradual segregation of the polymer in formation toward the water/oil interface is responsible for nanocapsule generation as the polymerization proceeds (Allouche, 2013).

Another interesting procedure that could be employed is the generation of interfacial polymerization and nanoemulsion simultaneously, using solvent diffusion. Alkyl cyanoacrylate monomers, for instance, can be introduced in the oil-dispersed phase that contains an organic solvent miscible in water (Chouinard et al., 1991). Accordingly, polymerization can be initiated when the fast diffusion of the solvent to the aqueous continuous phase occurs. Therefore, the nanocapsules are formed at the same time as the nanoemulsion is formed.

Finally, the third route is the most commonly used for the generation of nanocapsules, and involves introducing reactive species of different solubilities into the continuous and dispersed phases, resulting in an interfacial reaction. Of course, the nature of the final polymer shell depends on the type of monomers added. For example, nanocapsules of polyamides (Bouchemal et al., 2006), polyuria (Lu et al., 2011), or polyurethanes (Gaudin and Sintès-Zydowicz, 2011) can be produced by this method. Some authors have shown that the polymer wall thickness does not depend on the lipophilic monomer concentration used, but it is affected by the amount of the hydrophilic monomer added (Bouchemal et al., 2006).

As can be seen, different methods have been developed for designing nanoparticles by interfacial polymerization, but the major problem of this method, which is the lack of particle size control, has not been solved.

In recent years, membrane reactors have been used to overcome this problem, since a more controlled addition of one reagent to another can be achieved. Furthermore, this versatile technique offers the possibility to prepare nanospheres or nanocapsules, allowing a selection of nanoparticle size by choosing both the appropriate membrane (pore radius, flow velocity, pore shape, transmembrane pressure, etc.) and the correct factors involved in the formulation (dispersed and continuous phase viscosity, surfactant type, etc.) (Allouche, 2013). However, the preparation of nanoparticles using membrane reactors is considered an expensive process, due to its complicated technological development, a drawback for its widespread use.

3.3.11 CONTROLLED/LIVING RADICAL POLYMERIZATION

Recently, controlled/living radical polymerization has emerged with the advantage of allowing better control of some parameters, such as mass distribution, macromolecular architecture, and end-functionalities (Le Droumaguet and Nicolas, 2010). This development was promoted by the industrial production of hydrophilic polymeric nanoparticles specifically designed for biomedical applications, as well as environmental awareness related to “green chemistry.”

Among the main methods of controlled/living radical polymerization include: nitroxide-mediated polymerization (NMP) (Nicolas et al., 2013), atom transfer radical polymerization (ATRP) (Matyjaszewski, 2012), and reversible addition and fragmentation transfer chain polymerization (RAFT) (Semsarilar and Perrier, 2010).

Both the type and the concentration of the mediating (control) agent are the main parameters that affect the nanoparticle properties. However, they also are influenced by the concentration of the monomer and its nature, as well as the initiator and surfactant used.

Using either NMP, ATRP, or RAFT and different formulations, several types of polymeric nanoparticles have been synthesized, such as poly(butylacrylate), poly(styrene), or poly(methyl methacrylate) nanoparticles. Typical nanoparticle size is in a 30–400 nm range (Allouche, 2013).

The elimination of the control agent, mainly for environmental purposes, causes additional difficulties and increases the cost of this process.

3.3.12 DESOLVATION

In this method, the macromolecular solution is destabilized and the polymer precipitates when a desolvation factor is slowly added. This desolvation factor could be a salt, alcohol, or a particle (Allouche, 2013). This method is similar to other nanoprecipitation methods, also based on a loss of solubilization of the polymer, and it

is often associated to biopolymeric nanoparticle generation. The desolvation process is generally employed for nanoparticle production of different kinds of proteins, such as human serum albumin ([Langer et al., 2008](#)), bovine serum albumin ([Raval et al., 2015](#)), gliadin ([Ramteke and Jain, 2008](#)), or gelatin ([Pawar and Pande, 2015](#)).

3.3.13 SUPERCRITICAL FLUID TECHNOLOGY

An alternative that has been investigated to prepare biodegradable micro and nanoparticles, is supercritical fluid technology, due to it being environmentally safe. A supercritical fluid is a solvent at a temperature that is above its critical point, condition at which the fluid remains in a single phase, regardless the pressure. CO₂ is the most widely used supercritical fluid (SC CO₂), mainly based on its mild critical conditions (T_c = 31.1°C, P_c = 73.8 bars), and because it is non-flammable, nontoxic, and has a low price.

Supercritical antisolvent (SAS) and the rapid expansion of supercritical solution (RESS) are the most common processing methods that use supercritical fluids. To dissolve the solute to be micronized, SAS technique employs a liquid solvent (e.g., methanol) that is completely miscible with the supercritical fluid (SC CO₂). Due to the solute being insoluble in the supercritical fluid, an instantaneous precipitation of the solute takes place, forming nanoparticles.

In the RESS process, the main difference with SAS process is that the solute is directly dissolved in the supercritical fluid (such as supercritical methanol). The solution is then abruptly expanded through a small nozzle to a reduced pressure. Accordingly, the solvent leaves its supercritical state and the solute eventually precipitates. One important characteristic of RESS is that it is a clean technique, due to the precipitate being solvent free ([Bhokare et al., 2015](#)). This technique was used to prepare trans-resveratrol SLN ([Ahmad et al., 2016](#)).

3.3.14 SPRAY-DRYING

A typical spray-drying process consists of the atomization of a liquid into very small droplets that are brought into contact with a hot drying gas to evaporate the solvent, forming a solid product, which is recovered via a cyclone separation unit ([Allouche, 2013](#)).

This technique has been used to prepare inhalable chitosan nanoparticles (CNPs) for sustained delivery of antitubercular drugs (isoniazid (INH) and rifampicin (RIF)) in the lungs ([Garg et al., 2016](#)).

3.4 LIMITATIONS/TOXICOLOGY OF NANOMEDICINES

Nanomedicine has varied applications and several benefits, but it is not a flawless medicine. As the transition from micro- to nanoparticles begins, the number of

surface atoms increase as the size range decreases greatly, and interparticular friction and sticking problems become significant. Furthermore, because of the small NP size, they may have a high clearance rate from the body, which prevents their use in diagnosis or in drug delivery (Singh and Nehru, 2008).

For NP hepatic targeting, the entrapment by the mononuclear phagocytic system is an advantage. But this property can become a problem if these nanostructures have been designed for drug action in other parts of the body. The mononuclear phagocytic system recognizes these particles, leading to a subsequent phagocytosis and final removal from the body.

Chemical reactivity of these NPs increased due to the increased surface area, creating uncertainty as to how these particles react under different conditions, and if they can cross cell membranes and enter into the cells. Conversely, the increased chemical reactivity of NPs produces ROS, which may cause oxidative stress, inflammation, and damage to proteins, DNA, and membranes, which ultimately leads to toxicity.

A major drawback of nanomedicine is that NPs have no common characteristic other than their size. Therefore, each particle must be evaluated individually. In addition, changes in shape and size can lead to different physical and chemical interactions: a substance that is nontoxic when its size is 100 nm, can become toxic when it reaches 1 nm, or vice versa.

Another drawback is the dependence of these particles on the surroundings. They may disintegrate or aggregate, resulting in size changes and causing toxicity. Other factors that may influence toxicity are the chemical composition, surface charge, surface structure, solubility, and the presence of NP functional groups (Shubhika, 2013).

Unexpected interactions of these particles can take place inside the body, leading to unpredictable reactions. Within the body, they can also behave of their own volition: enter capillaries, move from the injection site to other parts of the body, cross cell membranes, and enter undesirably into the BBB (Singh and Nehru, 2008).

According to reported studies, NPs can accumulate in the organs of several animals. In addition, it cannot be predicted whether organisms will be able to excrete NPs or will remain accumulated in the body. Usually, biodegradable NPs are excreted, but the nonbiodegradable NPs can be accumulated in the organs and potentially cause damage (Hui, 2005).

3.5 FUTURE TRENDS FOR NANOMATERIALS IN DRUG DELIVERY

In the last few years, several novel technologies have been developed for disease treatment. The majority of commercial nanoparticles applications in medicine have been geared toward drug delivery. The use of nanotechnology to develop

nanocarriers for drug release is bringing much hope and enthusiasm in the research of drug delivery systems. Scientists working in this field are searching for the combination of the ideal nanovehicle-ideal nanodrug to develop the delivery system. This could dramatically reduce drug dosage and improve its absorption, which also leads to the patient consuming a lower dose, having the same benefit.

Delivering the drug in the correct place in living systems, increases local drug concentration at the desired site and limits or eliminates the side effects. Nanocarriers can be conjugated to a ligand, such as an antibody, to favor a targeted therapeutic approach. Empty viral capsids are also being used to deliver drugs as a new therapeutic strategy. Therefore, nanoscale drug delivery systems can revolutionize the whole drug therapy strategy, bringing them into a leading position in the near future. As it stands today, the scope of this emerging field seems to be unlimited. However, the private sector and governments must adequately address the significant technological and financial obstacles before the full capacity of nanotechnology can be fully exploited. In addition, issues related to the toxicity of nanoparticle formulations should not be ignored. Complete test methods should be established to evaluate short- and long-term toxicity analysis of nanosized drug delivery systems.

Nanotechnology in biomedical sciences is expected to create innovations and play a vital role, not only in drug delivery and gene therapy, but also in molecular imaging, biomarkers and biosensors, changing the landscape of pharmaceutical and biotechnology industries in the near future.

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Target-specific delivery: An insight

4

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CHAPTER OUTLINE

4.1 Targeting.....	118
4.1.1 Background	118
4.1.2 Concept and Rationale	120
4.1.3 Target Identification.....	120
4.1.4 Levels of Targeting.....	122
4.2 Site-Specific Delivery.....	124
4.2.1 Receptors	124
4.2.2 Carriers	126
4.2.3 Ligands	140
4.3 Challenges and Obstacles.....	140
4.4 Gene Delivery	141
4.4.1 Introduction	141
4.4.2 Approaches for Gene Therapy	142
4.5 Gene Modification.....	143
4.5.1 Replacement Therapy	143
4.5.2 Corrective Gene Therapy	143
4.6 Gene Transfer Techniques	144
4.6.1 Physical Techniques	144
4.6.2 BIOLOGICAL (Viral Vectors)	146
4.6.3 CHEMICAL (Nonviral Gene Delivery)	146
4.7 Gene Transfer to Specific Cell Lines.....	146
4.7.1 Somatic Gene Therapy	146
4.7.2 Germline Gene Therapy.....	147
4.7.3 Delivery Systems in Gene Therapy.....	147
4.7.4 Candidate Diseases	149
4.7.5 Problems Associated With Gene Therapy	149
4.8 Conclusion	150
4.9 Future Perspective	150
References	151

This chapter includes following highlights:

- *Targeting*
- *Domains of targeted drug delivery systems*
- *Challenges of targeted delivery*
- *Gene delivery*
- *Approaches for gene delivery*
- *Hurdles associated with gene delivery*
- *Future Perspectives*

4.1 TARGETING

4.1.1 BACKGROUND

Drug delivery is the technique of delivering a pharmaceutical compound for a therapeutic outcome in humans and other living organisms. A drug delivery system is defined as a formulation or a device that enables the introduction of the therapeutically active substance in the body and improves its efficiency and safety by controlling the rate, time, and the place of release of drugs in the body. Some of the challenges for most drug delivery systems include: poor bioavailability, solubility, in vivo stability, intestinal absorption, prolonged and targeted delivery to site of action, therapeutic effectiveness, side effects, and plasma fluctuations of drugs which either fall under the minimum effective concentrations or exceed the safe therapeutic level. The very slow progress in the treatment of severe diseases has led to the adoption of a multidisciplinary approach to the site-specific delivery and release of drugs, underpinned by nanoscience and nanotechnology. Nanotechnology in drug delivery is an approach designed to overcome the challenges due to the development and fabrication of nanostructures at submicron and nanoscale which are mainly polymeric and have multiple advantages. Generally, nanostructures have the ability to protect drugs entrapped within them from hydrolytic and enzymatic degradation in the gastrointestinal tract; target the delivery of a wide range of drugs to various areas of the body for persistent release; and are thus able to deliver drugs, proteins and genes through the oral route. Nanostructures deliver drugs that are highly water insoluble and can bypass the liver, thereby avoiding the first pass metabolism of the encapsulated drug. They increase oral bioavailability of drugs due to their specialized uptake mechanisms, such as absorptive endocytosis, and are able to stay in the blood circulation for a longer time. This liberates the incorporated drug in a sustained and constant manner, leading to low plasma fluctuations and thereby minimizing side-effects caused by drugs. For targeted delivery, nanostructures can be conjugated with targeting moieties, such that the linkage between the polymer and the active substance can be modified to control the site and duration at which the drug is liberated. The linkage can be accomplished by inclusion of amino acids, lipids, peptides, or small chains as spacer molecules (Khanna, 2012; Kayser et al., 2005).

Targeting is signified when a site is differentiated from the other compartments, where toxicity may take place, and also if the active drug could be located predominantly in the proximity of target site. Targeted drug delivery is a method of delivering therapeutically active agent in a pattern that considerably enhances the concentration of the medication in some parts of the body in comparison to others. It implies selective and effective localization of a pharmacologically active moiety at a predetermined target in therapeutic concentration, while confining its access to nontarget cellular linings, thus minimizing toxic effects and maximizing therapeutic index. This mode of delivery is the establishment of nanomedicine, which plans to employ nanocarrier-associated drug delivery in order to overcome the demerits of conventional drug delivery. These nanocarriers would be entrapped with drugs and directed to defined regions of the body where there is exclusively diseased tissue, thereby avoiding interaction with healthy tissue. The goal of a site-specific drug delivery system is to extend, limit, target, and schedule a drug interaction with the infectious tissue. The merits of targeted release systems include: a decrease in the dose frequency, consistent performance of the drug, reduced side-effects, and decrease in the fluctuation of plasma drug levels. These systems are not cost-effective, resulting in poor productivity and decreased dose regulation. A targeted drug delivery system (TDDS) is based on a method that delivers a definite amount of a therapeutic agent for an extended period of time to the diseased area within the body. This helps to maintain the required plasma and tissue drug levels, thereby avoiding any damage to the healthy body organs from the drug. It is an integrated drug delivery system, which requires various disciplines, such as chemistry, biology, biotechnology, and genetic engineering to join efforts to optimize this system (Brunner, 2014).

In conventional drug delivery systems, such as oral and parenteral, the drug is diffused throughout the body via systemic blood circulation. For most drugs, only a small portion of them reaches the affected organ, such as in chemotherapy where roughly 99% of the drugs administered do not reach the tumor site. Targeted drug delivery seeks to accumulate the medication in the preidentified tissues, while promoting depletion in the concentration of the medication in the nontargeted tissues. By evading the host's defense systems and hindering nonspecific distribution in the liver and spleen, a system can reach the anticipated site of action in higher concentrations. Targeted delivery is believed to improve efficacy while minimizing undesired and detrimental effects. The objective of drug targeting is to achieve a desired pharmacological response at as elected site without undesirable interactions at other sites. Other objectives of targeted drug delivery are to ensure safety and to improve efficacy of drugs with good patient compliance. This can be attained by better control of plasma drug levels and less frequent dosing (Tiwari et al., 2012; Gokhan et al., 2011).

4.1.2 CONCEPT AND RATIONALE

Most drugs do not have special affinity toward disease sites in the body, hence they compromise healthy cell function nonspecifically and eventually cause harmful side effects. Selective targeting can address toxicity issues and decrease side effects originating from normal cell-drug nonspecific interactions. The rationale for targeted drug delivery is to modify the pharmacokinetics and pharmacodynamics of pharmacologically active moieties by using novel drug delivery systems or by altering the molecular structure and/or physiological parameters inherent in a selected route of administration. It is inevitable that the extent of drug action becomes more a design property of the drug molecule's inherent kinetic properties. If one wishes to conceptualize the ideal drug delivery system, two prerequisites would come to mind. First, it should transport drug at a rate dictated by the requirements of the body over the period of treatment. Second, it should conduit the active entity exclusively to the site of action. This may necessitate delivery to specific receptors (as in the case of H_1 and H_2 antagonists), localization to tumor cells, and to specific areas of the body, as for arthritis or gout. Drug targeting can be considered as a form of controlled release in that it exercises spatial control of drug release within the body. Following are the reasons for the intense interest of researchers in target drug delivery:

1. Recognition of the possibility of patenting successful drugs by applying the concepts and techniques of TDDSs, coupled with the increasing expense in bringing new entities to market, has encouraged the development of new drug delivery systems.
2. New systems are desirable to deliver the novel, genetically engineered pharmaceuticals i.e., peptides and proteins, to their sites of action without experiencing major immunogenicity or biological inactivation.
3. Treating enzyme deficient diseases and cancer therapies can be enhanced by better targeting.

Drug targeting is achieved by one of two approaches. The first approach involves chemical variation of the parent compound to a derivative which is activated only at the intended site. The second approach exploits nanocarriers such as nanospheres, nanoparticles, liposomes, microsphere, microballoon, carbon nanotubes, quantum dots, antibodies, cellular carriers and macromolecules to direct the drug to its site of action (Ochekpe et al., 2009; Robinson and Lee, 2009).

4.1.3 TARGET IDENTIFICATION

A target could be described as a cell or group of cells in minority, identified to be in need of treatment. Cell surface antigens, are cell surface receptors, which recognize and internalize the macromolecular ligands, associated carrier and cellular oncogenes, serve as molecular targets. Molecular targets represent the most specific form of protein and peptide drug targeting. Molecular targets provide a

means for “active” drug delivery; i.e., the site to which the drug is ultimately delivered depends on the molecular specificity of the targeting agent more than the physical characteristics of the drug or the drug delivery system (Pandey et al., 2004).

4.1.3.1 Drug targets in the body

The main targets in the body can be summarized as follows:

Receptors on cell membranes: Receptors on cell membranes allow specific interaction of drug carriers with cells, facilitating their uptake via receptor-mediated endocytosis. Folate receptors, which are distinctively overexpressed in cells of cancers with epithelial origin, are applied to tumor-specific drug delivery in cancers, including breast, ovary, brain, and lung malignancies. Furthermore, as peptide receptors are expressed in massive quantities in some tumor cells, peptides/peptide analogs are conjugated to a drug carrier to allow tumor-specific aiming of cytotoxic agents, ensuring interaction with peptide receptors.

Lipid components of cell membranes: Interaction of synthetic phospholipid complements with cellular membranes alters the lipid composition, membrane permeability, and flexibility, which ultimately affects the signal transduction mechanisms, inducing apoptotic cell death.

Antigens or proteins on cell surfaces: The ailing cells either express new proteins or exhibit disparate (under/over) expression of the proteins found on normal cells. Monoclonal antibodies are used against these proteins. A usable tumor-specific antigen for drug targeting is the one expressed solely and similarly by all tumor cells; erbB2, a growth factor overarticulated in 20%–30% human breast adenocarcinomas on the tumor cell surface, has been used for immunotherapy with liposomal doxorubicin coupled to anti-erbB2-antibody. The reason for its attractiveness is its ready accessibility, low expression on normal cells, and a steady delivery within the tumor.

There are several approaches to discovering new prospective drug targets. Two conceptually divergent approaches are: genomic and genetic approach.

4.1.3.2 Genomic approach

The genomic advances involves the distinct expression of mRNA in normal versus disease tissue or gene expression modulates after a compound has been added to the culture medium of cells or given to an animal. Many groups are now using microarray technology and proteomics to identify the changes in gene expression. This approach assumes that the expression of relevant targets will be diverse in diseased tissue and after treatment.

4.1.3.3 Genetic approach

One interesting approach in the post–human genome sequencing era is the genetic investigation of human and model organisms. The genetic approach is more long-term and exhaustive but has better potential.

4.1.4 LEVELS OF TARGETING

There are four levels of drug targeting, depending on the nature of target moiety. First-order targeting is also called organ level targeting as the target is tissue or organ. An example of such targeting is drug-eluting stents that deliver drug to the blocked artery.

In the case of second order targeting or cell-level targeting, the intended target is a definite cell. When an antibody is attached to a delivery system, it exclusively identifies and couples to a specific antigen on a cell surface. Most of the recent nanoparticulate systems are projected to target-specific cells. The third order targeting or intracellular-level targeting focuses on targeting the intracellular compartments. Fourth level of targeting or molecular-level targeting refers to locating a precise molecule inside the cell. Gene delivery systems are one of the examples of this type of targeting (Vyas and Khar, 2002).

Apart from having the knowledge of various levels of drug targeting one should also know about various schemes (Fig. 4.1) of vectoring the drug to the target site.

4.1.4.1 Passive targeting

This process utilizes the inherent route of biodistribution of the transporter system, through which it concentrates in the organ compartment(s) of the body. It is the result of the body's natural response to the physicochemical properties of the drug or drug-carrier system. The capability of some colloidal transporters to be taken up by the reticuloendothelial framework (RES), particularly in liver and spleen, has made them extreme vectors for latent hepatic focusing of medications of these compartments. This class of targetable tools incorporates medicines consisting of bilayer vesicular systems to cell transporters of micron or submicron size range. The compliant targetability of these frameworks is because of the acknowledgment of these exogenous particulates, either in integrated or in



FIGURE 4.1

Various schemes of target sites.

opsonized form, by the phagocytic cells of the RES. A noteworthy disadvantage of microparticulate carriers is that they cannot pass endothelial cell lines. For example, drug or drug carrier nanosystems can be passively selected, making use of the pathophysiological and anatomical opportunities which include targeting of antimalarial drugs for treatment of leishmaniasis, brucellosis, candidiasis (Chou et al., 2011; He et al., 2010).

4.1.4.2 Active targeting

The active targeting uses modification or manipulation of drug carriers to redefine its biological outcome. Active targeting is the facilitation of the binding of the carrier to anticipated cells through the use of ligands to enhance receptor-mediated localization of the drug and target-specific drug delivery. It can be attained by ligand-based targeting and physical targeting (trigger release).

Targeting components are leading molecules themselves (bioconjugates) or attached as ligands on delivery system. Colloidal carrier systems can be precisely designed using various biologically significant molecular substances, including polypeptides, antibodies, oligosaccharides, and viral-mediated proteins. The engineered carrier selectively delivers the drug to the cell or set of cells, referred to as the target. The cascade of events in ligand-associated drug delivery is termed ligand-motivated receptor-mediated targeting.

Physical targeting is selectively programmed and monitored externally with the help of physical means. In this, some uniqueness of the bioenvironment is used to direct the carrier to a definite site or to cause discriminating release of its contents. The foremost advancement reported is temperature-receptive liposomes, which were applied to tumors. The release of drug in the vicinity of tumor is then brought by serum components which, at phase transition, induce drug release. An example of active targeting is the use of monoclonal antibody for the treatment of cancer (Ruoslahti et al., 2010).

4.1.4.3 Reverse targeting

As the name implies, in reverse targeting, instead of the drug delivery system seeking its target, attempts are made to attract the target cells toward the drug delivery system. The concept is inspired from the natural process of immune activation at the site of injury through secretion of signaling molecules such as chemokines.

It is based on attempts to discourage RES for passive uptake of colloidal carriers, which may lead to the reversion of the biodistribution flow of the carrier; consequently the process is called reverse targeting. One strategy applied to achieve this is to restrain the function of RES by a preinjection of a large amount of colloidal carriers or macromolecules which leads to RES blockade.

4.1.4.4 Dual targeting

Dual targeting strategies can be divided into two types: those that directly act on target structures, like cell surface receptors; and others, which use dual targeting

for delivery of a therapeutically active agent. The first type includes binding and neutralization of two receptors or two ligands, activation of two receptors, and neutralization of a receptor and one ligand. The second type involves simultaneous targeting of liposomes and nanoparticles.

4.1.4.5 Double targeting

This can be defined as the combination of site- and time-controlled drug delivery. The combination results in an improved therapeutic index. For this phenomenon, two effects are responsible. First, if drug release or activation occurs locally at therapeutic sites, selectivity is increased by a multiplication of the spatial selectivity with local activation. The second effect is the improvement in the therapeutic index by a combination of a spatially discriminating delivery and a desirable release pattern for a drug, such as zero-order release for a longer period of time (Vyas and Khar, 2002).

4.2 SITE-SPECIFIC DELIVERY

For achieving site-specific drug delivery, novel constructs are to be designed which consists of following components: receptors, carriers, and ligands.

4.2.1 RECEPTORS

Cell surface receptors are complex transmembrane proteins, which intervene highly specific communications between cells and their extracellular milieu. Receptors are cellular markers and play an integral role in the regulation of cellular functions, including growth differentiation, metabolism, secretion, contraction, and migration. The two most common functions for receptors are to mediate the trafficking of their specific ligands and to transduce and control transmembrane signaling. Blood cell receptors are comprised of a diverse range of cell surface lectins, immunoregulatory molecules, and several macromolecular ligand–recognizing receptors.

Some types of receptors in the human body are as follows:

4.2.1.1 Carbohydrate-specific (lectin) receptors

Endogenous lectins are found on many normal and malignant cells, and are involved in various biological functions, acting as specific receptors and/or mediating endocytosis of precise glycoconjugates. The existence of membrane lectins as carbohydrate-specific receptors present in the cell membrane (which recognize carbohydrate epitopes present on membrane glycosphingolipids and glycoproteins) suggests their clear role in cellular adhesion and recognition processes. Membrane lectins usually distinguish specific complex oligosaccharides with an elevated affinity and avidity, while simple sugars do so with low avidity.

4.2.1.2 Asialoglycoprotein receptors

Asialoglycoprotein receptors (ASGPR) have also been termed mammalian hepatic lectin, galactose-specific receptors, or “galaptins” and are completely characterized as hepatocyte-specific receptors. Different liver cells express specific galactose receptors and interact with specific ligand class. Hepatocytes avidly take up molecular and small-sized particulate ligands via ASGPRs, whereas endothelial cells take up small- and intermediate-sized galactose-terminating ligands.

4.2.1.3 Mannose receptors

Cell surface receptors for mannose or *N*-acetyl galactosyl mannose-terminated (neo) ligands, including lysosomal hydrolysis, are expressed on sinusoidal cells and Kupffer cells of the liver. Mannose receptors are also found on alveolar and peritoneal macrophages, cultured circulating mononuclear cells and bone marrow cells. Coupling of mannose residues to the liposomal surface augments their systemic clearance. The rapid uptake of liposomes appended with mannose-terminating ligands by macrophages suggests them to be an ideal carrier system for macrophage targeting.

4.2.1.4 Lymphocyte-homing receptors

These belong to a class of proteinaceous lectins, which include the serum mannose-binding protein and mannose receptor on various tissue macrophages. They interact with tissue-specific adhesion molecules on high endothelial venule's direct recirculation of lymphocytes in a tissue-specific fashion. Homing receptor lectins on lymphocytes are probably the only member that undergoes phagocytosis and mediates the extravasation and extracellular release of drugs. Along with the aforementioned receptors, cell surface receptors expressed for immunoregulatory molecules are as follows:

4.2.1.5 Fc receptors

Fc receptors are situated on the surface of macrophages and neutrophils. Immunoglobulins clustered on the surface of the target cell expose their tail regions (Fc) to be recognized by the Fc receptors.

4.2.1.6 Interleukin receptors

Interleukins and interferons constitute a family of potent lymphokines/cytokines that activate cells of the immune system. It has been demonstrated that IL-2, IL-5, IL-7 and IL-12 have markedly different affinity for ligands.

4.2.1.7 Lipoprotein receptors

Lipoprotein receptors are instrumental for the transport of cholesterol and other lipids with their subsequent organization in the intracellular compartments.

4.2.1.8 Scavenger receptors

Scavenger receptors are expressed on endothelial liver cells and macrophages and mediate the uptake of a variety of polyanionic proteins/ligands or macromolecular complexes. They are capable of recognizing a number of structurally diverse polyanionic ligands, such as modified protein, polysaccharides, polynucleotides, polyvinyl sulfate, and bacterial lipopolysaccharide. The broad ligand specificity of scavenger receptors for polyanionic compounds make them striking entrant for mediating the bonding and intracellular uptake of negatively charged particulate systems.

4.2.1.9 Transferrin receptors

Transferrin is a major mammalian serum glycoprotein, transporting iron from sites of absorption and storage to tissue cells. It presents on all the growing cells, rendering these cells as target modules, selectively approachable through transferrins as carrier molecule. A wide range of cell types including hepatocytes, fibroblasts, and blood brain endothelial cell lines express the transferrin receptors.

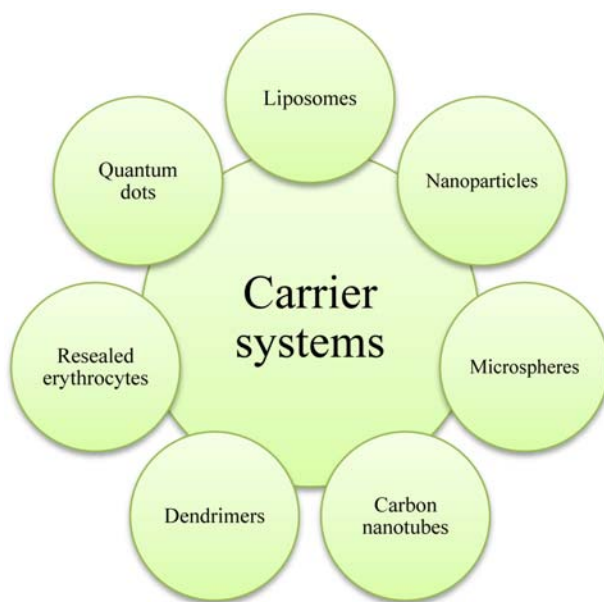
4.2.1.10 Folate receptors

A folate receptor is a glycosyl-phosphatidylinositol-bounded membrane protein. It mediates the cellular uptake of folate and the related coenzymes via receptor-mediated endocytosis. Ligand binding to folate receptors stimulates their endocytic uptake by a clathrin-independent pathway that has been used to negotiate a nonspecific uptake of macromolecules and particulate drug delivery systems (King and Feener, 1998).

4.2.2 CARRIERS

A carrier is one of the most significant entities required for successful delivery of the entrapped drug. They segregate, carry and hold drugs en route, while delivering it within a target. The perfect drug carrier developed as a targetable tool should possess the following hallmarks:

- capable of traversing anatomical barriers;
- it must be identified specifically and selectively by the intended cells and must conserve the avidity and precision of the surface ligands;
- the drug–ligand association should be stable in plasma, interstitial and other biofluids;
- carrier should not be toxic or immunogenic, easily biodegradable and, after recognition and internalization, the carrier system should liberate the drug molecule inside the target;
- bioactives used for carrier routing and site identification is not supposed to be common otherwise it may cross over the sites, failing the concept of targeting.

**FIGURE 4.2**

Types of carrier systems.

There are several carriers (Fig. 4.2) which are used for targeted delivery of drugs and bioactives. In upcoming sections, the carrier systems are discussed (Vyas and Khar, 2002).

4.2.2.1 Liposomes

Liposomes are vesicles constituted by an aqueous core surrounded by one or several phospholipid bilayers. Hydrophilic drugs or active components can be assimilated into the inner aqueous cavity and lipophilic drugs imbibed into the phospholipid layer. The pioneering work by Bangham demonstrated that phospholipids dispersed in water can be organized in multilamellar vesicles, identified as liposomes. Later, Gregoriadis instigated the concept of drug delivery using liposomes to carry enzymes, proteins, and drugs. Liposomes are spherical vesicles with particle size in nano to micro range. They are comprised of single or multiple lipid bilayers enclosing an aqueous core, where the polar head groups are aligned in the passage of the interior and exterior aqueous phases. Liposomes can entrap both lipophilic and hydrophilic components, circumvent degradation of the entrapped moiety, and liberate the therapeutic moieties at designated targets.

Depending upon their size and number of bilayers, liposomes are categorized as unilamellar vesicles and multilamellar vesicles. The vesicle consists of a single

phospholipid bilayer spheroid enclosing the aqueous solution. Typically, numerous unilamellar vesicles are formed on the inside of the other, making a multilamellar vesicles of concentric phospholipid globules separated by layers of water (Huang et al., 2014; Akbarzadeh et al., 2013).

A typical liposome and their methods of preparation are shown in Figs. 4.3 and 4.4.

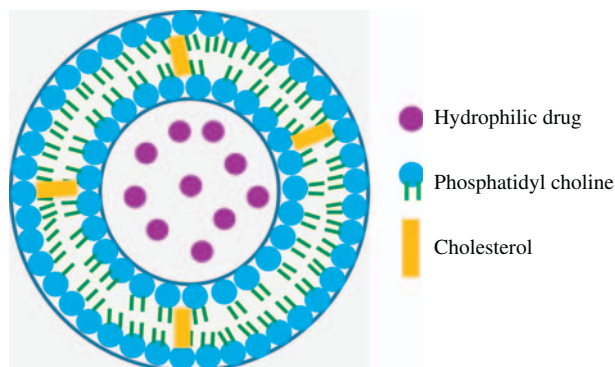


FIGURE 4.3

Diagrammatic representation of liposomes.

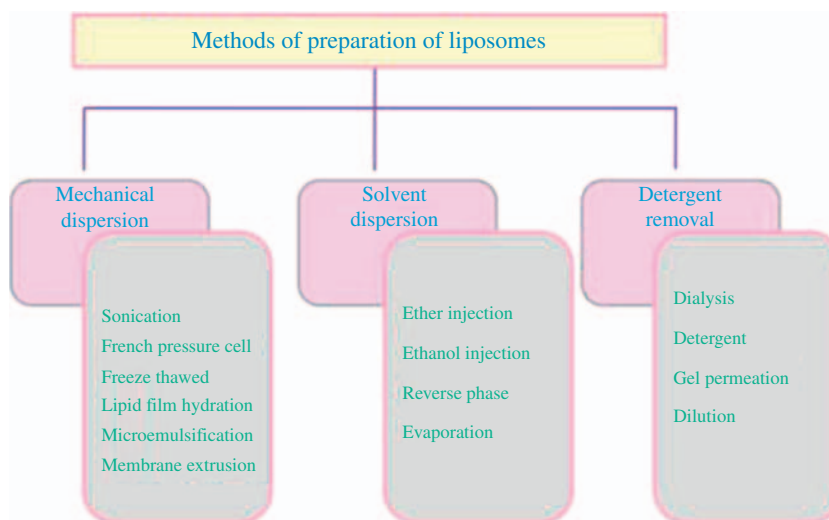


FIGURE 4.4

Flow chart of preparation of liposomes using several approaches.

4.2.2.2 Drug entrapment in liposomes

In liposomes, drug can be entrapped either passively (during formation of liposomes) or actively (after formation of liposomes). Lipophilic drugs can be loaded into liposomes during vesicle formation. Trapping efficiency of 100% can be achieved, but this solely depends upon the solubility of the drug in the liposome membrane. Passive entrapment of hydrophilic drugs depends on the ability of liposomes to entrap aqueous buffer, comprising a dissolved drug, during vesicle formation.

Trapping effectiveness (generally <30%) is restricted by solubility of the drug and the entrapped volume enclosed in the liposomes.

4.2.2.3 Liposomal transportation

The interaction of liposome drug carriers with cells and their fate after in vivo administration defines its constraints and benefits. The reported studies have indicated that the major interaction of liposomes with cells is through simple adsorption (through cell-surface components, electrostatic forces, or weak hydrophobic interactions) or engulfment (phagocytic cells of the RES such as macrophages and neutrophils). The other possible interaction is the swapping of bilayer component with cell membrane constituents ([Mozafari, 2005](#)).

4.2.2.4 Applications of liposomes

Liposomes have wide applications in pharmaceutical field, some of them are summarized here:

- drugs from liposomes can be delivered systemically as well as locally.
- they are ideal vehicles for macrophage targeting, which can be utilized in the delivery of drugs in parasitic diseases such as leishmaniasis.
- they possess versatile multifunctional properties due to which they are most preferable drug carriers for passive as well as active targeting.
- they also have promising potential in delivery of peptides, proteins and, oligonucleotides.
- drugs loaded in liposomes display reduced toxicities and preserve improved efficacy, compared with free counterparts ([Allen and Cullis, 2013](#)).

4.2.2.5 Dendrimers

A dendrimer is a symmetrical three dimensional structure which offers a high degree of surface area for functionalization.

The first concerted effort to create and design dendritic structures by organic synthesis was carried out by Vogtle et al. in 1978. However, in the early 1980s Donald Tomalia and his coworkers had worked in this field at Dow Chemicals. The first synthesized dendrimers were polyamidoamines (PAMAM) (Starburst Dendrimers). They possess three separate architectural components, namely: an initiator core; interior layers called generations, composed of repeating entities, radically attached to the initiator core; and exterior (terminal functionality)

anchored to the outermost interior generations. Dendrimer generation is the hyper-branching from the core of the dendrimer toward the periphery, resulting in homostructural layers packed between the branching points. The number of branches in the direction of the dendrimer surface is known as the generation number. If a dendrimer molecule possess four focal points while moving from the center to the periphery, it is denoted as a fourth generation dendrimer.

Dendrimer drug delivery systems are getting wide interest as an advantageous solution for delivering bioactives, such as drugs and gene. They provide a base for the attachment of drugs or genes and their release through several mechanisms. Various applications of dendrimers have been explored during the last three decades. Development in controlled polymerization and synthesis techniques have led to the emergence of well controlled dendrimer structures with a large number of surface groups that can be utilized to display a range of biological molecules including peptides, proteins, sugars and targeting agents. The high loading capacity of dendrimers renders them highly attractive as carriers for the delivery of chemotherapeutic agents. PEGylated and non-PEGylated dendrimers proved to encapsulate hydrophobic drug molecules into the void of their branching architecture, which enhanced the aqueous solubility and stability of the encapsulated drug molecules (Malik et al., 2012; Barbara and Maria, 2001; Tripathy and Das, 2013).

4.2.2.6 Drug-loading techniques

Simple encapsulation: The ellipsoidal or sphere shape, empty internal cavities, and open nature of the architecture of dendrimers make it feasible to directly enclose new molecules into the macromolecule interior. These empty internal cavities are hydrophobic in nature, which make it suitable to interact with poorly soluble drugs through hydrophobic interactions. Moreover, the nitrogen or oxygen atoms in the internal cavities can interact with the drug molecules by hydrogen bond formation (Brownlie et al., 2004).

Electrostatic interaction: The huge bulk of functional groups, such as amine groups and carboxyl groups, on the surface of dendrimers have potential applications in enhancing the solubility of hydrophobic drugs by electrostatic interaction. The G3 PAMAM dendrimer with an ammonia core is taken as an example. It has a much elevated amino group density when compared with conventional linear polymers (Neerman et al., 2004).

Covalent conjugation: The drug molecules can be covalently attached to the periphery of the dendrimer to form dendrimer prodrugs. Dendrimers can be used as dendritic boxes and unimolecular micelles for the incorporation of hydrophobic/hydrophilic molecules by host–guest interactions inside their empty cavities present around core. The micellar structure of these molecules is maintained at all the concentrations because the hydrophobic segments are covalently connected, which is not possible with polymeric micelles (Neerman et al., 2004; Madaan et al., 2014).

4.2.2.7 Mechanism for drug transport

There are broadly two mechanisms for drug delivery. The first one is by in vivo degradation of drug dendrimer covalent bonding, which depends on the incidence of appropriate enzymes or an environment capable of cleaving the bonds. The second one is by releasing the drug due to changes in physical environment, such as pH or temperature. This approach is independent of external factors and takes place in the outer shell of the receptor (exoreceptor) or cavities of the core (endoreceptor) (Abbasi et al., 2014).

4.2.2.8 Applications

Transdermal drug delivery: Dendrimers have been found to improve solubility and plasma circulation time via transdermal formulations and to transport drugs competently. PAMAM dendrimer complexes and NSAIDs (e.g., ketoprofen, diflunisal) have been reported to improve drug access through the skin as penetration enhancers.

Oral drug delivery: Studies for oral drug delivery, using the human colon adenocarcinoma cell line, Caco₂, have specified that low generation dendrimers cross cell membranes, through a combination of two processes: paracellular transport and adsorptive endocytosis.

Ocular drug delivery: Dendrimers can be employed for ocular drug delivery by synthesizing hydrogels, cross-linked networks that increase in volume in aqueous solution and are more like living tissue than any other synthetic compound. Furthermore, by adding polyethylene glycol groups to the dendrimers, these hydrogels have applications including production of cartilage tissue and for sealing ophthalmic injuries (Spataro et al., 2010).

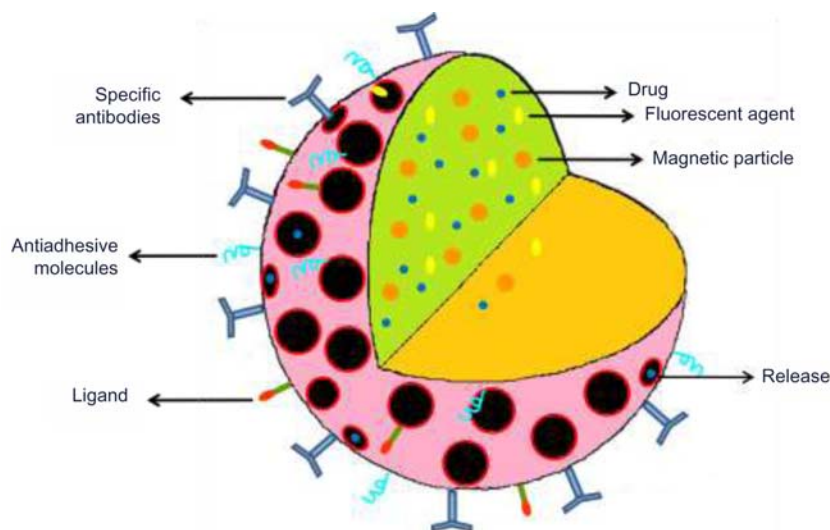
As nanodrugs: Dendrimers possess some antiviral properties due to their functional groups. Some studies indicated that poly(lysine) dendrimers reformed with sulfonated naphthyl groups have antiviral potential

Transfection

Dendrimers have potential to perform as vectors in gene therapy. A transfection reagent called SuperFect, consisting of activated dendrimers, is commercially available (Medina and Mohamed, 2009; Yang and Kao, 2006).

4.2.2.9 Nanoparticles

Nanoparticles are solid, colloidal particles composed of macromolecular substances that vary in size from 10 to 1000 nm. However, particles >200 nm are not heavily pursued and nanomedicine often refers to devices <200 nm (i.e., the width of microcapillaries). Some advantages of nanoparticles are: improved bioavailability by escalating aqueous solubility, enhancing half-life for clearance, increasing selectivity for its cognate receptors, and drug targeting to specific location in the body. For specific requirements of targeted delivery, they can be

**FIGURE 4.5**

Schematic representation of surface-modified nanoparticles.

surface-modified easily (Rodrigues et al., 1995). The Fig. 4.5 represents surface-modified nanoparticles.

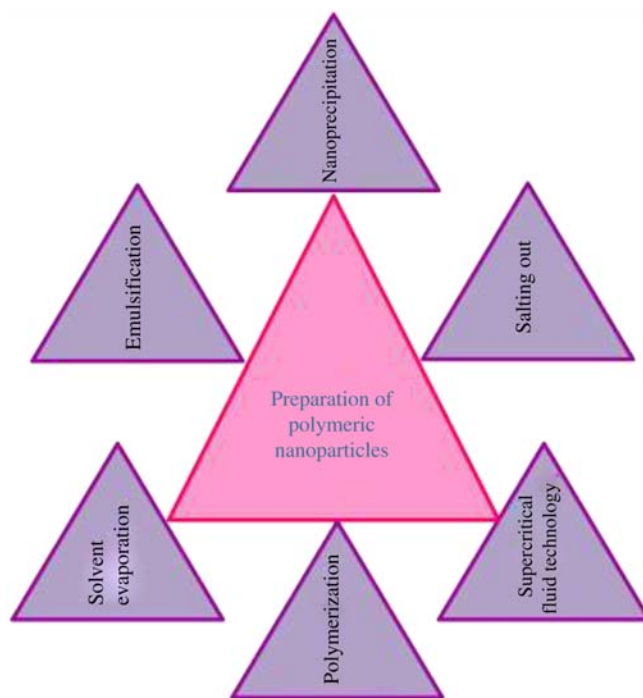
A schematic representation of method of preparation of polymeric nanoparticles is shown in Fig. 4.6.

4.2.2.10 Release mechanism from nanoparticles

The mechanisms responsible for drug release from nanoparticles are controlled diffusion or abrasion from the interior across the polymeric matrix. The membrane coating acts as a barrier to release, so it can be concluded that the solubility and diffusivity of drug in polymer membrane are determining factors in drug release. For the development of a successful nanoparticulate system, both drug release and polymer decomposition are essential consideration factors. Conclusively, drug release rate is a function of solubility, surface adsorption, diffusion through the nanoparticle grid, nanoparticle matrix abrasion, and combination of erosion/diffusion process. Accordingly, solubility, dispersal, and biodegradation of the matrix ingredients regulate the release process (Allemann et al., 1993; Fessi et al., 1989).

4.2.2.11 Applications

Promising drug delivery system for peptides and proteins: An increasing number of new molecules of biotechnological origin, such as hormones, monoclonal antibodies, and vaccines and their therapeutic potential, make protein delivery an important area of research. Protein stability is the balancing result between

**FIGURE 4.6**

Preparation methods of nanoparticles.

destabilizing and stabilizing forces. The development and stability of the secondary, tertiary, and quaternary structures of proteins are based on weak noncovalent interactions (e.g., hydrogen bonding, electrostatic attractions, van der Waals forces, and hydrophobic interactions). Interference in any of these interactions will swing this delicate balance and destabilize the proteins. Therefore, the chemical and physical stability of proteins can be compromised by environmental factors, such as temperature, pH, high pressure, nonaqueous solvents, ionic strength, metal ions, detergents, adsorption, agitation, and shearing.

In dermatology: Nanoparticles have great potential in dermal applications like controlled drug release to skin and skin appendages, targeting of hair follicle-specific cell populations, transcutaneous vaccination, and transdermal gene therapy.

Dermal drug delivery: Dermal drug delivery with lipid nanoparticles is of particular interest in hair follicle diseases, in order to enhance the local bioavailability of API at their drug target. Several targets were recognized for drugs in the hair follicle. For instance, isotretinoin causes a cell cycle arrest and apoptosis in sebocytes. Regarding the regularity aspect, topical application is relatively unproblematic. The major advantages for topical products are the defensive properties of SLNs for chemically labile drugs against degradation, and the blocking effect due to film formation on the skin.

For gene delivery: Nanoparticles laden with plasmid DNA could also serve as an efficient sustained release gene delivery system, due to their quick escape from the degradative endolysosomal compartment to the cytoplasmic compartment.

In ocular delivery: The basic problem of ophthalmologic formulation is the fast removal from the eye, which implies clearance of the applied drug through the nose. It could be shown that an increased adhesiveness is available for nanoparticles, leading to higher drug levels at the desired site of action. However, the problem was that the nanoparticles are of limited toxicological acceptance.

As oral drug delivery: Per oral administration forms of nanoparticles may include aqueous dispersions or nanoparticle-loaded conventional dosage forms, e.g., tablets, pellets, or capsules. The microclimate of the stomach aids particle aggregation due to the high ionic strength and acidity. It can be expected that food will have a large impact on performance.

Implantable delivery systems: Implantable drug-delivery systems provide new means for attaining therapeutic drug values over entire treatment durations in order to augment drug action. Magnetic nanoparticles are good candidates for being implanted and used for the targeted delivery of antitumor agents. They can be localized on a desired region, reducing collateral effects, and improving the efficiency of the chemotherapy.

Pulmonary drug delivery: Due to large surface area and the negligible barriers hampering access to the lung periphery, the organ is a suitable portal for a variety of therapeutic interventions. Nanoparticles can be designed in both, dispersed liquid droplet dosage forms such as metered dose inhalers and nebulizers, and dry powder formulations. Nanoparticle formulations possess benefits over conventional dosage forms, such as improved dissolution properties and the potential for intracellular drug delivery. Precisely, drug nanoparticles conjugates, polymeric nanoparticles, and drug-loaded liposomes offer some promising outcomes for delivering drugs to and through the lungs (Bailey and Berkland, 2009).

Drug discovery: Nanoparticles help in identification and validation of target by recognizing the protein present on the target surface. Nanoparticles will enhance drug delivery process, through miniaturization, automation, momentum and consistency of assays.

Molecular diagnostics

Molecular diagnosis includes: representation, characterization and quantification of subcellular biological processes include gene expression, protein-protein interaction, signal transduction, and cellular metabolism. Nanoparticles are used in magnetic resonance, optical, ultrasonic, and nuclear imaging. They are also employed in specific labeling of cells and tissues, useful for long-term imaging, multicolor multiplexing, dynamic imaging of subcellular structures, fluorescence resonance energy transfer, and magnetic resonance imaging (MRI).

Biosensor and biolabels: Biosensors are employed for recognition and estimation of various pathological proteins and physiological-biochemical indicators

linked with disease or interrupted metabolic conditions of body. A biosensor is a measurement system which has a probe with a sensitive biological recognition element or bioreceptor, a physiochemical detector component, and a transducer to amplify and transducer these signals into measurable form (Chen et al., 2002, 2014; Peltonen et al., 2002; Gomez et al., 1998).

4.2.2.12 Quantum dots

The quantum dots are semiconductor and coreshell nanocrystals, containing an interface between different semiconductor materials. The size of quantum dots can be constantly adjusted from 2 to 10 nm, which, after polymer encapsulation, generally increases to 5–20 nm in diameter. Particles <5 nm are rapidly cleared by renal filtration. Semiconductor nanocrystals have distinctive and captivating optical properties and become a vital module in biomedical research, for multiple, quantitative, and long-term fluorescence imaging and detection. The core of quantum dots serves as the structural scaffold, and the imaging contrast agent can be entrapped between the inorganic core and the amphiphilic polymer coating layer. This fully integrated nanostructure may act as panacea that will not only identify, but bind to diseased cells and treat it (Choi et al., 2007; Michelet et al., 2005).

4.2.2.13 Applications

As carriers with integrated utilities: In the quantum dot core, small molecule of lipophilic drugs can be implanted between the inorganic core and the amphiphilic polymer coating. Hydrophilic therapeutic agents like antibodies, peptides, aptamers, small interfering RNA (siRNA) and antisense oligodeoxynucleotide (ODN) can be trapped onto the hydrophilic side of the amphiphilic polymer.

Tumor cell markers: Quantum dots offer excellent traceable drug delivery. In such type of delivery, labeling a conventional drug carrier such as poly(lactic-co-glycolic acid) and polyethyleneimine (PEI) with quantum dots serve as photostable fluorescent reporters. They have been used to label both organic and inorganic drug carriers and potentially even bacteria and viruses, with a significant expansion of activity in the area of ODN and siRNA delivery.

Bimodal molecular imaging: Paramagnetic quantum dots were functionalized by conjugating them with cyclic RGD peptides and were successfully targeted to human endothelial cells in vitro. This nanoparticulate bimodal contrast agent may be of great use for the detection of (tumor) angiogenesis (Ghasemi et al., 2009).

Recognition of pathogen and toxin: Several different pathogens have been targeted to date, including *Cryptosporidium parvum*, *Giardia lamblia*, *Escherichia coli*, *Salmonella typhi* and *Listeria monocytogenes*. Quantum dots also attach to molecular structures that are exceptional to the virus coat and the cells that it infects. Rapid and sensitive diagnosis of respiratory syncytial virus (RSV) is important for controlling infection and development of antiviral therapy. Antibody-conjugated quantum dots swiftly and sensitively detect RSV. Quantum dot systems can detect the occurrence of particles of the RSV in a matter of hours (Jamiesona et al., 2007).

Surgical guidance: Quantum dots have a potential surgical utility by providing optical regulation that can result in the reduction of cancer metastases. They offer sensitivity and stability superior to conventional methods.

Detecting apoptosis: By combining a quantum dot with a novel transporter of the MRI agent, gadolinium, which can recognize apoptosis, using both MRI and fluorescence imaging is designed. This unit can offer anatomical information using MRI and cellular level information using fluorescence imaging. Imaging programmed cell death in the body could provide an indication that an antitumor therapy is indeed killing cancer cells.

Tumor cell markers: There are two approaches by which quantum dots locate and mark tumor cells. Quantum dots can be coupled with tumor-specific active binding sites to attach themselves to tumor cells in active targeting. Immunofluorescent probes are manufactured with antibodies to detect these tumors. In passive targeting, the quantum dot probes do not have the tumor-specific active binding sites. Instead, certain properties of the tumor cells are used. The growth rate of tumor cells greatly surpasses that of normal cells and thus the membranes of such cells are more permeable (Cheki et al., 2013; Modani et al., 2013; Alireza et al., 2012; Wang and Chen, 2011).

4.2.2.14 Carbon nanotubes

Carbon, one of the most magnificent elements has revolutionized material science because of its unique qualities viz. strongest fibers, electrically conducting (e.g., graphite electrodes), carbon–carbon composites, porous gas absorbers (activated carbon), best solid lubricant (graphite), an essentially amorphous impermeable stuff (vitreous carbon), the hardest material (diamond), and now the most intriguing substance, the fullerene. Fullerenes are currently synthesized and used in research; they include buckyballs, carbon nanotubes, carbon nanobuds, and nanofibers. Carbon nanotubes are tiny tubes $\sim 10,000$ times thinner than a human hair, composed of spun sheets of carbon hexagons. They are the rigid and sturdy fibers, have significant electronic properties, and many other unique characteristics such as high flexibility, specific surface area, low density, and superior optoelectronic characteristics. Nanotubes are categorized into two types, namely single wall carbon nanotubes (SWNTs) and multiwall nanotubes (MWNTs). The salient features of these nanotubes are briefly viewed in Table 4.1.

The bonding system between the carbon atoms in CNTs is similar to the arrangement in graphite. Carbon nanotubes are hollow nanostructured materials consisting of carbon atoms bonded with three neighboring atoms via sp^2 bonds which are stronger than the sp^3 bonds present in diamonds, giving the structure its unique strength. Carbon nanotubes are considered to be rolled up graphene sheets. Carbon occurs in many forms and the properties of the substance depend mainly on the structure and arrangement of the atoms. The structural arrangement of the carbon tubes in CNTs is responsible for the unique mechanical and chemical properties (Klumpp et al., 2006).

Table 4.1 Comparison Between Single Wall and Multiwall Carbon Nanotubes

Single Wall Carbon Nanotubes	Multiwall Carbon Nanotubes
Composed of a single cylindrical graphene layer	Comprise several concentric cylinders of graphitic shells, each one forming a SWNT
Outer diameter: 0.6–2.4 nm	Generally have a larger outer diameter (2.5–100 nm) with an interlayer separation of about 0.34 nm
Better defined structure	Have structural defects, resulting in a less stable nanostructure
Purification results in low yield and defect formation	Purification is relatively easy and effective
Catalyst is required for synthesis	Catalyst not necessarily required
Highly toxic	Relatively less toxic

4.2.2.15 Cellular uptake

The striking attributes of CNTs facilitate their use for therapeutic delivery through cells, proved as a boon in the field of biomedicine and biotechnology, hoping to make an incredible move in the recognition, monitoring, and therapy of diseases. A number of groups have shown that CNTs can be used to deliver therapeutic and diagnostic molecules (micro/macromolecules) through cellular binding, internalization, and intracellular trafficking by diverse cell types. The exact mechanism remains hard to pin down but the determination of the cellular internalization of CNTs is measured as a great quest in their continued development. The type of CNTs utilized is of much significance and their interactions with cells must be performed using biocompatible CNTs, acquired by either covalent or noncovalent surface functionalization to produce water soluble CNTs. The surface chemistry of CNTs does not influence their internalization. Relatively, their dimensions widely influence their cellular uptake, as short nanotubes competently pierce the cell membrane (nanoneedles) than longer nanotubes, which are often aggregated ([Scheinberg et al., 2013](#)).

4.2.2.16 Applications

Drug delivery: A drug delivery system is generally designed to improve the pharmacological and therapeutic profile of a drug molecule. The ability of f-CNT to penetrate into the cells offers the potential of using f-CNT as vehicle for the delivery of several drug molecules. Carbon nanotubes exhibit great potential for the delivery of cytotoxic, antiinflammatory, cardiovascular, and antiinfective agents.

Gene delivery: Gene delivery is based on the development and use of viral and nonviral vector systems. Precisely, gene therapy may be described as the delivery of nucleic acids by means of a vector, thereby correcting genetic defects or altering cellular genetic makeup. However, its major drawback is the instability

of exogenous nucleic acids (undergoing rapid degradation). To overcome this problem, intracellular translocation was carried out in association with (self-assembled) DNA, RNA, other nucleic acids, and nonviral gene transfer systems (polymers, liposomes, cationic lipids, and nanoparticles). These nonviral gene transfer systems offer several advantages, including easy up-scaling, flexibility in terms of the size of nucleic acid to be conveyed, and minimized immunogenicity, in comparison to viral gene. Different types of f-CNTs were designed and were found to have the ability to condense DNA to varying degrees; capable of delivering plasmid DNA to cells successfully, leading to efficient gene expression.

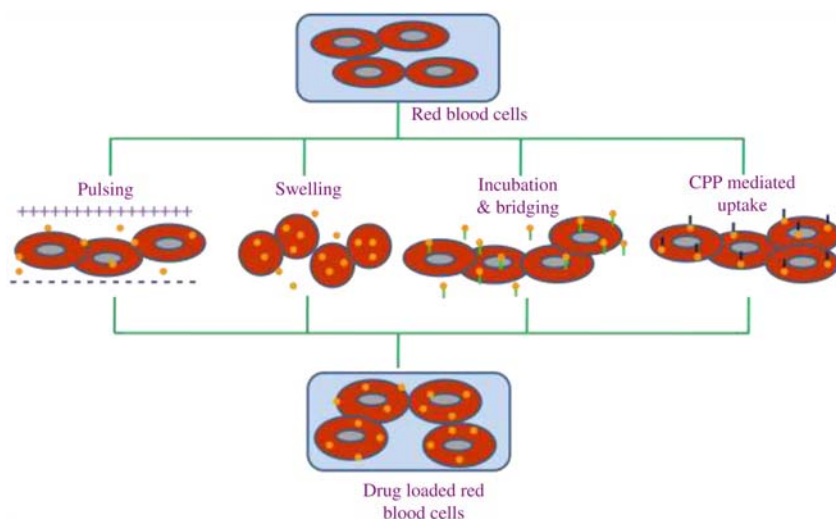
CNTs as theranostics: Theranostics are chemically/physically modified molecules that serve the dual properties of therapeutic as well as diagnostic agents. The objective of such systems is to provide specific and efficient therapies for various diseases (especially cancer). Nanotechnology has emerged as a promising field for theranostics delivery. Many nanosystems such as iron oxide nanoparticles, quantum dots, carbon nanotubes, gold nanoparticles, and silica nanoparticles have performed the role of both therapeutics and imaging (diagnosis) (Wang and Arash, 2014; De volder et al., 2013; Hong et al., 2011; Pauluhn, 2010).

4.2.2.17 Resealed erythrocytes

Erythrocytes have great carrier potential for drug delivery. Erythrocytes are biocompatible, biodegradable, possess long circulation half-lives, and can be laden with diverse active components using varied chemical and physical methods. Resealed erythrocytes are prepared by collecting blood from the interested organism, separating erythrocytes from plasma, trapping the drug in erythrocytes, and resealing the resultant cellular carriers. In general, the method is based on the behavior of these cells under osmotic conditions. Drug-loading in resealed erythrocytes (Fig. 4.7) is depicted in the following scheme:

4.2.2.18 Drug uptake mechanisms

Phagocytosis and diffusion through the cell membrane are principal drug uptake mechanisms from resealed erythrocytes. Using a specific transport system, the rate of diffusion depends upon the pace at which a particular molecule pierces through a lipid bilayer. Many substances enter cells by precise membrane protein organization, as they have many properties analogous to that of enzymes, emphasizing specificity, e.g., nucleotides and nucleosides. The drug release from erythrocytes is rapid, followed by constant release profile, and the rate of exit is comparative to the instantaneous intracellular drug concentration (first-order kinetics). By assimilating polymers into erythrocytes, the release pattern may be modified. The drug however, could be released from macrophages after engulfment if the linkage is vulnerable to lysosomal enzymes. Potential advantages and disadvantages (Table 4.2) can be summarized as follows:

**FIGURE 4.7**

Drug-loading in resealed erythrocytes.

Table 4.2 Advantages and Disadvantages of Resealed Erythrocytes

Advantages	Disadvantages
Biocompatibility, no triggered immune response	Limited potential as carrier to non phagocyte target tissue
Biodegradable with no toxic byproducts	Comparatively larger amount of test material is desired than that for the microcapillary method
Uniform size and shape of carrier	Possibility of clumping of cells and dose dumping may be there
Ability to circulate throughout the body	Direct injection into the cell nucleus is not feasible

4.2.2.19 Applications

Carrier for drugs: Erythrocytes have been utilized as circulating depots for the persistent delivery of anticancer, antiprotozoals, antibiotics, vitamins, steroids, and cardiovascular drugs.

Enzyme delivery: One method to outclass these issues is by employing enzyme-loaded erythrocytes. An injection of resealed erythrocytes loaded with amino levulinate dehydrogenase [ALA-D] to lead-intoxicated animal significantly reduces toxic manifestations.

Proteins and macromolecule delivery: The various approaches to modify the surface characteristics of erythrocytes include surface modification with antibodies, glutaraldehyde, carbohydrates such as sialic acid, and sulfhydryl surface chemical cross-linking (Harisa et al., 2011; Sprandel and Way, 1997).

Table 4.3 Types of Ligands

Endogenous	Immunological	Glycoconjugate	Antibody Conjugate
Transferrin	Interferons	Lectins	Polyclonal Ab
Lipoprotein	Interleukins	Glycolipids	Haptens
Folate	Major histocompatibility complex (MHC) peptide	Asialoglycoproteins	Fab' or F(ab) ₂
Insulin	Recombinant gp120, gp 41	Neoglycoprotein	Immunotoxins

4.2.3 LIGANDS

Ligands are carrier-associated surface groups, which can selectively direct the carrier to the prespecified site(s), accommodating appropriate receptor units to serve as a “homing device” to the carrier/drug. The carrier system interactions serve to assist presentation of ligands to their respective receptors localized on the cellular surface. The ligand confers recognition and specificity upon carrier/vector and endows them with the ability to approach the relevant target selectively and transport the drug. A ligand is neither a shield nor a motor, thus it needs prolonged carrier/system circulation in order to make certain the target site accessibility for receptor ligand identification and communication. Carrier target identification is a prerequisite for ligand-mediated targeting and provides a basis for using cell-specific ligands, anchored to the carrier surface as a means of encouraging identification and specificity. Ligands are frequently covalently anchored or noncovalently associated with the surface of the carrier in such a way that the carrier tends to approach the specific cells, those expressing a surface receptor, with a ligand-specific affinity. The endogenous or exogenous ligands can be conjugated with either the drug or drug-bearing delivery systems using noncovalent and covalent techniques.

Various ligands investigated are summarized in [Table 4.3](#).

4.3 CHALLENGES AND OBSTACLES

A number of crucial aspects should be considered for the design of drug delivery systems to accomplish this objective such as target, carrier, ligand(s), and physically modulated components. Following are some major challenges associated with targeted drug delivery:

- They have not been broadly characterized in a scientific condition, i.e., pharmacokinetics, biodistribution, and toxicity.
- Each system is exclusive and must be assessed as a new formulation. Advances of appropriate screening methodologies for determining optimal

uniqueness of nanocarriers are unclear. Consequently, successful targeting strategies must be determined on a case-by-case basis, which is arduous.

- Major developmental concerns must be resolved, such as particle size uniformity, control of drug release rate, formulation stability, and bulk manufacturing of sterile preparations prior to become therapeutically relevant.
- The development process is time-consuming due to its distinctive requirements, thereby making overall cost high.
- The correlation of in vitro tests of activity, uptake, discrimination, and adverse effects of focused systems with in vivo studies is still an area of focus and a significant amount of work is needed before clinical application of such systems.

4.4 GENE DELIVERY

4.4.1 INTRODUCTION

Gene therapy is an investigational technique for correcting defective genes that are liable for disease progression. A functional gene is incorporated into the genome to substitute an abnormal, pathogenic gene.

The phrase “gene therapy” was first presented at the International Congress of Genetics. At the end of the 1970s, the milieu of most of genetic disorders was understood, and gene therapy came to the fore. The first gene therapy trial in humans was performed in the 1970s, and it was observed that naturally occurring DNA and RNA tumor viruses successfully delivered new genetic information to the genomes of mammal cells. In the 1980s, gene transfer to mammalian cells came to the fore after the development of retroviral vectors. Retrovirus-based gene therapies brought significant advantages, as they could stably integrate their genomes to host-cell chromosomes. After the 1980s, DNA was defined as a genetic material. Later, it was structurally analyzed and further advances allowed the manipulation of genetic code. In the 20th century, it was understood that diseases such as hemophilia, colon cancer, diabetes, and retinoblastoma were genetic-based diseases. These discoveries of genetic material made cloning possible. Melanoma was successfully treated in 2006 for the first time. The treatment used a retroviral formulation of melanoma antigen (MART-1)-specific T-cell receptor (TCR), which encodes α - and β -chains. Currently, great advances have been made in gene therapy, ranging from somatic cloning and completion of human genome project, to the finding of microRNA-based gene-regulating systems.

4.4.1.1 Gene delivery vectors

The mode for obtaining the new gene or replacing a gene into a patient’s target cells is the greatest challenge for gene delivery. A carrier moiety, called a vector,

is suitable for the purpose. Vectors facilitate the transfer of genetic information into a cell. Following are characteristics for ideal vectors:

- It should be specific, capable of proficiently delivering genes of the dimension needed for clinical purpose.
- A vector should be harmless for the patient.
- It should not induce any allergic or hypersensitivity reactions.
- A vector should possess the capability to target precise cell types.
- It should be able to communicate the gene for required time period.

Gene delivery systems use various methods to allow uptake of the gene that has been chosen to target the cell. The successful design of a gene delivery system requires complete knowledge of the interaction mechanism between the target site and delivery system. A deep knowledge of intercellular traffic and targeting phenomenon is the most important factor in designing a more effective gene delivery system. Cell targeting refers to delivery of the therapeutic agent to a specific organelle of the cell. After cellular uptake of the carrier system by endocytosis, cellular release takes place to initiate DNA transcription and translation, and to construct the related protein. A successful gene delivery procedure involves reducing potential inhibitory inflammatory reactions while also overcoming certain barriers at each step of the gene delivery procedure, in order to optimize gene activity (Jin et al., 2014; Wang and Gao, 2014; Dixit and Vyas, 2011).

4.4.1.2 Prerequisites

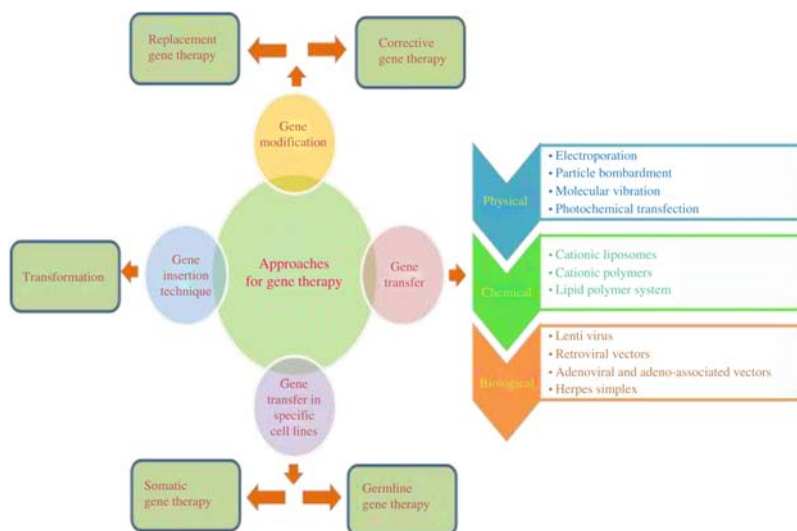
Prerequisites for gene therapy include: finding the best delivery system for the gene, demonstrating that the transferred gene can express itself in the host cell, and establishing that the procedure is safe. Some clinical trials of gene therapy in humans have given satisfactory results. As the delivery system fails to reach cells often or the genes are not expressed by cells, improved gene therapy systems are being developed by using nanotechnology.

Before the patient is subjected to gene therapy, the following prerequisites should be fulfilled:

- It must be possible to isolate the appropriate gene and to define its major regulatory regions.
- Identification and harvesting of appropriate target cells and development of safe and efficient vectors with the new gene could be introduced.
- Clear evidence of experimental details on the adequate functioning of inserted gene, life span of the recipient cell and that no untoward effect exists, should be ensured.
- The patient or their family must be fully counseled.

4.4.2 APPROACHES FOR GENE THERAPY

Various approaches (Fig. 4.8) for gene therapy broadly fall into four categories:

**FIGURE 4.8**

Various approaches for gene therapy.

4.5 GENE MODIFICATION

4.5.1 REPLACEMENT THERAPY

Gene replacement therapy is the technique of recognizing a faulty gene, applying a piece of DNA in its correct form through a viral vector (known as the carrier molecule) to the gene, thus overriding the identified faulty gene with the correct copy. The success of therapy is contingent on directing a gene to the correct cells. While the current delivery models and approaches continue to be intensely explored, the most common approach is via viral vectors. Viral vectors are carrier molecules containing a virus. Because viruses are able to adhere or enfold to healthy cells, viral vectors are genetically modified to represent normal DNA and are reinjected to compromised cells, which in turn generates restoration to the cell to a healthy or normal state.

4.5.2 CORRECTIVE GENE THERAPY

This requires replacement of a mutant gene or a part of it with a normal sequence. This can be achieved by recombinant technology. This approach is useful when the defective gene produces a disease-causing protein. This can be utilized for dominant disorders after extensive study of a specific gene. To make the approach effective and useful, site-specific insertion is required.

4.6 GENE TRANSFER TECHNIQUES

4.6.1 PHYSICAL TECHNIQUES

- *Electroporation*

This is a method of physical transfection for generating tiny pores in cell membranes to insert macromolecules, by creating an electrical field of the cell for microseconds and milliseconds; their size varies according to the amplitude and duration of pulses. Electroporation involves controlled electric application to increase cell permeability. Foreign genes can be introduced into the cell by electric pulses using this technique. However, longer exposure of high frequency electrical fields can cause local tissue damage and inflammation. An important application of electroporation is to boost plasmid-mediated gene expression in skeletal muscle, which might be applicable for inherited muscular dystrophies. It is a very reliable and efficient method, although it suffers with some drawbacks. High temperature due to high voltage application can cause permanent and harmful tissue damage.

- *Particle bombardment*

Particle bombardment is also alternatively known as gene gun technology or ballistic microprojectile method. A gene gun is a suitable handy device that provides fast gene transfer into a range of targets under in vivo or in situ conditions. The method involves liberation of DNA layered on heavy metal, from a target area, at a specific momentum. Generally tungsten, gold or silver microparticles are utilized as the gene carriers. Particle size, dose frequency, and gas pressure are key factors in the determination of extent of tissue injury and penetration efficacy of the application. The method is an extensively trialed practice for intramuscular, intradermal, and intratumoral genetic immunization. The gene gun approach does not use harmful chemicals or complicated biological systems. The method is reproducible and delivery is attained without the need for a receptor (Al-Dosari and Gao, 2009).

- *Ultrasound mediated transfection*

Ultrasound is a promising method for introducing foreign DNA or other macromolecules into a cell. Ultrasound offers several attributes of an ideal transfer system for gene therapy. It is noninvasive and well tolerated, with an excellent safety record over a broad spectrum of frequency, intensity, and high levels of public acceptance and cognizance. Furthermore, there are highly sophisticated, flexible, cost-effective, and easily available diagnostic and therapeutic systems that can achieve site-targeted transfer of ultrasound energy anywhere in the body. The progress of ultrasound enhanced gene transfer (UEGT) began when a number of researchers hypothesized that the physical perturbations induced by an ultrasound ray would facilitate plasmid DNA uptake and transgene expression.

- *Molecular vibration*

Molecular vibration-mediated transfection has several unique and advantageous properties, compared to some widespread transfection methods.

The method is cost-effective, as special chemicals or reagents are not required, just a physiological saline solution. The procedure also elicits minimal exogenously introduced cytotoxicity. This is a one-step, efficient procedure, with no special containment of cells, and the entire procedure can be performed within seconds. The transfection method appears to be applicable to an ample variety of cell types.

Photochemical transfection

Synthetic gene delivery vectors are taken up inside the cell by endocytosis. Ineffective escape of the transgene from endocytic vesicles often is a major obstacle for gene transfer by these vectors. To improve endosomal release, photochemical internalization is used. It is based on photochemical reactions commenced by photosensitizing compounds confined in endocytic vesicles, on light exposure. Photochemical internalization (PCI) is a technique which utilizes the photochemical properties of photodynamic therapy (PDT) for the enhanced delivery of macromolecules inside the cell. These macromolecules lack the ability to naturally permeate intracellular barriers. Therefore, PCI a physical targeting technique has been used in combination with adenovirus transduction, introducing up to 20-fold increase in transgene expression in photochemically treated cells. The concept of PCI is based on using photosensitizers, which will concentrate in the membranes of the endocytic vesicles. After application of light, the photosensitizers will react with oxygen and cause the membrane to break apart, releasing all of the entrapped macromolecules so more genes will be released into the cells, in spite of being degraded by lysosomes.

- ***Hydrodynamic therapy***

Hydrodynamic gene delivery utilizes the hydrodynamic pressure generated by injecting a large volume of solution of DNA with blood pressure in veins. Accordingly, the permeability of the endothelial cells increases and pores form in the plasma membrane surrounding parenchyma cells. Molecules remain in the cells as pores close after some time. A contender gene in plasmid or DNA fragment, with regulatory sequence for its expression, can be hydrodynamically injected into an animal (mouse or rat) holding a target disease. The therapeutic effect of the gene product (protein or RNA) on disease development or regression is evaluated with respect to time. Compared to a conventional biotech approach that relies on purified protein for therapeutic assessment, hydrodynamic-based protocol makes the liver the manufacturing site for therapeutic proteins, eliminating the steps and cost for protein production and characterization.

- ***Naked DNA***

A gene (2–19 kb) can be relocated into skin, thymus, cardiac muscle, and especially skeletal muscle and liver cells after direct injection of naked DNA. Although a naked DNA injection is a less harmful and uncomplicated method, its efficiency is low and therefore has limited application, such as DNA

vaccination. Naked DNA gene transfer has been mainly utilized for genetic immunization studies. Recent studies showed that naked plasmid DNA (pDNA) can be administered proficiently to cells *in vivo* via electroporation, or intravascular delivery, and has immense prospects for basic research and gene therapy (Nayerossadat et al., 2012).

4.6.2 BIOLOGICAL (VIRAL VECTORS)

Viruses have developed a way of encapsulating and transferring their genetic material to humans in a pathogenic way. Biotechnologists have attempted to utilize this ability and alter the viral genome, substituting them with functional human gene. This manipulated virus can relocate genes into cells with great efficiency. Viral vectors typically contain strong promoters to support a high level of transgene expression. Viral DNA vectors can also include tissue-specific promoters to exclude expression in a place other than the target tissue. Another means of targeting include the possibility to introduce precise recognition series in the surface structures of the virus coat to enable infection of specific cells/tissue. In addition to disabling viruses from replicating in host cells and producing progeny, it has been observed that deletion of viral genes has significantly reduced the cytotoxicity on host cells and therefore improved the host-cell survival.

Many gene therapy clinical trials rely on retro viruses or adenoviruses to deliver the preferred gene. Most common viruses used as vectors include adeno-associated viruses, lentiviruses, pox viruses, alpha viruses, and herpes viruses. Adenovirus vectors are probably the most frequently used viral vectors, mainly because of the architecture of packaging cell lines for generating high-titer virus stocks and the high-level gene expression obtained in a broad range of host cells (Lundstrom and Boulikas, 2003; Kay et al., 2001; Stone et al., 2000).

4.6.3 CHEMICAL (NONVIRAL GENE DELIVERY)

Viral-mediated delivery suffers with several drawbacks, as it is difficult to target to certain cells. The delivery results in low DNA transfer. Production of high-titer viral vectors is also difficult.

To evade these disadvantages chemical approaches were introduced for gene delivery. This approach is also known as nonviral gene delivery (Elsabagy et al., 2011; Ganta et al., 2008; Niidome and Huang, 2002).

4.7 GENE TRANSFER TO SPECIFIC CELL LINES

4.7.1 SOMATIC GENE THERAPY

The technique of somatic gene therapy involves incorporating a normal gene into the suitable cells of an individual affected with a genetic disease, thereby

permanently correcting the disorder. The therapy is based on the fact that therapeutic genes are conveyed into the somatic cells, the effects are limited to the targeted cells, and are not heritable. These genes are short-lived because the cells of tissues are continuously replaced by new cells. Somatic gene therapy can be performed *ex vivo* and *in vivo*. However, there are some major technical obstacles that must be overcome before somatic gene therapy is likely to work. The first is acquiring the human gene by the patient's cells. The second hurdle is insertion of gene into the right cells. The final obstacle is making sure the gene is active. This means it must be under the control of the sequence of DNA that is responsible for switching the gene on.

4.7.2 GERMLINE GENE THERAPY

This involves incorporation of functional genes, which are integrated into their genomes. This would allow the therapy to be heritable and passed onto later generations. The therapy is highly efficient in counteracting genetic disease and hereditary disorders. Gene therapy on germline cells generates a lot of controversy because any changes become heritable since the progeny receive the manipulated DNA.

4.7.3 DELIVERY SYSTEMS IN GENE THERAPY

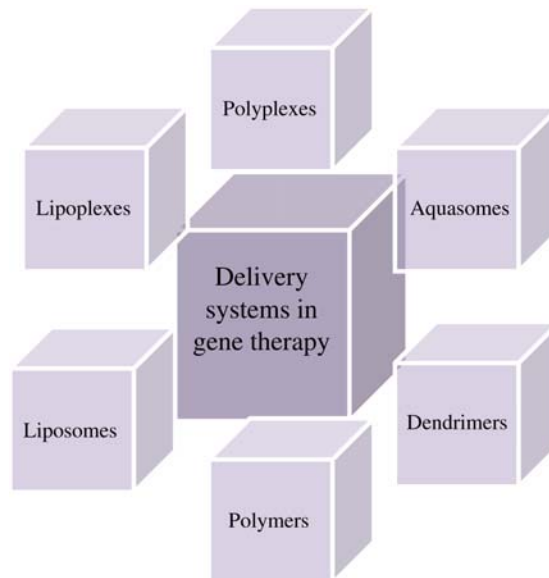
Among various approaches for gene therapy, the nonviral approach of delivery system is widely used as it offers several advantages. Antigen-specific immune responses are less harmful as in case of viral vectors. Large scale production of vectors is easy and size of gene does not affect the efficiency of these systems. The [Fig. 4.9](#) represents some delivery systems which are discussed below:

4.7.3.1 Polymers

Polymers are one of the promising carriers for gene delivery. Polymers should pack DNA in small dimensions for successful and effective delivery. Accordingly, stability of DNA is improved and cellular uptake by endocytosis facilitated. Once DNA reaches inside the nucleus, the active form of it can be released within the nucleus. The physical properties of a polymer carrier include: hydrophilicity, surface charge, permeability and diffusibility, and biocompatibility with tissue and blood. Polymeric gene transporters deliver genetic material through electrostatic interactions with nanosized polyplexes for gene therapy ([Yue and Wu, 2013](#); [Pack et al., 2005](#)).

4.7.3.2 Liposomes

These comprise of lipid bilayers surrounding an aqueous vesicle, and can be used to deliver foreign DNA inside the target cell. Intracellular trafficking of DNA, following endocytosis by cells, have also been demonstrated using pH-sensitive liposomes and proteoliposomes. pH-sensitive liposomes are known to fuse with the lipid membranes in the acidic environment of the endosomes, thereby

**FIGURE 4.9**

Various delivery systems in gene therapy.

enhancing the endosomal liberation of encapsulated gene expression systems into the cytoplasm of transfected cells. A crucial step is the transfer of plasmid DNA from the endosomal compartment to the nucleus of the target cell.

4.7.3.3 Lipoplexes

DNA must be protected from damage and its entry into the cell must be facilitated. Plasmid DNA can be enveloped with lipids in an organized structure, such as a micelle or a liposome conjugated with DNA, called a lipoplex. Three types of lipids can be used for preparation of lipoplexes. Anionic and neutral lipids were used for the construction of lipoplexes for synthetic vectors. There was a possibility of adapting them to be tissue-specific. As they are complicated, cationic versions were therefore adapted. Cationic lipids, due to their positive charge, naturally complex with the negatively charged DNA and interact with the cell membrane. The cationic lipids also protect against degradation of the DNA by the cell ([Wasungu and Hoekstra, 2006](#)).

4.7.3.4 Cationic lipid compatible systems

The aim of cationic lipid-based gene therapy is to transport plasmid DNA to the cell and thus to achieve the preferred protein/peptide transcription and translation. DNA-lipid complex is administered into the cell through the vesicular passage, which is followed by the release of cell cytoplasm of DNA from the lipoplex.

Strong DNA-cationic lipid complexes are not enough for gene transfer alone; however, DNA release before or after transfer to the nucleus is crucial. Lipid-cationic lipid ratio, cationic lipid–DNA ratio, and DNA build-up presented to the cell are critical parameters that significantly influence transfection efficiency. The most significant disadvantages of cationic lipids are: instability and heterogeneity, inactivation in blood, low efficiency, and weak targeting ability, when compared to other systems (Morille et al., 2008; Uddin, 2007).

4.7.3.5 Polyplexes

Polyplexes are referred to as complexes of polyelectrolyte formed by self-congregation of DNA with cationic polymers such as poly-L-lysine or polyethylenimine. A major difference compared to lipoplexes is that polyplexes cannot release their DNA load into the cytoplasm.

4.7.3.6 Aquasomes

A self-assembled molecular carrier, aquasomes, is formed with preformed carbon ceramic nanoparticles and self-assembled calcium phosphate dehydrate particles with a glassy carbohydrate coating, and can be studied for the delivery of genes. A marked retention of biological activity was observed with surface immobilized DNase on the solid phase of a colloidal calcium phosphate nanoparticle coated with polyhydroxyloligomeric films (Cotton and Wagner, 1993).

4.7.4 CANDIDATE DISEASES

The various diseases (Table 4.4) like cancer, neurodegenerative disorders, cardiovascular diseases etc. are candidates for gene therapy.

4.7.5 PROBLEMS ASSOCIATED WITH GENE THERAPY

- DNA mutations: The new gene might be inserted in the wrong location in the DNA, which may result in damaging mutations to the DNA.
- Immune response: The body's immune system may see the newly introduced viruses as intruders and the defense system may cause inflammation, toxicity, as well as organ failure in severe cases.
- Viral spread: As a virus can affect several cell types at once, it is possible that the viral vectors may infect cells beyond just those containing mutated or missing genes. As a consequence, healthy cells may become damaged and cause other serious diseases.
- Reversion in virulence: After insertion in the body the virus may recover their original ability to cause disease.
- Risk to progeny: The new DNA may affect reproductive cells; egg cells in women and sperm cells in men. This may cause genetic changes affecting children born after treatment.
- High cost

Table 4.4 Candidate Diseases for Gene Therapy

S. No.	Candidate Disease	Gene Transfer Technique
1.	Cancer	Physical, nonviral, viral <i>vaccinia</i> virus recombinant nonreplicating fowlpox virus encoding GM-CSF (TRICOM) Prostac-VF vaccine
2.	Neurodegenerative disorders	
	Alzheimer's disease	Recombinant adeno-associated virus encoding tau protein
	Parkinson's disease	Lentivirus mediated delivery
3.	Cardiovascular disorders	Viral-mediated method using retrovirus, sendai virus, adenovirus nonviral techniques using liposomes, microinjection, mechanical, implantation
4.	Cystic fibrosis	Adenoviral-mediated delivery of CFTR gene
5.	Hemophilia B	Recombinant retroviral and adenoviral encoding canine factor IX
6.	Insulin-dependent diabetes <i>mellitus</i>	Recombinant retroviral vector encoding complete sequence for preproinsulin
7.	AIDS	Lentivirus encoding the triple shRNAs

4.8 CONCLUSION

As a concept, drug targeting is to localize the therapeutically active ingredient at the site of its action so that dose-dependent side effects and adverse effects to the healthy organs can be avoided. Gene delivery is the most advanced form of site-specific delivery as it defines cellular level of targeting. Although, it is a very complex approach, involves a cascade of cellular events and designing of an efficient delivery system, the approach offers several advantages and results of such research are motivating. The expansion of TDDS is being pursued devotedly by various research groups worldwide. These are being studied *in vitro* for release pattern and *in vivo* for pharmacokinetics, but less frequently for effectiveness. There is a lack of data on clinical outcomes and therapeutic value of the TDDS in patients. It is essential that pharmacologists should be involved in the exploration of pharmacokinetics and pharmacodynamics of TDDS if the products have reached their meaningful outcome: clinical use.

4.9 FUTURE PERSPECTIVE

Targeted therapy remains an unachieved objective, however the idea has encouraged a long progression of experiments that hypothesized the values of targeting of drugs and genes. Although we have the necessary information, true targeted

drug delivery is still beyond our grasp because the unpredictability of cellular behaviors creates complexities in terms of optimizing true targeting. The reality, however, is far away from that ideal scenario. It has attracted researchers toward the problems and prospects associated with the concept. Nonetheless, ongoing research shows promising results, especially in the treatment of life-threatening diseases. The TDDS may prove specifically valuable to enable the use of a drug seems to be ineffective or toxic, if delivered systemically. At the current rate of gene modification, recombinant protein production, and discovery of new drug molecule, many more site-specific drug delivery products will be clinically investigated and implemented in the near future.

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Stimuli-responsive nanosystems for drug-targeted delivery

5

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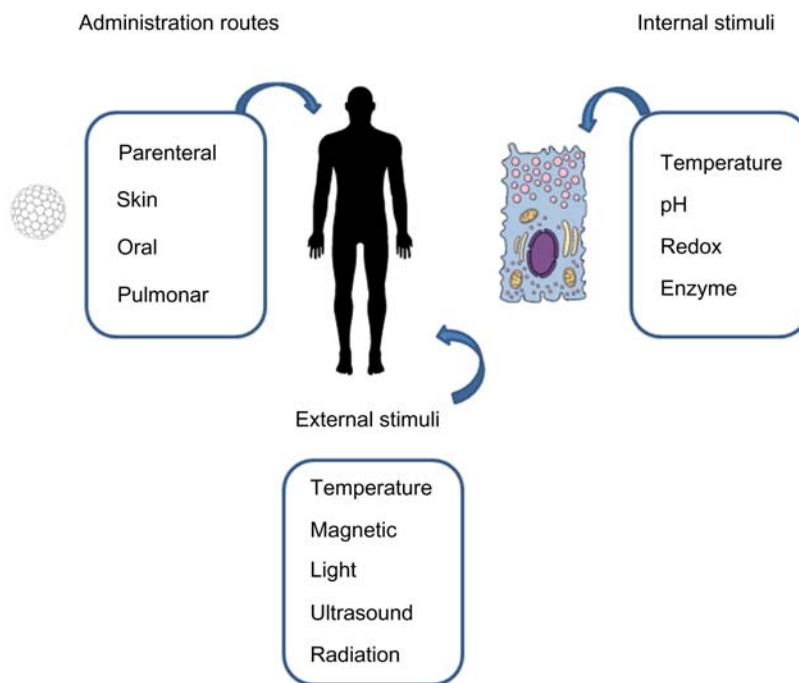
CHAPTER OUTLINE

5.1 Introduction	155
5.2 Stimuli-Responsive Nanosystems	160
5.2.1 Physical Stimuli-Responsive Nanosystems	160
5.2.2 Chemical Stimuli-Responsive	171
5.2.3 Dual and Multistimuli Responsive Nanosystems	177
5.3 Conclusions and Future Perspectives	193
Acknowledgments	194
References	194

5.1 INTRODUCTION

In the area of drug delivery system, the research community has made a huge effort to develop novel tools and technological innovative approaches that satisfy the therapeutic needs and overcome some limitations associated with the performance of conventional dosage forms (e.g., physical and/or chemical drug degradation, poor drug bioavailability at the disease site, undesired side effects). With the aim to enhance drug release at the target site (spatial release control) and/or at the specific time (temporal release control), several environment-sensitive nanosystems that undergo conformational changes or phase transitions (e.g., dissolution, swelling, collapse, protonation, or hydrolytic cleavage) in response to an internal or an external stimulus have been actively investigated in recent years (Fig. 5.1) (Lopes et al., 2013; Gong et al., 2013; Guragain et al., 2015; Lehner et al., 2012). The stimulus is responsible for the activation process which is, in almost all cases, caused by the presence of an intelligent polymer in the composition of the sensitive system.

In the internal stimuli, i.e., systems that are sensitive to specific endogenous/physiological stimuli such as a decrease in interstitial pH or an increase of

**FIGURE 5.1**

Internal and external stimuli promoting activation process in sensitive responsive systems. Internal stimuli are common in certain pathological conditions such as cancer and inflammation: increased temperature, acidic environment, differential redox potential and metalloprotease overexpression. External stimuli are extracorporeal and need deep tissue penetration. Internal stimuli are less damage for the healthy tissues than external stimuli.

glutathione concentration, a self-control of the drug delivery and release occurs. Therefore, the internal stimuli might respond to a molecular mechanism highly specific for a certain pathological condition, which can diverge from one patient to another, making the control of internal triggers intrinsically complicated (Lehner et al., 2012). Much of the preclinical research on stimuli-responsive nanocarriers is based on internal (micro)environmental changes that occur at the cellular level (e.g., intracellular lysosomal pH) or at the tissue level (e.g., in neoplastic and inflammatory diseases or infections) (Mura et al., 2013). Despite some nanoformulations sensitive to internal stimuli having come into clinical trials, their clinical benefit has not yet been proved (Tagami et al., 2011). The external stimuli, also termed extracorporeal stimuli, needs an exogenous stimulus in order to control drug release. This control could be done by remote equipment that uses a switched “on” and “off” (e.g., ultrasound, light). Such devices have the advantage of external regulative application properties, offering a spatial and temporal precision, as well as dose control of the drug/contrast imaging agent release,

making them more promising (Chan et al., 2013). This type of stimuli could be very useful where a target cell is in the basis of pathogenesis and is at a specific location (e.g., cancer stem cells in a cancer tissue). Despite this, improvements in both tissue-penetration depth and retention, specifically in the pathological site and/or target cells in order to mitigate the side-effects, are the principal challenges for externally controlled nanosystems. Additionally, the potential of tissue damage and/or disruption of biological membranes due to the interaction of the external energy source (e.g., temperature, light, electromagnetic fields) with biomacromolecules (e.g., membrane proteins), not only in the target tissue but also in healthy bystander tissues, is also a limiting factor of externally responsive nanocarriers (Lehner et al., 2013). The main advantages in the literature using stimuli-responsive nanosystems include (Lehner et al., 2012): improvements in efficacy, safety, targeted specific nonviral drug delivery, cellular uptake, and intracellular stability and activity.

The first scientific publication on stimuli-responsive drug delivery nanosystems was in the late 1970s, where Yatvin and collaborators (1978) developed thermosensitive liposomes for local release of drugs by hyperthermia. To enhance therapeutic outcome, stimuli-responsive nanosystems can also be used to release contrast agents in diagnostic imaging, such as magnetic resonance imaging (MRI) and ultrasonography, for accurate imaging and detection (Chen et al., 2012a; Zhou et al., 2013).

The use of nanosystems seems to be a smart idea once important advantages as delivery carriers are revealed, such as (Ganta et al., 2008): improving the target-to-nontarget concentration ratios, increased drug residence at the target local, and improved cellular uptake and intracellular stability. Therefore, when nanocarriers are associated to a stimuli-responsive delivery approach, additional benefits could be obtained, since they become an active participant in the process of delivery instead of being a passive vehicle.

Several types of nanocarriers with diverse sizes, architectures, and surface properties have been designed for stimuli-responsive drug delivery, including (Lehner et al., 2012): liposomes, polymer nanoparticles, nanogels, micelles, dendrimers, quantum dots, carbon nanotubes, and a variety of inorganic nanoparticles.

Ideally, a well-designed stimuli-sensitive nanosystem should present the following features (Li et al., 2011; Lehner et al., 2013): biocompatibility; specifically accumulating in the organ or tissue of action (i.e., present high target specificity) and subsequently penetrate inside target cells; intracellularly delivering its load; controlled stimuli-responsiveness; and stealth capacities to escape recognition by the immunological system. The literature reported different methodologies to improve the in vivo performance of nanocarriers in terms of pharmacokinetics and biodistribution, and to achieve high accumulation in the required site. Some such approaches are based on the physicochemical properties of nanosystems, e.g. composition, size optimization, and surface functionalization (Mura et al., 2013). The first challenge of controlled drug delivery is the particle size

that influences its pathway and distribution in the organism, its clearance, as well as its content. Small particles (i.e., with a diameter between 1 and 30 nm) are rapidly cleared by the kidney. Particles larger than 30 nm are cleared by the reticulo-endothelial system (RES), which includes macrophages located in the liver and spleen. To increase their effectiveness, the nanosystems must persist in the bloodstream for the time required to induce a therapeutic response, and this can be assured by size control (Gaumet et al., 2008). The process of attaching some hydrophilic polymers, of which polyethylene glycol (PEG) is the most frequently used, on the surface of nanocarriers induces steric repulsion of blood opsonins (i.e., molecules that enhance phagocytosis), increasing nanocarrier blood circulation time, improved drug tolerance, and providing the ability to extravase through the endothelium of inflammatory tissues (i.e., the enhanced permeability and retention effect), termed passive targeting (Torchilin, 2011). Furthermore, cell-targeted specificity can be reached by either anchoring biologically active ligands (e.g., monoclonal antibodies, aptamers, folic acid, and transferrin) or using sensitive responsive system (the so-called active targeting process). Passive and active targeting are represented in Fig. 5.2.

The main nanosystems suitable for stimuli response are (Fig. 5.3) (Khodabandehloo et al., 2016; Lehner et al., 2013):

Liposomes: spherical bilayered phospholipid vesicles. Their main advantages are the ability to include a variety of drug molecules with different solubilities, lack of immune response, and diversity of composition and morphology;

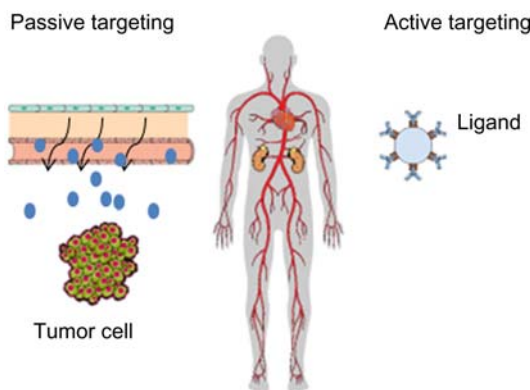
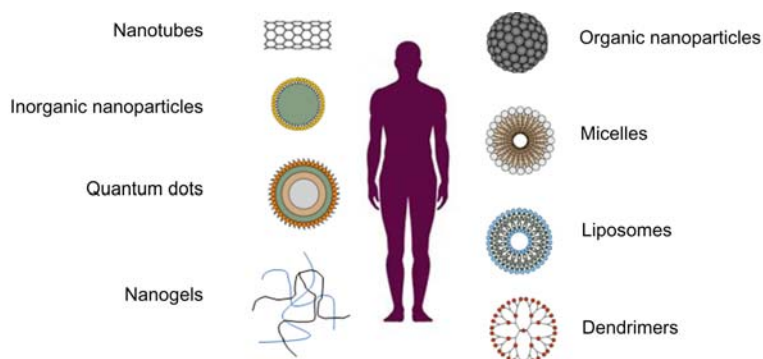


FIGURE 5.2

The distribution of nanosystems depends firstly on their small size to achieve prolonged circulation half-life. Passive targeting depends on the Enhanced Permeability and Retention effect promoted by the high vascular fenestration during tumor angiogenesis. Active targeting depends on the specific biological active ligands that interact with target surface cell receptor.

**FIGURE 5.3**

Main nanosystems suitable for stimuli response.

Micelles: made of amphiphilic compounds that are organized in closed structures that encapsulate drug molecules. Their major advantage is the diversity of moieties (hydrophobic or hydrophilic core) allowing an encapsulation of a wide range of molecules and provide an improvement of solubility;

Dendrimers: branched macromolecules with a regular chemical structure and a hydrophobic inner for including poor water soluble drug molecules. Dendrimers present a more stable structure than micelles and liposomes and can reach smaller sizes but have lower loading capacity;

Organic nanoparticles: usually based in natural components that provide biocompatibility, biodegradability, and low toxicity. They can be water soluble (e.g., composed of chitosan, starch or other water soluble polymers) or liposoluble (e.g., solid lipid nanoparticles) allowing the encapsulation of a wide variety of molecules. Loading capacity and size control are the focal challenges;

Inorganic nanoparticles: composed of iron oxide, gold, or silver particles. Magnetite and maghemite exhibit magnetic properties when a magnetic field is applied. They are used as drug delivery carriers, imaging agents, and for inducing hyperthermia. Their cytotoxic activity has been proven;

Carbon nanotubes: present a tubular structure of benzene rings arranged in a single-walled graphene tube or multiwalled concentric graphene layers. They have specific properties such as high mechanical strength, high electrical conductivity, high thermal conductivity, metallic behavior and high surface area. As a main disadvantage, carbon nanotubes have toxicity problems to solve;

Nanogels: a three-dimensional network of cross-linked hydrophilic polymers. They can swell in contact with water, entrapping drug molecules in their interior. For specific targeting, nanogel surfaces can be chemically modified with different ligands;

Quantum dots: semiconductor nanocrystals with an inorganic core (e.g., Cd or Se) and a metallic shell (e.g., ZnS). Their surface can be modified by ligands or polymer encapsulation. Due to their fluorescent properties, they can be used in imaging and drug labeling.

In the area of sensitive responsive nanosystems, the engineering of biocompatible macromolecular polymers, termed “smart” or “intelligent,” or even stimulus-responsive polymers, has a crucial role to develop promising nanosystems that can be modulated by one or more activation processes. Regarding the distinctive characteristics of smart polymers, such as conductivity, viscoelasticity, and transparency, they present the ability to modify some properties (e.g., solubility, shape, and surface) in response to certain stimuli (Medeiros et al., 2011; Joglekar and Trewyn, 2013).

The responsible stimuli that activate the nanosystems can be subdivided into two main types according to the nature of the applied process or the type of energy used (Delcea et al., 2011; Lehner et al., 2012; Lopes et al., 2013): physical (e.g., light, magnetic or/and electric field, ultrasound, temperature, radiation), chemical (pH, redox potential, temperature, enzymes and endogenous receptors). In our chapter, we adopted this systemic classification criteria. We highlight some examples of the most recent promising developments in stimuli-responsive nanosystems for drug or diagnostic agent delivery application, focusing on their basic action mechanism, drug release performance, and therapeutic and/or diagnostic purposes. The research examples give in this chapter are emphasized in studies using cell cultures and in vivo assays.

5.2 STIMULI-RESPONSIVE NANOSYSTEMS

5.2.1 PHYSICAL STIMULI-RESPONSIVE NANOSYSTEMS

5.2.1.1 Magnetic field stimulation

Magnetic systems consist of a carrier (e.g., tablets, nanoparticles) and materials with responsiveness to a magnetic field (e.g., magnetite (Fe_3O_4), magnetite ($\gamma\text{Fe}_2\text{O}_3$), iron, cobalt, nickel, samarium-cobalt) (Sun et al., 2008). The main property of magnetic systems is their ability to utilize magnetic force to trigger the drug for a specific region and/or activate their payload release at the site of action by remote application of a magnetic field (Bajpai et al., 2008; Corchero and Villaverde, 2009; Gröning et al., 1998; Medeiros et al., 2011; Nyberg et al., 2007; Paasonen, 2010). These systems have an interesting advantage of prolonging retention time at the site of action, which allows for modifying the extent of drug release and absorption and optimizing its bioavailability, without causing toxic effects on the adjacent tissues or throughout the organism (Corchero and Villaverde, 2009; Hafeli, 2004). These advantages make magnetic systems quite promising for various therapeutic and diagnostic applications, such as controlled drug release, gene therapy (Ortner et al., 2012; Pan et al., 2008; Prijic et al., 2012), cancer treatment (Prijic et al., 2012; Motoyama et al., 2008), MRI (Benyettou et al., 2009), and drug delivery (Laulicht et al., 2011).

Magnetic carriers have been used to transport various drugs, such as anti-inflammatory agents (e.g., diclofenac, ibuprofen) (Dutta and Sahu, 2012; Xing

et al., 2012), proteins, and therapeutic peptides (e.g., Herceptin, known as trastuzumab) (Huh et al., 2005). Although oral administration has not been particularly explored, magnetic transporters are already widely used intravenously in the treatment of cancer to carry chemotherapeutic agents (e.g., cisplatin, gemcitabine, methotrexate, mitoxantrone, mitomycin C, etoposide, paclitaxel, or oxaliplatin) (Alexiou et al., 2000; Hafeli, 2004; Rudge et al., 2000) or radioisotopes (e.g., ^{111}In , ^{188}Re) (Hafeli et al., 1995, 2001, 2003).

The magnetic properties of a material result from the magnetic susceptibility, namely the ratio of the induced magnetization to the applied magnetic field. Based on this principle, the materials can be classified as diamagnetic, paramagnetic, ferromagnetic, and superparamagnetic. In the first case, the magnetic moment is antiparallel to the applied magnetic field, which causes a small negative susceptibility (-10^{-6} to -10^{-3}), so when the external magnetic field is removed, the material does not retain the magnetic properties. In paramagnetic materials, the magnetic moment aligns in parallel with the applied magnetic field, forming susceptibility in the order of 10^{-6} to 10^{-1} . In the case of ferri- or ferromagnetic materials, the magnetic moment also aligns in parallel with the magnetic field; however, the interactions of the connections between the electrons of the material give rise to ordered magnetic states, which translates into magnetic domains and a large spontaneous magnetization. If ferromagnetic materials are small, in the order of tens of nanometers (e.g., nanoparticles), they exhibit superparamagnetic behavior, i.e., they become a single magnetic domain with a large magnetic moment, but influenced by thermal fluctuation. At suitably high temperatures, the thermal energy is sufficient to induce free rotation of the particles, causing the loss of magnetization when the magnetic field is removed. This property allows materials when exposed to a magnetic field to be magnetized, but not permanently because the field is turned off. Therefore, the superparamagnetic materials have superior susceptibility to paramagnetic materials; they maintain stability and prevent the colloidal aggregation (Corchero and Villaverde, 2009; Sun et al., 2008). The applied material determines the reaction in the body, so it must be nontoxic, biocompatible, biodegradable, and nonimmunogenic (Medeiros et al., 2011; Hafeli, 2004; Anal, 2007). Various in vitro and in vivo studies reported the biocompatibility and tolerability of magnetic particles (Huh et al., 2005; Jain et al., 2008; Lubbe et al., 1996; Kohler et al., 2006; Teply et al., 2008). In addition, it is believed that the excretion of excess iron from the body is efficient and the application of magnetic field is harmless to biological systems and suitable to any part of the body (Corchero and Villaverde, 2009; Jain et al., 2008).

Aiming for the targeting and releasing the drug at the target site, different magnetic carriers have been developed, such as magnetic nanoparticles or magnetic liposomes. Magnetic nanoparticles are amorphous or semicrystalline structures (Corchero and Villaverde, 2009). These nanostructures have a variable size which, according to Berry and colleagues, can differ between 10 and 500 nm (Berry et al., 2004). Due to the reduced size of the nanoparticles, their uses in

drug delivery exhibited various advantages, such as increased drug solubility rate and promoting bioavailability (Wang and Zhang, 2012). These colloidal vectors are generally constituted of an inorganic nucleus, composed of the magnetic nanoparticle, and coated by a biocompatible polymer responsible for its stabilization, to which functional ligands can be attached. The high surface-to-volume ratio allows a high load capacity. Based on their magnetic characteristics, magnetic nanoparticles offer a high potential for accumulating the therapeutic agent at the site of action. The properties of these colloidal carriers can be altered and optimized according to certain parameters: composition, morphology, size, and chemical surface (Corchero and Villaverde, 2009; Sun et al., 2008). Taking into account the composition, synthetic magnetic nanoparticles can be divided into: nanoparticles of colloidal iron oxide, metallic nanoparticles, and bimetallic nanoparticles (Sun et al., 2008).

The nanoparticles of colloidal iron oxide constitute nanocrystalline magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$), coated by a polymer (e.g., alginates, PEG, polyvinyl alcohol, dextran based coatings) (Sharifi et al., 2012; Sun et al., 2008). These materials are the most studied and used in therapeutic applications, since they are less sensitive to oxidation and less toxic (Sharifi et al., 2012). The oral administration of this type of nanoparticle is already described in the scientific literature. As an example, Sahu and Dutta (2011) developed a hybrid nanostructure through the encapsulation of nanoparticles of magnetite and pectin by crosslinking to calcium ions. This system was developed based on the potential application to deliver the drugs to the colon that can be administered orally. Therefore, the magnetic behavior, the gastric degradation of the nanostructure, and the loss of the magnetic material under stomach conditions ($\text{pH} = 1.2$) were tested. The results showed that the system is not degraded, is impermeable under acidic conditions with minimal loss of magnetic material and, when subjected to the application of a magnetic field, presents considerable superparamagnetic behavior, allowing it to direct and protect the drug (Sahu and Dutta, 2011).

Superparamagnetic iron oxide nanoparticles were implanted in 6-hydroxydopamine (6-OHDA)-lesioned rat model of Parkinson's disease. The exposure to a magnetic field resulted in a significant enhancement of mitochondrial function and attenuation of lesion volume, and also on feeding behavior, gait, and postural stability in all the intervention groups (Umarao et al., 2016).

Several diseases were the focus for magnetic nanoparticle development aiming for imaging and controlled drug release, including atherosclerotic plaque (Dong et al., 2016); cardiac lesions ablation (Nguyen et al., 2016); tissue inflammation (Santos et al., 2016); ovarian cancer (Zhang et al., 2016a); hepatocellular carcinoma (Chen et al., 2016c); temporal lobe epilepsy (Fu et al., 2016); and brain glioma (Zhang et al., 2016b), among others.

The metallic nanoparticles are composed of nickel, cobalt, or iron, commonly coated with silica or gold. This coating acts as a protection against the formation of oxides, often impairing their therapeutic application due to toxicity problems. Despite this limitation and the complexity of the production process, researchers

continue to focus on their development. Metal nanoparticles have the ability to prolong superparamagnetism in larger particles because of their high magnetization (Sun et al., 2008). Gold nanoshells encapsulated in silica layers with iron oxide and conjugated with neutrophil gelatinase-associated lipocalin can specifically target pancreatic cancer in an in vivo xenograft model (Chen et al., 2014).

The bimetallic nanoparticles are composed of two metals (e.g., FePt and FeCO), whose interaction significantly increases the chemical stability of the particle, relative to pure metal nanoparticles (Ferrer et al., 2007; Lu et al., 2005; Sun, 2006). Several studies have been carried out in order to maximize the metal and coating ratio of the nanoparticles and increase their stability, superparamagnetic properties, water solubility, and to prevent corrosion and oxidation (Bai and Wang, 2005; Du et al., 2009; Hong et al., 2005; Reiss and Hutten, 2005; Seo et al., 2006).

Magnetic liposomes, called magnetoliposomes, are closed spherical microvesicles composed of one or more aqueous inner nuclei surrounded by one or more lipid bilayers with incorporated magnetic particles. These colloidal carriers are composed mainly of phospholipids, cholesterol, and magnetite. The magnetoliposomes can be produced by different processes: (1) incorporation of liquid iron into the core of the liposomes (in most of the processes) or (2) by covalent attachment of ligands to the surface of the vesicles (Akhtar et al., 2009; James, 2006). In an alternative methodology, magnetic nanoparticles may precipitate in situ of the phospholipid vesicle, which acted as a nanoreactor (Akhtar et al., 2009). Magnetoliposomes, specially functionalized for specific targeting, are being studied for imaging agents (Meier et al., 2015; Ketkar-Atre et al., 2014; Martins et al., 2014) and therapeutic drug delivery (Ye et al., 2014), or both (Calle et al., 2015; Yan et al., 2013).

Magnetic microparticles consist of aggregate crystalline nanometric particles that form particles of micrometric size. They are constituted of biodegradable polymers (e.g., poly(lactic-co-glycolic acid), PLGA) and added magnetic components (e.g., micromagnets) characterized by superparamagnetic properties (Silva et al., 2009; Teply et al., 2008). With the aim of drug delivery at a specific site of the gastrointestinal tract, microparticles are safer and more effective than nanoparticles. Their characteristics allow protection of the drug from the enzymatic and acidic degradation to which it is subjected until the small intestine, releasing the drug only at the site of action. In addition, magnetic microparticles present a low rate of diffusion, low reactivity, and poor interaction with the immune system. Silva et al. (2009) developed magnetic polymer microparticles administered orally for local release of an antibiotic agent, amoxicillin. The system contained a core of magnetite particles coated with a solution of amoxicillin and Eudragit S 100 formed by coprecipitation and spray-drying techniques. The results showed that the particle size was 17.2 nm, presenting superparamagnetic properties when exposed to an external magnetic field. Consequently, the system was directed to the site of infection and its removal rate decreased, modifying the extent of drug

absorption. This magnetic system has been shown to be very promising, allowing the treatment of infections caused by *Helicobacter pylori*.

Magnetic resonance navigation with biodegradable microparticles loaded with iron-cobalt nanoparticles and doxorubicin was achieved in vivo using a rabbit model. Endovascular steering of magnetic microparticles and tumor targeting was improved in deep tissues (Pouponneau et al., 2012).

Despite magnetic systems seeming to be very promising, there are some limitations in their application, such as: (1) the approach to this strategy requires a specific magnet for the direction of the system, (2) the need for monitoring techniques and experts; and (3) the compatibility and stability of magnetic materials in body tissues (Anal, 2007). In addition, a constant gradient across the magnet is necessary to avoid overdose of the drug and a large and shaped magnetic force to activate the system.

5.2.1.2 Temperature stimulation

External or internal thermal stimulus can be used to either promote drug release or as a therapeutical method: hyperthermia to induce apoptosis.

The temperature-activated systems are composed of thermosensitive polymers, capable of modifying their structure in response to the local temperature or to an external heat source. According to Makó and collaborators, the temperature variation results in a phase transition process of the system, which can be: (1) variation from the soluble to the insoluble state, (2) the conversion of the solution to the gel state, (3) oscillations from the crystalline state to the amorphous state, or (4) transition from the crystal phase to the liquid state (Mako et al., 2009). Based on these changes, various thermosensitive pharmaceutical dosage forms have been studied and developed for targeted delivery and controlled drug release, such as hydrogels (Fan et al., 2009; Karg et al., 2006; Karg and Hellweg, 2009; Lu and Ballauff, 2011; Ma et al., 2010), liposomes (Kono et al., 2010, 2011), micelles (Lo et al., 2009), microemulsion gels (Zhao et al., 2007) and nanoparticles (Kumar and Mohammad, 2011).

Saint-Cricq et al. (2015) developed core-shell $\text{Fe}_3\text{O}_4/\text{SiO}_2$ mesoporous silica nanoparticles coated with a thermodegradable polymer. The nanosystem allowed the release of a model drug by augmenting temperature caused by a high frequency oscillating magnetic field. The thermodegradable polymer was made of poly(ethylene glycol) (PEG) functionalized with azo bonds that break with an elevation of temperature. The heat is generated by an external magnetic field and produced locally within a nanoscopic volume, preventing damage to surrounding tissues (Saint-Cricq et al., 2015).

Rejinold et al. developed chitosan-graft-poly(*N*-vinyl caprolactam) nanoparticles containing iron oxide nanoparticles and loaded with curcumin for targeting breast cancer cells. Controlled local drug delivery was performed by radiofrequency, which activated the thermoresponsive nanoparticles and improved tumor targeting (Rejinold et al., 2016).

Thermosensitive materials can be used in various applications due to their biodegradability, biocompatibility, and easy handling. The use of temperature-sensitive systems for pulsatile drug release has aroused a special interest in the treatment of diseases accompanied by fever. The presence of pyrogens or pathogens is often accompanied by a change in body physiological temperature (i.e., a febrile response), which stimulates the activation of the system and consequently the release of the encapsulated drug (Anal, 2007; Mako et al., 2009).

Nanocarriers can also be used for targeting energy deposition. In cancer therapy, hyperthermia, or the increase of local temperature environment of a tumor, can change the physiology of cancer cells, leading them to apoptosis. Moderate hyperthermia (41–46°C) can induce several effects on cells such as: protein denaturation, protein folding, aggregation, and DNA cross-linking. Hyperthermia is complementary to chemotherapy or radiation therapy but may have some adverse effects, like the heating of healthy tissues resulting in burns and discomfort, and the limited penetration of heat into body tissues by external means (i.e., microwave, laser, or ultrasound), resulting in thermal under-dosage in the target region if they are deep tissues. Magnetic energy can be transformed into heat by alternative magnetic field. The use of magnetic nanoparticles has some advantages compared to conventional hyperthermia therapy: the targeting of the heat to specific cancer cells avoiding other healthy tissues; increase of therapeutic effectiveness; nanoparticles can penetrate through the brain barrier, allowing the use of thermal therapy in brain tumors; nanoparticles enable the use of different routes of administration; nanoparticles can be used simultaneously to drug delivery. Iron nanoparticles and metallic nanoparticles act as heating agents for hyperthermia when exposed to alternative magnetic field (Kumar and Mohammad, 2011).

Superparamagnetic iron oxide nanoparticles were studied in subcutaneous Ehrlich carcinoma in mice, by inducing magnetic resonance hyperthermia in tumor cells. After 20 min of maximum temperature of $47 \pm 1^\circ\text{C}$ in the tumor center, apoptotic cells and a decrease of tumor growth rate was observed (Elsherbini et al., 2011). Sadhukha and colleagues developed an epidermal growth factor receptor–targeted, inhalable magnetic nanoparticles for magnetic hyperthermia of nonsmall cell lung cancer; antitumor efficacy was studied in a mouse orthotropic cancer model. The authors obtained 60% higher concentration of nanoparticles in the tumor area, an enhanced retention time and a significant inhibition of in vivo lung tumor growth (Sadhukha et al., 2013). Clares et al. prepared magnetoliposomes loaded with 5-fluorouracil and demonstrated the ability of magnetoliposomes to induce hyperthermia. These nanosystems also presented high 5-fluorouracil-loading capability and hyperthermia-triggered burst drug release (Clares et al., 2013). Solid lipid nanoparticles containing an iron oxide core were studied in vitro using human HT29 colon adenocarcinoma cells. The results demonstrated a decrease in cell viability by 40 min at 46°C of maximum temperature (Munoz de Escalona et al., 2016).

Theranostics combines therapy and diagnostics, and represents a recent research field in stimuli-responsive nanosystems. Since the physical basis of

magnetic particle imaging and magnetic hyperthermia are similar, magnetic nanoparticles can be efficacy used to obtain specific targeting of therapeutic heat. Al-Ahmady et al. developed liposome-nanoparticle hybrids loaded with doxorubicin whose release is triggered by external heat. Metallic nanoparticles were incorporated into lipid bilayers maintaining loading and retention properties and allowing imaging at the same time (Al-Ahmady et al., 2016). Ferreira and collaborators prepared a magnetic nanoparticle core and encapsulated the anticancer drug, gemcitabine, in a phospholipid bilayer (magnetoliposomes), aiming for a double therapeutic effect of drug controlled release and hyperthermia treatment both induced by alternative magnetic field. With an encapsulation efficiency of 66%, drug release profile at 37°C showed that 17% of the gemcitabine was released after 72 h and after a 5 min of thermal-induced stimuli drug release from magnetoliposomes reached 70% (Ferreira et al., 2016).

Doxorubicin-loaded PLGA microspheres with magnetic nanoparticles electrostatically assembled on the surface were investigated for *in vitro* assays using 4T1 breast cancer cells. After being submitted to an alternative magnetic field, magnetic nanoparticles can induce both heat therapy and a 7-times higher drug release (Fang et al., 2015).

The surface modification of magnetic nanoparticles can be useful in the specific targeting of cancer cells. Superparamagnetic iron oxide nanoparticles coated with 2-deoxy-D-glucose can be captured by cancer cells. These nanosystems were used in an *in vitro* U-251 human glioma cell assay demonstrating internalization capacity, MRI improvement, and magnetic inductive heating characteristics (Zhao et al., 2016). Other authors also modified magnetic nanoparticles to obtain specificity in thermotherapy and/or drug release: MUC1 glucoprotein aptamer to target adenocarcinomas (Guo et al., 2016); peptides to target nucleus or mitochondria human liver cancer cells (HepG2) (Wang et al., 2016); glucose to target high metabolic activity cells, such as mesenchymal-like cancer cells (Venturelli et al., 2016); herceptin to target HER2 + metastatic breast cancer cells (Almaki et al., 2016); atherosclerotic plaque-specific peptide-1 to target colorectal cancer cells (CT26-IL4R α) (Kuo et al., 2016); and hydroxyapatite to target bone cancer (Sneha and Sundaram, 2015).

In vivo studies are few; however, Traikov et al. used glucose-conjugated iron nanoparticles to target breast cancer cells in female Wistar rats with the goal of high concentration in high metabolic activity cancer cells (Traikov et al., 2015). Dextran-coated lanthanum strontium manganese oxide nanoparticle-mediated hyperthermia significantly reduced tumor growth by 84%, increased life survival by 50% and reduced tumor proliferation in melanoma-bearing C57BL/6J mice (Haghniaz et al., 2016).

Magnetic nanoparticles were functionalized with nucleolin antagonist multivalent pseudopeptide Nucant (N6L) and/or doxorubicin. N6L targets a nucleolin-receptor complex overexpressed selectively at the cell surface of tumor cells, and induces lethal effects to cancer cells after internalization. An intratumor injection in subcutaneous MDA-MB-231 tumor-bearing female athymic nude mice resulted

in substantial tumor growth inhibition and reduced proliferative activity when all multifunctional strategies were used: ligand, doxorubicin, and induced heat by alternative magnetic field (Kossatz et al., 2015).

Yang et al. prepared magnetic iron oxide bovine serum albumin nanospheres functionalized with folic acid to target folate receptors in nasopharyngeal cancer cells. They were also loaded with doxorubicin, obtaining an encapsulation efficiency of 80%. After intratumor injection in xenograft nasopharyngeal tumor mice, apoptosis and inhibition of cell proliferation were induced by heat treatment temperature (43°C), resulting in an increase of drug release and hyperthermia simultaneously (Yang et al., 2014).

Other in vivo studies achieved satisfactory results in theranostics by specific tumor cell- targeting, MRI, or other imaging methods (e.g., radiolabeling) and hyperthermia treatment, using functionalized magnetic nanocarriers (Baldi et al., 2014; Radovic et al., 2015; Kim et al., 2014; Mojica Piscioti et al., 2014; Tang and Chen, 2014; Wadajkar et al., 2013; Hayashi et al., 2014).

5.2.1.3 Photostimulation

Photostimulation uses light energy to trigger responsive delivery systems such as nanoparticles, liposomes, or dendrimers, conjugated to a light-sensitive chemical group to control the drug release. The main disadvantage is the production of reactive oxygen species (ROS) during photodynamic therapy, creating molecular damage in the lipids and proteins of biological membranes. Light penetration in tissue is another drawback, since it is very difficult to reach deep tissues. The use of infrared light instead of UV light allows an increase of penetration in tissue, reducing its damage (Lehner et al., 2012).

Phototherapy includes photothermal therapy and photodynamic therapy. Photothermal therapy uses electromagnetic radiation (generally infrared) to promote heat and a biocompatible photothermal agent with large absorption coefficient in the near-infrared regions is required, as well as an infrared light source. Functionalized nanomaterials of carbon, metals, and semiconductors with infrared absorption can be used as photothermal agents. The increase in the temperature depends on the infrared absorption wavelength and the power of the excitation light (Shibu et al., 2013). Gold or silver nanoparticles or their inclusion on silica nanoparticles surface are the most used nanocarriers for photothermal nanotherapy. These metals undergo a resonance (namely, surface plasmon resonance) when interacting with light that depends on the size, shape, interparticle distance, type of metal, and the local dielectric constant (Shibu et al., 2013). Nanocarriers for photothermal therapy include: gold nanorods, gold nanoparticles, core-shell nanoparticles, gold nanoshells, gold nanocubes, gold nanocages, silver triangles, carbon nanotubes, and graphene oxide. Gold nanorods were tested in cancer mice model and demonstrated selective high temperature in internal tumor, when compared to the surface. It was also observed that temperature difference increased with nanoparticle concentration, decreased with laser power, and was not influenced by treatment time (Mooney et al., 2015). Macrophage cell membrane

vesicles coated with gold nanoshells for near-infrared adsorption demonstrated active targeting to tumor endothelium and significantly enhanced in vivo blood circulation time and local tumor accumulation. When exposed to near-infrared laser irradiation, the local heat achieved is high efficiency to suppress tumor growth and selectively ablated cancerous cells within the illuminated zone (Xuan et al., 2016). Gold nanocages conjugated to a cationic mammalian-membrane disruptive peptide, as an anticancer agent, were studied in an in vivo cancer mouse model. Irradiation resulted in cancer cells ablation either by photothermal effects or target drug release. No skin damage due to irradiation was observed (Piao et al., 2016). A gold-ferrite nanocomposite was developed and tested in a melanoma cancer mice model. After intratumor injection and laser irradiation, significant differences in histological necrosis extent when compared to control groups were found (Heidari et al., 2016). Robinson et al. prepared gold nanorods and nanocages and tested them in a prostate cancer mice model. Despite both nanocarriers being photothermal agents, they differ in shape. Nanocages required 18.4 times fewer particles and approximately half the gold mass of gold nanorods to achieve the same heating profile. The tumoral uptake and biodistribution profiles also differed, with the nanocages residing in the liver, kidneys, and spleen for less time than the nanorods. Nanocages were excreted in higher percentage than nanorods (Robinson et al., 2015). Wu et al. developed PEGylated silica-core gold nanoshells aiming for cancer ablation by photothermia after exposure to external near-infrared laser irradiation, especially targeting ovarian cancer cells by conjugation with antihuman CD47 monoclonal antibody. The intraperitoneal administration in mouse ovarian cancer models showed specific target to cancer cells and ablation effect without damage of normal tissues (Wu et al., 2015).

Photodynamic therapy uses a photosensitizer agent that can be excited by a specific range of the light spectrum and trigger a chain of photochemical reactions that originates reactive oxygen intermediates, resulting in molecular desegregation or heat production. The photosensitizer agent must have a high absorption coefficient, in the 650–850 nm region, high yield of $^1\text{O}_2$, solubility under physiological conditions, large tumor targeting, poor damage of healthy tissues, and non-mutagenic or carcinogenic effects. The main advantages of photodynamic therapy over conventional radiation therapy and chemotherapy are: the specific delivery of photosensitizer agents and their stimulation with visible or near-infrared light and minimal damage of healthy tissues. The photosensitizer agents can be classified as porphyrins and nonporphyrins. Toxicity, accumulation in tissues, hydrophobicity, and poor tumor selectivity are the main limitations of porphyrin-based photosensitizer agents. Nonporphyrin photosensitizer agents include hypericin, methylene blue, cyanine dyes, and rhodacyanine dyes, aminolevulinic acid, porphycenes, and phthalocyanines. Quantum dots, silica nanoparticles, gold nanoparticles or nanorods, carbon nanomaterials, polymer nanoparticles, liposomes, and micelles are combined with photosensitizer agents to deliver site-specific photodynamic therapy (Shibu et al., 2013). Protoporphyrin IX is a well-known photosensitizer with aggregation problems. Basoglu et al. included protoporphyrin IX

in magnetoliposomes of Fe_3O_4 nanoparticles (Basoglu et al., 2016). After 5 min of LED light stimulation, all MCF-7 human breast cancer cells that were exposed to concentrations of 250 nM and higher protoporphyrin IX died within 24 h. Yue et al. prepared blended nanoparticles to target mitochondria (ROS producers) and to maximize photodynamic therapy. In the formulation, the authors included: zinc phthalocyanine as a photosensitizer agent, camptothecin as a chemotherapeutic agent, and triphenylphosphine as a selective targeting agent to mitochondria. A thioketal linker is ROS-responsive and was introduced to provide drug release as ROS were produced. Efficient subcellular delivery of the photosensitizer to mitochondria was observed and, after laser irradiation, nanoparticles had a good ROS-responsive effect to release camptothecin (Yue et al., 2016).

Despite cancer treatment being the main goal for phototherapy, infections also have been studied. Sherwani et al. developed gold nanoparticles conjugated with photosensitizers methylene blue or toluidine blue O for the treatment of *Candida albicans* infection biofilm in both skin and oral sites. Using a mouse model, the combination of gold nanoparticles with both photosensitizers significantly depleted the hyphal *C. albicans* burden against superficial skin and oral *C. albicans* infection, revealing a synergetic effect (Sherwani et al., 2015).

Combination of both phototherapies is also possible, as claimed by recent studies (Chu et al., 2016; Chen et al., 2016a; Qin et al., 2015; Chang et al., 2015), as well as with magnetic hyperthermia (Espinosa et al., 2015; Yang et al., 2015).

5.2.1.4 Radiation stimulation

The first clinical trials on radiotherapy to control drug delivery started in 1999 at Vanderbilt University. These initial studies exploited the findings that platelets are activated in tumor blood vessels after high-dose irradiation, as used in radio-surgery (Hallahan et al., 2001).

Electromagnetic radiation can be used to control drug delivery to specific tissues. This is most applicable to cancer, in which cytotoxicity and tissue destruction should be limited to cancer cells. These systems consist of innovative compositions and methods for targeting tissues with very high doses of prodrugs or nanoparticle-caged prodrugs, which are locally released as active drugs by either localized or wide field/total body low-dose radiation. The prodrug includes a nanoparticle and a drug which is nontoxic and nonactive, ineffective, until activated by radiation. The inclusion of ligands can also facilitate crossing of natural barriers, such as the blood–brain barrier.

Ionizing radiation is advantageous for many reasons. High-energy photons can easily penetrate deep tissues, which makes it suitable for the treatment of uneasily accessible tissues such as bones. Photons predominantly interact with water molecules, forming hydroxyl radicals that initiate DNA strand breaks, which are incompatible with cellular survival and are primarily cytotoxic in rapidly dividing cells such as tumor cells. Furthermore, radiation can be directed to a specific tissue using external (linear accelerator) or internal sources (encapsulated seeds and radioimmunoconjugates (RICs)). Emerging technologies (stereotactic,

brachytherapy, and intensity-modulated radiation therapy) improve the feasibility of radiation-induced drug delivery. Internal emitters also provide a strategy to envelope the entire tumor with cytotoxic therapy, even when the biodistribution is diffuse (e.g., prostate seed implants) (Wahl, 2003).

RICs have also been utilized to achieve tumor-specific cytotoxicity (Khodabandehloo et al., 2016). Examples include RICs to non-Hodgkin's lymphoma (Johnson and Press, 2000; Rao et al., 2005) and leukemia (Cheson, 2006; Nemecek and Matthews, 2003). Similar therapeutic models were developed for the treatment of colon carcinoma and other solid tumors (Ram and Buchsbaum, 1994). Tumor-specific drug delivery has the potential to minimize toxicity to normal tissues and improve the bioavailability of cytotoxic agents to tumors (Arap et al., 1998; Hallahan et al., 1995; Kasahara et al., 1994).

5.2.1.5 Ultrasound stimulation

The traditional use of ultrasound in medicine is for diagnostic imaging (which occurs at low average intensities and high frequencies) and for tissue heating (which occurs at higher intensities and higher frequencies). However, an application in drug delivery takes advantage of the remarkable ability of ultrasound to induce cavitation, which consists of the formation and/or activity of gas-filled bubbles in a medium exposed to ultrasound (Barnett et al., 1994).

Ultrasonication and cavitation can be applied in drug delivery by several mechanisms, the simplest of which, derives from the oscillatory motion of the insonated fluid and can occur in the absence of cavitation. The oscillating fluid increases the effective diffusion of molecules; thus the transport of any drug, whether free or bound to a carrier, will also be increased in a process often called "acoustic streaming" (Pitt et al., 2004). The second major contribution of ultrasound toward drug delivery consists of the disruption of drug carriers by ultrasound. Nanoparticles more dense than the surrounding fluid will be attracted to the shear field surrounding an oscillating bubble. If the shear stress exceeds the strength of the nanoparticle, it will rupture and release its contents. In the case of a liposome, the vesicle will probably reshape, often at a smaller size. The case of a liposome resizing into a number of smaller vesicles presents an interesting method of drug release. This means that some inner content must be released during the fracture event. Other mechanisms of vesicle disturbance come from collapse cavitation. The collapse event produces a shock wave that produces a shear stress at the vesicle surface that can rupture the vesicle if the critical stress is exceeded (Guzman et al., 2003; Sundaram et al., 2003).

Gene delivery using insonated microbubbles was first reported in 2000 (Lawrie et al., 2000; Shohet et al., 2000) and has been highly studied since then. The microbubbles can be injected upstream of the target region, and that target region is then easily imaged at low intensity by the presence of the bubbles. When the imaging demonstrates that the ultrasound is precisely focused on the target tissue, then the ultrasonic intensity can be increased to create collapse cavitation. This permeates the vessel walls and provides pathways for extravasation of

DNA that is freely floating along with the bubbles, or that is associated with the bubble surface (Miura et al., 2002).

Liposomes have shown their high utility for decades as drug carriers, and recently as gene carriers, particularly cationic liposomes. During the past decade, ultrasound applied in combination with gene-carrying liposomes has enhanced the transfection rate, both in vitro (Lawrie et al., 1999; McCreery et al., 2004a) and in vivo (McCreery et al., 2004b).

5.2.2 CHEMICAL STIMULI-RESPONSIVE

5.2.2.1 pH stimulation

The development of pH-activated release systems has a wide range of applications, since pH differences are permanent between healthy and pathological tissues (such as inflammations, infections, ischemia, and tumors) and in the gastrointestinal tract. Along the digestive tract, there is an increasing pH gradient: in the stomach it ranges from 1 to 2.5, in the proximal small intestine is 6.6, and 7.5 in the distal, except in the colon where the pH is 6.4 in the proximal zone and 7 in the distal zone (Sousa et al., 2008). Therefore, the development of pH-sensitive systems is one of the most promising systems in the research field.

Minimal pH variations in the surrounding environment cause changes in the conformation of the pH-sensitive intelligent polymers of the carrier, allowing the targeting and controlled release of drugs at the site of action (Ganta et al., 2008). Accordingly, these systems constitute a promising strategy to promote absorption and avoid the degradation of drugs with low permeability and solubility in sites such as the intestine (Barea et al., 2010). However, intersubject pH differences can sometimes cause drug targeting and release at unpredictable and unwanted sites, which is a gap in such systems (Barea et al., 2010).

There are several developed pH-sensitive drug carriers, such as hydrogels, nanoparticles, polymer micelles, among others (Lehner et al., 2013). Generally, drug-targeting systems for oral delivery aim to protect drugs from gastric pH and release them in the small intestine. So, materials must respond to an increase of pH values to release the drug.

According to Wang and Zhang (2012), nanoparticles are colloidal carriers ranging in size from 10 to 1000 nm, usually smaller than 200 nm. Due to the inherent advantages of their small dimensions, already mentioned above, they have been used in pH-dependent drug release. The matrix of polymeric nanoparticles can be composed by: (1) linear polymers, such as polycations (e.g., chitosan), polyanions (e.g., Eudragit, alginate, MAA), polycations, and polyanions, or (2) crosslinked polymers, (crosslinked) compounds composed of polyacrylic acid, polymethacrylic acid, chitosan, polyethylene glycol with crosslinking agents or by free radical polymerization reaction. According to the matrix, they may have distinct mechanisms of action for drug release: dissolution and/or swelling at a determined pH. Typically, the pH-sensitive nanoparticles release the drug by a

breakdown mechanism, resulting from the dissolution of the carriers. As an example, at stomach pH the nanoparticles consisting of polycarboxylic acid release only a small amount of drug. After reaching the small intestine, with the change to neutral pH (6–7.4), its carboxyl groups become deprotonated and the polymer dissolves, releasing the drug (Wang and Zhang, 2012). These authors also referred to one other release mechanism, which is the use of swellable polymers (e.g., nanogels) in the formation of nanoparticles. In this case, e.g., at acidic pH the crosslinked polymers have a compact conformation in which the porosity of the reduced matrix prevents significant diffusion of the drug from the nanogel. As the pH increases, nanogel particles swell, increasing their porosity which decreases the diffusion resistance of the drug and allows its release.

Some nanoparticles may conjugate both release mechanisms (Li et al., 2006; Wang and Zhang, 2012). Makhlof et al. studied the use of stable chitosan nanoparticles at acidic pH for enteric insulin administration. Chitosan nanoparticles were formulated with a pH-sensitive and acid-insoluble polymer, hydroxypropyl methylcellulose phthalate (HPMCP), in order to protect insulin from gastric degradation. In acidic medium, the system demonstrated good physical stability without changing the size and poor solubility of chitosan. Nanoparticles have been shown to protect the drug, improve mucoadhesion and intestinal penetration, and further control insulin release, increasing the hypoglycemic effect, compared to oral administration of an insulin solution (Makhlof et al., 2011).

Ahmad et al. also tried to improve insulin oral bioavailability. These authors reported the preparation of pH-responsive hydrogel microparticles using bacterial cellulose-*g*-poly(acrylic acid) which exhibited *in vitro* pH-responsive release and decreased the proteolytic activity of trypsin up to 60%. Oral administration showed higher hypoglycemic effect, when compared to insulin solution, and enhanced the relative oral bioavailability of insulin up to 7.45 times (Ahmad et al., 2016). Verma and colleagues produced calcium phosphate nanoparticles using vitamin B12 as a pH-sensitive and targeting ligand conjugated with chitosan. *In vivo* Caco-2 cell tests and diabetic rats revealed paracellular (through tight junctions) and vitamin B12 receptor-mediated uptake. Oral insulin bioavailability increased fourfold and sustained hypoglycemic effects up to 12 h after administration (Verma et al., 2016).

Liposomes are vesicular structures composed of one or more phospholipid bilayers. Due to their composition, they are able to carry liposoluble drugs in the lipid bilayer and water soluble drugs in the nucleus (Felber et al., 2012). In order to expand the applicability of these systems, studies of pH-sensitive liposomes for oral administration have been performed (Barea et al., 2010). These authors developed a modified lipid bilayer by the addition of pH-sensitive polymers for directing drugs to the colon mucosa. The cationic liposomes were coated by Eudragit S100, an anionic polymer with side carboxylic acid groups which exhibited solubility at pH 7.0. The drug release was evaluated in media with different pH: pH 1.4 (stomach condition), pH 6.3 (small intestine), and pH 7.8 (ileocecal junction). The study demonstrated that drug release occurred mostly in the colon,

decreasing early release in the stomach and small intestine. However, the presence of bile salts affected the polymer barrier and the integrity of the liposomes.

Micrometric pH-sensitive systems, such as microspheres and microcapsules, are other systems whose development has been widely approached by researchers. These systems are used in a variety of therapeutic applications (e.g., diabetes *mellitus*, inflammation, musculoskeletal problems) because of their small size and the ability to incorporate a large number of drugs, such as ibuprofen (Lin et al., 2005), insulin (Kim and Peppas, 2003), and calcitonin (Lamprecht et al., 2004). Kendall et al. (2009) reported that systems smaller than 100 μm allow gastric emptying to be improved and surface area/volume ratio increased, which optimizes drug delivery at the site of action, making it faster.

Alhnan et al. (2011), with the aim of improving the oral bioavailability of cinnarizine at the target site, also developed enteric microparticles of Eudragit L, highly sensitive to pH. Cinnarizine, an antihistamine used in the treatment of tinnitus, vertigo, vomiting, and nausea present in Ménière's disease, presents high acid solubility. The Eudragit polymer has a dissolution threshold at pH 6.0, allowing dissolution and drug release under conditions of the small intestine. In vitro and in vivo studies demonstrated that, unlike control (suspension of cinnarizine), the microparticles prevented the early release of the drug into the gastric medium and remained intact. Dissolution and drug release occurred rapidly, only in the upper small intestine, immediately after gastric emptying. This system demonstrated an optimization of the cinnarizine bioavailability; a value of 223% compared to the control (Alhnan et al., 2011).

Besides oral delivery, other administration routes and therapies have attracted researchers' attention. Unlike the oral systems, inflammation, infections, and tumors promote an acidic medium that can be utilized to trigger nanocarriers drug release.

Smart liposomes were prepared using pH-responsive lipids such as 2,3-dimethylmaleic anhydride and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, loaded with paclitaxel. As the pH decreases, liposome surface undergoes a charge reversion (from negative to positive) facilitating the cellular internalization and endosome escape of the nanocarrier. The anticancer activity efficiency was observed in an in vivo xenograft breast cancer model (Jiang et al., 2016).

Sericin nanoparticles conjugated with folate for targeting cancer cells with folate receptors were prepared through a pH-sensitive hydrazone-serine bond, and loaded with doxorubicin. The acidic environment of pH 5 successfully triggers doxorubicin release fivefold higher than the neutral condition of pH 7.4. Nanoparticles specifically target folate-receptor-rich KB cells and endocytosed into lysosomes, which are acidic organelles, promoting the rapid release of the anticancer drug (Huang et al., 2016). Cell-targeting, drug internalization, and lysosomal/endosomal pH-responsiveness is an approach widely used (Liu et al., 2016; Scheeren et al., 2016; Nogueira et al., 2015, 2016; Hwang et al., 2015; Chen et al., 2015). Cholesterol-PEG solid lipid nanoparticles loaded with doxorubicin were developed as pH-responsive nanocarriers for overcoming multidrug

resistance breast cancer cells. pH-controlled drug release of 63.4% at pH 4.7 and 25.2% at pH 7.4 was observed. In vivo study demonstrated that the doxorubicin delivered by the nanoparticles accumulates predominantly in tumor cells, with the inhibition of the tumor growth and no side effects (Chen et al., 2015).

Taking advantage of the acidic microenvironment of solid tumors (6.5–7.2) compared to the normal tissues (7.4), some pH-triggered nanocarriers were studied (Min et al., 2010; Deng et al., 2011; Qiu et al., 2016; Hu et al., 2015a).

Another interesting pathological condition in which pH-sensitive nanosystems can have an important role is in infections. Bacterial infection promotes a local acidic environment, due to its metabolism. Radovic-Moreno et al. prepared poly(D,L-lactic-co-glycolic acid)-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) nanoparticles loaded with vancomycin that switch surface charge as the pH changes. As the pH decreases, pH-sensitive nanoparticle surfaces suffer protonation and bind to the bacteria, delivering the drug. This systems demonstrated better efficacy and lower minimal inhibitory concentration than free vancomycin (Radovic-Moreno et al., 2012).

Other less common nanocarriers are also used to develop pH-responsive systems, such as dendrimers (Li et al., 2016), quantum dots (Zhu et al., 2015), and polymeric micelles (Gao et al., 2015).

5.2.2.2 Redox activity stimulation

Redox-responsive nanomaterials switch their properties as the redox potential changes, which ranges from 100 to 1000 between oxidizing extracellular medium and reducing intracellular medium (Lehner et al., 2012). Disulfide bonds, also called an S-S bond, or disulfide bridge, can be broken down by glutathione, whose concentration is higher in the intracellular compartment and lower in the extracellular compartment. It is also found in tumor tissues. Several anticancer agents were loaded in conjugated nanocarriers through a disulfide bond, revealing an effective intracellular delivery, including PEG-disulfide-paclitaxel conjugate nanoparticles (Chuan et al., 2014) and poly(ϵ -caprolactone)-disulfide-poly(ethylene phosphate)-doxorubicin nanoparticles (Wang et al., 2011b).

Camptothecin prodrugs with different functionalization conjugated to porous silica nanoparticles through disulfide bond were prepared and their intracellular release was proved. Drug precursor incorporated into the nanoparticles modulated both cell uptake rate and cytotoxicity, according to the different functionalization (Botella et al., 2016).

However, an improvement of therapeutic efficacy could be achieved with specific cell-targeting. For that, specific bioactive ligands should be included for targeting medication. Mesoporous silica nanoparticles were prepared and conjugated with heparin (coating agent) by a disulfide bond and loaded with doxorubicin. Lactobionic acid was also included to serve as a target ligand for HepG2 human cancer cells. The system inhibited the in vivo tumor growth with minimal side effects (Dai et al., 2014).

Dual-loaded anticancer drug nanoparticles were developed to target both cancer breast cells and cancer stem cells (Hu et al., 2015b). Nanoparticles loaded with doxorubicin (chemotherapeutic agent) and cyclopamine (cancer stem cell inhibitor) were functionalized with a hydrophobic poly(lactic-co-glycolic acid) head and a hydrophilic hyaluronic acid segment, linked by a disulfide bond. While hyaluronic acid targets the nanoparticles to CD44-overexpressing cancer cells and stem cancer cells, the system demonstrated a reduction of the number and size of both tumor and stem cancer cells. In vivo studies in a mammary fat pad tumor growth model showed a synergistic antitumor effect and prolonged survival, compared to monotherapy.

Redox-sensitive carriers may also be triggered by ROS production, as in inflammatory tissues. Chitosan nanoparticles conjugated with a copper chelator through a disulfide bond were coated with hyaluronic acid and loaded with doxorubicin. Hyaluronic acid specifically targets nanoparticles to CD44 overexpressing cells and the disulfide bond are cleaved by intracellular glutathione releasing the copper chelator that induces the ROS production. In turn, doxorubicin is released by ROS stimulus. When tested in human colon adenocarcinoma cells (HT-29 cell line), this system presented active targeting and specific cytotoxicity for the CD44-overexpressing HT-29 cells (Lin et al., 2016). Camptothecin was also included in dual glutathione and ROS-responsive micelles that demonstrated release in cancer cells with high ROS or glutathione and high in vivo antitumor efficacy (Chiang et al., 2015).

5.2.2.3 Enzymes stimulation

Several pathological conditions, such as cancer, arteriosclerotic plaque, or inflammation alter extracellular enzyme production (i.e., proteases, glycosidases, or phospholipases). Metalloproteases (MMP) are endoproteases containing zinc and are responsible for extracellular matrix protein degradation. Enzyme-responsive nanosystems are triggered by the MMP overexpression during certain diseases. Silica mesoporous nanoparticles are the most studied enzyme-responsive nanosystems once they can be coated by a biodegradable material that release the drug via enzyme activity stimulation. Liu et al. developed mesoporous silica nanoparticles sealed by phenylboronic acid—conjugated human serum albumin by a MMP-2 peptide substrate as a linker, loaded with doxorubicin. The system induced in vitro cell apoptosis and efficiently inhibited in vivo tumor growth with minimal side effects (Liu et al., 2015a). Using a similar approach with a different MMP peptide substrate and including lactobionic acid as a target cancer cell molecule, this doxorubicin-loaded system also promoted the in vivo tumor growth inhibition with minimum side effects (Liu et al., 2015b).

Hyaluronidases are produced during some bacterial infection, like *Staphylococcus aureus* and *Escherichia coli*, to promote bacterial invasion. Baier and colleagues prepared hyaluronic acid—based nanocapsules containing the antimicrobial agent polyhexanide. This nanosystem revealed stability and effectiveness as an enzyme-triggered antibacterial agent (Baier et al., 2013). During

wound healing, it is a prophylactic measure to administer antibacterial drugs to prevent wound infection, despite them often impairing wound regeneration. Hyaluronan nanocapsules containing polyhexanide biguanide and poly-L-lactic acid nanoparticles loaded with octenidine were developed as a preventive antimicrobial in wound infections. Proteases and hyaluronidases produced by bacteria stimulate the drug release after cell uptake. In vitro primary human dermal microvascular endothelial cell studies showed no sign of inflammation or toxicity. Based on the results, the authors concluded that this system could be used as a biocompatible prophylactic agent for wounds ([Grutzner et al., 2015](#)).

Nguyen and collaborators developed spherical micellar nanoparticles conjugated with a peptide-polymer amphiphiles that responds to MMP-2 and MMP-9 overexpressed in heart tissue post-myocardial infarction ([Nguyen et al., 2015](#)). The specific target process is due to the nanoparticle-MMP interaction, resulting in a morphological transition from discrete micellar nanoparticles into network-like scaffolds that remain at the local site delivering the drug. After intravenous injection of the nanoparticles at the site of acute myocardial infarction in a rat model, the system demonstrated targeted accumulation and long term retention (about 28 days) at the administration site.

Enzyme-responsive strategy is also a smart option for intracellular achievements, such as internalization of the system and intracellular enzymes activity. Mondragón et al. developed silica mesoporous nanoparticles conjugated with polymer ϵ -poly-L-lysine and loaded with camptothecin cytotoxic drug ([Mondragon et al., 2014](#)). In the presence of intracellular proteases, the amino group of the polymer hydrolyzed. The system revealed efficient cellular uptake and cell-death induction. In another study, mesoporous silica nanoparticles were conjugated with a multifunctional peptide (azido-GFLGR7RGDS) for tumor targeting, membrane-penetrating, and cathepsin B-responsive functions ([Cheng et al., 2015](#)). The nanoparticles demonstrated cancer cell internalization and drug release by the activity of cathepsin B overexpressed in endosomes and lysosomes of tumor cells. The system also showed high growth inhibition against $\alpha v \beta 3$ -positive HeLa cancer cells. The activation process by cathepsin in sensitive nanosystems was also explored in dendrimers. Lee and colleagues prepared a dendrimer-methoxy poly(ethylene glycol) (MPEG)-doxorubicin conjugate responsive to cathepsin B ([Lee et al., 2015](#)). In vivo CT26 tumor xenograft model tests revealed that the volume of the CT26 tumor xenograft was significantly inhibited when compared to controls.

Polymeric nanoparticles with a surface MMP-2 peptide substrate were conjugated to the surface of porous silicon microparticles. In the presence of MMP-2, the polymeric nanoparticles released the drug into the tumor interstitium. In a mouse melanoma lung metastasis model, systemic delivery of the system created an increase in intracellular accumulation of drug at cancer cells, compared to delivery of nonstimulus-responsive nanoparticles ([Mi et al., 2016](#)).

5.2.3 DUAL AND MULTISTIMULI RESPONSIVE NANOSYSTEMS

In recent years, a great deal of research has been carried out on dual and multistimuli responsive nanosystems that are sensitive to more than one stimulus. They respond simultaneously to a combination of two or more signals (e.g., pH/temperature, double pH, temperature/enzyme, pH/redox/magnetic, or temperature/redox/guest molecules) in order to better target drug release to disease sites, consequently maximizing therapeutic efficacy and minimizing adverse side effects/toxicity caused by drugs to healthy tissues and cells (Yavuz et al., 2009). These nanosystems are capable of responding to stimuli with superior accuracy, leading to more robust interactions. The responsive behavior mainly depends on the chemical composition of the materials used to prepare such dual or multistimuli-sensitive systems.

This approach is useful, e.g., in many pathological conditions where there is the coexistence of specific changes (e.g., a pH gradient, an oxidative environment, an elevation of temperature). The most published studies with dual and multistimuli nanosystems focus to enhance intracellular delivery and release their cargo (in most cases, anticancer drugs). The difference in intrinsic biological environments between extracellular and intracellular compartments in tumor regions, such as pH, concentration of glutathione and active redox/reduction atmospheres, can be used for triggering stimuli-responsiveness in favor of rapid drug release within tumor cells (Yu et al., 2014; Deng et al., 2015; Liu et al., 2014) which justifies the intensive research in this area. For example, tumors possess an acidic pH value due to the low oxygen in intercellular environment (Svastova et al., 2004), and an increase of temperature.

The drug release from these systems occurs either simultaneously at the target site (i.e. same place) or in a sequential manner in diverse settings, according to the nanosystems transporting pathways and/or cellular compartments (Cheng et al., 2013). Despite the theoretical versatility benefits of dual and multiple stimuli-responsive systems, their translation to the clinical practices appear more complexed than the single stimuli-sensitive nanosystems, once evidence of the control of the response to each stimulus is required (Mura et al., 2013).

Tables 5.1 and 5.2 give some recent examples of dual and multiple stimuli-responsive nanosystems, respectively, summarizing the types of stimuli, the type of nanosystems, the drug/contrast agent loaded, and the most relevant effects obtained. For this, we preferentially used example investigations that include cell line assays and in vivo animals studies.

5.2.3.1 Dual stimuli-responsive nanosystems

Dual stimuli-responsive nanosystems can be engineered to respond to different combinations of stimuli, which are briefly explored in this section and some of the most recent example research studies (i.e., published since 2010) are summarized in Table 5.1.

Table 5.1 Examples of In Vitro Cell Culture Assays and In Vivo Studies Using Dual-Stimuli Responsive Nanosystems

Stimuli	Nanosystem/Drug Loaded	Most Relevant Effects	Reference
Temperature/pH	Block copolymer P(NIPAAm-co-DMAAm)- <i>b</i> -PLA, and (P(NIPAAm-co-DMAAm)- <i>b</i> -PLC) micelles/ADR	Biodegradable micelles; Phase transition temperature of 39 and 40.5°C, respectively; Enhanced in vitro drug release rate and degradation of the hydrophobic core of micelles under pH 5.3 and 40°C; Higher intracellular uptake of micelle–ADR complex than a commercial formulation of ADR (Taxotere) at 40°C; Micelles are safer than Taxotere and can control ADR release; No cytotoxicity of blank micelles; Higher in vitro cytotoxicity using N-87 cancer cell (human stomach cancer cell) at 40°C compared to studies at 37°C.	Li et al. (2011)
Temperature/pH	Methoxy-PEG- <i>b</i> -P(n-(2-hydroxypropyl) methacrylamine dilactate)-co-(N-(2-hydroxypropyl) methacrylamide-co-histidine) micelles/DOX	Biodegradable micelles; DOX release in acidic pH and at body temperature (i.e., tumor pH-triggered drug release); Strong effects on the viability of different cancer cell lines—HeLa (human cervical cancer cells), ZR-75-1 (mammary tumor cells), MCF-7 (breast cancer cell), and H661 (monoclonal anti-HIF-1β antibody produced in mouse); Tumor-specific accumulation, enhanced permeation through the tumor site; Higher in vivo antitumor activity compare to free DOX using Balb-c/nude mice bearing human cervical tumor.	Chen et al. (2012b)
Temperature/pH	Methoxy-PEG- <i>b</i> -P(n-(2-hydroxypropyl) methacrylamine dilactate)-co-(N-(2-hydroxypropyl) methacrylamide-co-histidine) micelles/rapamycin	Hot shock protocol did not require toxic solvents or harsh surfactants to stabilize the hydrophobic drug; Faster in vitro drug release under acidic conditions (pH 6.5 and 5.0) and higher temperature (37°C vs 25°C) due to the deformation of the core structure of micelles; Delivered the drug efficiently to the cells through endocytosis; Placebo micelles was noncytotoxic; Enhanced in vitro (HCT116 cancer cells) and in vivo (Balb-c/nude mice bearing human colon tumor) cytotoxicity of drug.	Chen et al. (2013b)

Temperature/pH	Poly(NIPAAm)- <i>b</i> -poly(L-histidine) micelles/DOX	Stable under extracellular conditions but destabilized in acidic tumor environment High DOX-loading efficacy.	Johnson et al. (2013)
Temperature/pH	Hollow nanogels synthesized from an interpenetrating polymer network of PAA and PNIPAAm/INH	Much faster in vitro drug release at pH 1.2 than at physiological pH; Interesting for stomach-targeted delivery.	Xing et al. (2011)
Temperature/pH	Hollow nanogels synthesized from self-assembling of P(AA-co-MEA) grafted with either PNIPAAm alone or both PNIPAAm and mPEG/DOX	In vitro faster drug release at acidic pH (5.0) than normal physiological pH (7.4) at 37°C due to extensive disruption of complexes and marked shrinkage of nanogels; At 4°C, the drug release was restricted, mainly at pH 7.4 once drug molecules remain strongly bound to nanogels; After internalized by HeLa cells, rapidly release the drug within endosomes or lysosomes into the nuclei; Comparable cytotoxicity to free DOX in HeLa cells.	Chiang et al. (2012)
Temperature/pH	Photoluminescent poly (β-amino ester) dendrimers/DOX	Degradable nanosystems; Prepared without organic solvents; pH/temperature sensitivities occur due to a phase change of surface groups; In vitro faster drug release at acidic pH (e.g., at the lysosomal pH 4.0–5.0) than at physiological pH and 37°C; Presented strong photoluminescence; Intensive DOX fluorescence in MCF-7/ADR breast cancer cells, in contrast with no DOX fluorescence in cells cultured with free DOX.	Shen et al. (2011)
Temperature/pH	Poly(ionic liquid-co-NIPAAm) and deoxycholic acid ionically assembled nanoparticles/DOX	Sensitive to dual pH and temperature stimuli due to the deprotonation/protonation process of deoxycholic acid and highly thermo-responsive p(NIPAAm); Structural collapse of nanoparticles and higher DOX release occurred with low pH (5.2) and high temperature (43°C); Comparable inhibitory effect on cell growth using HeLa cervical cancer cells to the free DOX; Minor cytotoxicity in cells incubated with only nanoparticles, indicating their biocompatibility.	Cui et al. (2012)

(Continued)

Table 5.1 Examples of In Vitro Cell Culture Assays and In Vivo Studies Using Dual-Stimuli Responsive Nanosystems
Continued

Stimuli	Nanosystem/Drug Loaded	Most Relevant Effects	Reference
pH/redox	Synthesized RPAE— PEG copolymer micelles/DOX	Faster in vitro DOX release in a weakly acidic environment (pH 6.5) than at normal physiological (pH 7.4) and/or with a high concentration of reductive agent (5 mM DTT) (i.e., similar to the pH and reduction conditions in endosomes); In vitro drug release is more effective in a scenario of both stimuli; Higher cytotoxicity to HepG2 tumor cells (human liver cancer cell line) than free DOX at higher concentrations; Placebo micelles had a very low cytotoxicity to the tumor cells; Micelles can be quickly internalized, effectively deliver the drugs into nuclei, and inhibit cell growth.	Chen et al. (2011b)
pH/redox	PEG-SS-PTMBPEC block copolymer micelles/DOX	Low cytotoxicity and biodegradable of placebo micelles; Adequately stable with low in vitro drug release under physiological conditions (pH 7.4, 37°C); Fastest in vitro drug release occurred under 10 mM GSH and pH 5.0 conditions; Drug release activated during the whole intracellular trafficking process, i.e., not only in the mildly acidic endosomal compartments but also under the highly reducing cytoplasm and cell nucleus; Fast and maximum intracellular drug release in HeLa cancer cells; Higher antitumor activity than reduction-insensitive controls.	Chen et al. (2013a)
pH/redox	CMCH-SS-NA micelles/ DOX	Favorable stability of micelles in normal physiological environment and triggered rapid drug release in acidic and/or reduction environment using murine breast cancer 4T1 cells; DOX-loaded micelles improve target efficiency and high cytotoxicity to tumor cells after subcutaneous injection in tumor-bearing mice (in vivo studies)	Chen et al. (2016b)
pH/redox	mPEG-PAsp (MEA9_Paso(DIP) micelles/DOX	Stable at neutral pH; Faster in vitro drug release at pH 5.0 and even faster in the presence of reductive condition (10 mM DDT); Reduced premature in vivo (nude mice bearing the Bel-7402 xenograft) drug release in systemic circulation and improved therapeutic effects than DOX-loaded PEG-PCL micelles and free DOX.	Dai et al. (2011)

pH/redox	PCL-b-P(OEGMA-co-MAEBA) shell crosslinked micelles/ CPT	De-crosslinked micelles and subsequently accelerated in vitro drug release at acidic (pH 5.0) and/or reductive environment (10 mM DTT); Drug release inhibited at pH 7.4; High antitumor activity in HepG2 cells.	Hu et al. (2013)
pH/redox	PEG-SS-PDEAEMA polymersomes/ cytochrome C	Efficient loading protein under mild conditions with minimal protein denaturation and inactivation; Higher and faster in vitro protein release at pH 6.0 compared to at pH 7.4 or under a reductive condition (10 nM DTT); Greater improve of apoptosis of MCF-7 cancer cells over free Cytochrome C.	Zhang et al. (2012)
pH/redox	PEG-PAA-PDEA polymersomes/ cytochrome C	Good stability; Rapidly dissociated in response to reducing medium (10 mM glutathione) and at neutral or mildly acidic conditions; Potent cancer cell apoptosis for MCF-7, HeLa, and 293T cells.	Sun et al. (2014)
pH/redox	PDS-g-PEG/cRGD graft nanoparticles/DOX	Enhanced in vitro DOX release at pH 5.5 and/or in the presence of GSH (reducing glutathione); Comparable or higher anticancer activities in HCT-116 colon cancer cells than free DOX.	K et al. (2012)
pH/redox	PEG-nanoparticles/DOX and ODN	Controlled in vitro DOX and ODN release in accordance with DTT concentration and pH value, respectively; Both induced cell apoptosis, enhancing antitumor effect against A549 cells (adenocarcinomic human alveolar basal epithelial cells).	Yoon et al. (2013)
pH/redox	Hollow polyelectrolyte nanocapsules functionalized silica spheres/BSA	Protected protein from destruction by gastric acid; Faster in vitro BSA release under pH 6.8 and reduction conditions (10 mM GSH) (simulating the extracellular pH 9 compared to insignificant drug release at pH 1.4 (simulating the pH in the stomach); Good biocompatibility demonstrated by cell viability using Caco-2 cells.	Shu et al. (2010)

(Continued)

Table 5.1 Examples of In Vitro Cell Culture Assays and In Vivo Studies Using Dual-Stimuli Responsive Nanosystems
Continued

Stimuli	Nanosystem/Drug Loaded	Most Relevant Effects	Reference
pH/redox	PMAA-based nanohydrogels/DOX	High-loaded DOX into the nanohydrogels due to the strong electrostatic interactions between drug and nanohydrogels; Low in vitro drug release at pH 7.4, accelerated at pH 5.0 and in a reducing environment (10 mM GSH); Loaded DOX could be quickly released in the presence of GSH, but not in DTT, probably due to the synergic effect of reduction and charge exchange of GSH at a low pH; Fast uptake by human glioma (U251MG cells) via endocytosis, and then biodegraded to release the loaded drugs; Comparable antitumor activity to free DOX.	Pan et al. (2012)
pH/redox	Magnetic and silver nanocrystals deposited onto PMAA _{s-s} hollow microspheres/DNR	High encapsulation efficiency of drug ($\approx 96\%$); Low in vitro drug release at pH physiological (7.4) while under acidic conditions (pH 5.0) a significantly higher amount of drug was released due to ionization degree changes that result in conformational alternations and phase transitions; Complete drug release at acidic pH and in the presence of reducing agent (TCEP) proving synergic effect of pH and reductive environment; No cytotoxicity of empty carriers on the MCF-7 cells was observed, whereas the cytotoxicity of the DNR-loaded magnetic and silver hollow microspheres was comparable to that of free DNR.	Chatzipavlidis et al. (2013)
pH/magnetic	pH sensitive charge-conversional peptide mimic shell crosslinked Fe ₃ O ₄ magnetic nanocarriers/DOX hydrochloride	High DOX-loading capacity; Higher and complete in vitro release rate of DOX at lower pH (4.0 and 5.0) than at physiological pH (7.3); Sustained drug release profile and cellular internalization; Transformed from a negatively charged to a positively charged form in the acidic environment (i.e., pH of subcellular lysosomal compartments of tumor cells); Magnetic-field induced heating (i.e., hyperthermia effect); Enhanced in vitro cytotoxicity activity in HeLa cells under a magnetic field.	Barick et al. (2012)

pH/magnetic	Fe ₃ O ₄ magnetic nanocarriers/DOX (conjugate 1) and RGD Fe ₃ O ₄ magnetic nanocarriers/DOX (conjugate 2)	<i>Conjugated 1:</i> Higher in vitro anticancer activity against HeLa cells than free DOX; Improved the drug efficacy by magnetically guided delivery of nanocarriers (HeLa cells) due to increased local drug concentration in the region of cancer cells and consequently enhanced cellular drug uptake. <i>Conjugated 2:</i> Recognized the specific cells effectively (U-87 MG cells—human primary glioblastoma cell line that overexpresses $\alpha_v\beta_3$ integrin) compared to MCF-7 cells (breast cancer cell with low $\alpha_v\beta_3$ integrin expression); Increased cytotoxicity under external magnetic fields compared to free DOX	Zhao et al. (2012)
pH/magnetic	Superparamagnetic Fe ₃ O ₄ nanoparticles coated with MPEG- <i>b</i> -P (MAA- <i>co</i> - <i>n</i> BMA)- <i>b</i> -PGMA) and FA-PEG- <i>b</i> -PGMA)/ADR	Higher in vitro release rate of drug at acidic pH (mimicking endosome/lysosome conditions) compared to slow drug release at pH 7.4 (simulating the blood environment) due to the disruption of the ionic bond caused by protonation; Higher cellular uptake of folate-conjugated magnetic nanoparticles via folate-receptor endocytosis process in HeLa cells overexpressing folate than nonfolate-conjugated nanoparticles; Folate conjugation significantly increased nanoparticle cytotoxicity using HeLa cells.	Guo et al. (2011)
pH/magnetic	pH-responsive nanogels/anti-GFP siRNA and iron oxide nanoparticles	Boosted cellular uptake of nanogels using HeLa cells by the application of magnetic field (iron oxide nanoparticles used as magnetofection agents); Iron oxide nanoparticles used also as probes to track the intracellular trafficking and drug release by electron microscopy.	Curcio et al. (2012)
pH/magnetic	Anchoring superparamagnetic Fe ₃ O ₄ nanoparticles on the pore outlet of MSNs/dexamethasone	In vitro drug release strongly inhibited at pH 5–8 due to effective capping of the pores; Rapid in vitro drug release at pH 2–4 due to hydrolysis of the borooester bond; Drug release in a pulsinate manner at pH 3–7 once Fe₃O₄ could be switched “on” and “off”; Displayed good magnetic property and high cell biocompatibility and cellular uptake using MC3T3-E1 cells (osteoblastic cell line).	Gan et al. (2011)

(Continued)

Table 5.1 Examples of In Vitro Cell Culture Assays and In Vivo Studies Using Dual-Stimuli Responsive Nanosystems
Continued

Stimuli	Nanosystem/Drug Loaded	Most Relevant Effects	Reference
pH/magnetic	Superparamagnetic Fe ₃ O ₄ nanoparticles capped MSNs ensemble/ dexamethasone or DOX	In vitro dexamethasone release inhibited at physiological pH (7.4); Very fast in vitro dexamethasone release at pH 5–6; Good magnetic properties, high cell biocompatibility, efficient endocytosis and delivery of DOX in the MC3T3-E1 cell line.	Gan et al. (2012)
pH/pH	Polyphosphoester pH-sensitive nanoparticles/ DOX	Reversed surface charge from negative at pH 7.4 to positive at tumor extracellular pH (~6.8) due to acid-responsive cleavage, which facilitated tumor cell internalization; Promoted acid-triggered drug release; Enhanced cytotoxicity in drug resistant SK-3rd cancer stem cells.	Du et al. (2011)
pH/pH	Poly([2,2'-(propane-2,2-diylbis(oxy))bis(ethane-2,1-diyl) diacrylate]-co-[hexane-1,6-diyl diacrylate]-4,4'-trimethylene dipiperidine), poly-beta-aminoester ketal-2 nanoparticles/BSA	Excellent stability in healthy physiological pH; At pH 5 (endosomal level), nanoparticles underwent a rapid degradation probably due to increase the osmotic pressure in the endosomal compartments followed by concomitant release of encapsulated payloads; No significant cytotoxicity of placebo nanoparticles; Higher uptake of dual pH responsive polymeric nanoparticles encapsulated labeled BSA than to PLGA nanoparticles encapsulated fluorescent BSA using RAW 264.7 macrophage cells.	Sankaranarayanan et al. (2010)
pH/cis diol	Boronate crosslinked micelles/PTX	Slower in vitro drug release from boronate crosslinking micelles compared to that from noncrosslinked micelles; Accelerated drug release after addition 100 mM mannitol or adjusting pH value at 5.0; Enhanced in vitro stability when administered to nude mice; Accumulated preferentially in SKOV-3 ovarian cancer cells.	Li et al. (2012)
Temperature/enzyme (DNase I)	Attached duplex DNA to the outlet of MSNs/CPT	In vitro triggered drug release by increasing temperature to 50°C, leading to thermal denaturation of the DNA duplex, or addition of deoxyribonuclease I (DNase I that cleave the DNA with high selectivity); Significant higher cytotoxicity activity to HepG2 cancer cells than CPT-loaded MNs or free CPT.	Chen et al. (2011a)

Redox/enzyme	Hyaluronic acid-conjugated MSNs/DOX	In vitro drug release was markedly restricted in pH 7.4 and pH 5.0, while it was significantly accelerated upon the addition of glutathione (GSH) or hyaluronidases (HAase); Accelerated drug release in the presence of both GSH and HAase; Higher cellular uptake via CD44 receptor-mediated endocytosis in human HCT-116 cells; Higher cytotoxic against HCT-116 cells than NIH-3T3 (CD44 receptor-negative).	Zhao et al. (2015)

Abbreviations: ADR, adriamycin; BSA, bovine serum albumin; CMCH-SS-NA, carboxymethyl chitosan-cysteamine-N-acetyl histidine; CPT, camptothecin; DTT, dithiothreitol; DNR, daunorubicin; DOX, doxorubicin; FA-PEG-b-PGMA, the folate-conjugated block copolymer folate-poly(ethylene glycol)-b-poly(glycerol monomethacrylate); INH, isoniazid (antitubercular drug); mPEG, monomethoxypoly(ethylene glycol); MPEG-b-P(MAA-co-nBMA)-b-PGMA, methoxy poly(ethylene glycol)-b-poly(methacrylic acid-co-n-butyl methacrylate)-b-poly(glycerol monomethacrylate); MSNs, mesoporous silica nanoparticles; ODN, anti-bcl-2 oligonucleotide; PAA, poly(acrylic acid); P(AA-co-MEA), poly(AA-co-2-methacryloyl ethyl acrylate); PCL, poly(ϵ -caprolactone); PCL-b-p(OEGMA-co-MAEBA), PCL-b-poly(oligo(ethylene glycol) monomethyl ether methacrylate-co-p-(methacryloxyethoxy)benzaldehyde); PDS-g-PEG/cRGD, poly(2-(pyridine-2yl)disulfanyl) ethyl acrylate-g-PEG/cRGD; PEG, poly(ethylene glycol); PEG-PAA-PDEA, poly(ethylene glycol)-poly(acrylic acid)-poly(2-(diethyl amino)ethyl methacrylate); PEG-SS-PDEAEMA, poly(ethylene glycol)-SS-poly(2-(diethylamino)ethyl methacrylate); PEG-SS-PTMBPEC, poly(ethylene glycol)-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate); PLGA, poly-lactic-co-glycolic acid; PMAA, poly(methacrylic acid); PMAA_{s-s}, poly(methacrylic acid-co-N,N'-methylenebis(acrylamide).co-poly(ethylene glycol) methyl ether methacrylate-co-N,N'-bis(acryloyl)-cystamine); P(NIPAAm-co-DMAAm)-b-PLA, poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide-b-lactide); P(NIPAAm-co-DMAAm)-b-PLC, poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide-b-e-caprolactone); PTX, Paclitaxel; RGD, arginine-glycine-aspartic acid; RPAE, reducible poly(β -amino esters); TCEP, tris(2-carboxyethyl) phosphine.

Table 5.2 Examples of In Vitro Cell Culture Assays and In Vivo Studies Using Multiple-Stimuli Responsive Nanosystems

Stimuli	Nanosystem/Drug Loaded	Most Relevant Effects	Reference
Temperature/pH/redox	P(OEGA-co-DMDEA) nanogels containing acid labile ortho ester bond and BADS/DOX, PTX	Fast thermo-responsive property and shrank upon increase temperature to 37°C; Nanogels swelled quickly in weakly acidic media (pH 4–6); In vitro drug release was low at pH 7.4 (at normal physiological pH medium) and greater and more rapid drug release in pH to 5.0 or 6.0; Accelerated in vitro drug release by combining three stimuli (i.e., plus the presence of dithiothreitol (DTT), a reductive condition); PTX-loaded multiresponsive nanogels presented a concentration-dependent cytotoxicity to MCF-7 cells.	Qiao et al. (2011)
Temperature/pH/magnetic	P(NIPPAAm-co-MAA) coated magnetic mesoporous silica nanoparticle/DOX.HCl	In vitro drug release was low at pH 7.4 (at normal physiological pH medium) at 37°C and greater and more rapid drug release in pH to 5.0 or 6.5 under otherwise the same conditions; Similar cytotoxicity to HeLa cells as free DOX; Both overcome of the lack of selectivity of anticancer agents and EPR/magnetic-targeting allow increasing the therapeutic efficacy.	Chang et al. (2011)
pH/redox/magnetic	Magneto-fluorescent theranostic gold nanoshells (TGNS) conjugated with antibodies targeting NGAL in AsPC-1-derived xenografts in nude mice	Specifically targeted pancreatic cancer cells in vitro using NGAL-expressing pancreatic cancer cells (AsPC-1 cells) and in vivo (in nude mice with AsPC-1 xenografts) providing contrast; Highly specific cancer cell death via NIR photothermal therapy in vitro (AsPC-1 cells).	Chen et al. (2014)

Temperature/redox/ guest molecule	Supramolecular vesicles based on host–guest complex formation between C4AS and MVC12/DOX.HCl	In vitro rapid and complete drug release upon increasing temperature or addition of competitive guest, the cyclodextrin, due to disruption of the vesicular structure; High antitumor activity in cell experiments using HepG2 cells, i.e., human liver cancer cell line.	Wang et al. (2011)
Temperature/pH/ guest molecule	Supramolecular binary vesicles based on the host – guest complexation WP6 and SAINT molecule/DOX	Efficiently drug released either by adjusting acidic pH or by adding a certain amount of Ca^{2+} due to the structure disruption; Reduce cytotoxicity for normal cells using NIH3T3 cells (normal cells); Comparable cytotoxicity effect for MCF-7 cancer cells as free DOX.	Cao et al. (2014)
Temperature/pH/ guest molecule	Complexation of cucurbit(8)uril methylviologen-functionalized PNIPAAm and naphthalene-terminated PDMAEMA micelles/DOX	Enhanced in vitro drug release in response to decreasing temperature, lowering pH or addition of competitive guest molecules (e.g., adamantanamine) due to the collapse or swelled of the micelles; Nearly instantaneous and complete drug release with the combination of two stimuli (e.g., temperature and adamantanamine); The combination of external stimuli, cytotoxicity activity could be fine-tuned using HeLa cells.	Loh et al. (2012)
Magnetic/pH/redox	Superparamagnetic iron oxide (Fe_3O_4) and paramagnetic manganese oxide (MnO_x) loaded onto exfoliated GO nanosheets/DOX	Biocompatible nanopatform; MnO_x nanoparticles disintegrate in mild acidic and reduction environment generating a strong positive contrast enhancement which can be useful in diagnostic imaging process; DOX release from GO-based nanoaggregates is inhibited at neutral pH but accelerated in mildly acidic medium, mitigating toxic side-effects and enhancing the therapeutic outcomes; Inhibit the metastasis of cancer cells (using highly metastatic human breast cancer cell MDA-MB-231) by downregulating the expression of main proteins related to the metastasis; DOX loaded in nanographene oxide-based multifunctional triple stimuli-responsive systems demonstrated higher cytotoxicities than free DX against MCF-7/ADR cells (human mammary adenocarcinoma cells) and reverse the multidrug resistance of cancer cells.	Chen et al. (2014)

(Continued)

Table 5.2 Examples of In Vitro Cell Culture Assays and In Vivo Studies Using Multiple-Stimuli Responsive Nanosystems
Continued

Stimuli	Nanosystem/Drug Loaded	Most Relevant Effects	Reference
pH/redox/light	Hollow MSNs modified with PDEAEMA/DOX	High loading capacity; Drug release in acid and reduction intracellular environment; Enhanced drug release by external UV irradiation; Greater controlled drug delivery and release compared to single stimulus responsive nanocarriers; In vitro cytotoxicity assay using HeLa cells confirmed the augment with UV irradiation.	Zhang et al. (2015)

Abbreviations: BADS, bis(2-acryloyloxyethyl)disulfide; C4AS, p-sul-fonatocalix (4)arene; DMDEA, 2-(5,5,-dimethyl-1,3-dioxan-2-yloxy) ethylacrylate; DTT, dithiothreitol; GO, graphene oxide; MSNs, mesoporous silica nanoparticles; MVC12, asymmetric viologen; OEGA, monomethyl oligo(ethylene glycol) acrylate; PDEAEMA, poly(ethylene glycol)-SS-poly(2-(diethylamino)ethyl methacrylate; P(NIPPAAm-co-MAA), poly(N-isopropylacrylamine-co-methacrylic acid); PTX, paclitaxel; NGAL, neutrophil gelatinase-associated lipocalin; SAINT, molecule of pyridinium amphiphiles with one pyridinium group and two alkyl chains; WP6, water-soluble pillar[6]arene.

The nanosystems activated simultaneously by pH and temperature stimuli are among the most investigated dual sensitive nanosized formulations. The design and development of pH- and temperature-responsive polymers, preferably with biodegradable requirements, are generally based on the incorporation of pH-sensitive components, such as weak acids (e.g., deoxycholic acid and propylacrylic acid) into thermosensitive polymers such as poly(*N*-isopropylacrylamide) (PNIPAAm) (Cheng et al., 2013; Li et al., 2011; Xing et al., 2011; Cui et al., 2012; Ta et al., 2010; Johnson et al., 2013). Therefore, investigators developed various pH- and temperature-responsive polymers due to their versatility and straightforwardness. In these nanosystems, a slight pH change could provide a phase transition with subsequent drug release. Such effect has been explored to develop functionalized nanosystems for response to internal stimuli, using the biological characteristic of the tumor cell or the tumor-specific microenvironment and consequently improve the efficacy and specificity of anticancer agents (Chen et al., 2012b; Li et al., 2011; Chiang et al., 2012). Tumor tissues present an acidic pH value (5.3) and a hyperthermia associated with inflammation process ($\approx 40^{\circ}\text{C}$) which can be explored by pH and thermoresponsive nanosystems. For example, Li and colleagues prepared pH- and temperature-responsive micelles composed with types of thermosensitive biodegradable block copolymer poly(*N*-isopropylacrylamide-*co-N,N*-dimethylacrylamide-*b*-lactide), P(NIPAAm-*co*-DMAAm)-*b*-PLA), and poly(*N*-isopropylacrylamide-*co-N,N*-dimethylacrylamide-*b*- ϵ -caprolactone), (P(NIPAAm-*co*-DMAAm)-*b*-PLC), that presented a phase transition temperature of 39°C and 40.5°C , respectively (Li et al., 2011). The hydrophobic core degradation, intramicelle collapse of micelles, and consequently the *in vitro* release of adriamycin (an anticancer agent), was greatly enhanced at simulated tumor cell condition (pH 5.3 and 40°C), compared to release in normal physiological conditions (pH 7.3 and 37°C). The change of temperature also influenced the intracellular uptake of drug, probably due to hydrophobic interaction between the micelles and cell membrane. The cytotoxicity assay against human stomach cancer cell (N-87 cancer cells) was also superior at 40°C . Chen et al. also demonstrated the proof-of-concept through the results obtained in the *in vivo* studies using Balb-c/nude mice bearing human tumors which have been administered dual temperature- and pH-responsive micelles (Chen et al., 2012b, 2013b). The authors reported a specific targeting efficiency and an enhancement of *in vivo* cytotoxicity, compared to the administration of free doxorubicin. Shen et al. also prepared dual temperature and pH stimuli-responsive photoluminescence dendrimers without the use of organic solvents (Shen et al., 2011), which represents an advantageous approach. The strong photoluminescence property of dendrimers is useful for monitoring the level of drug loading and release. In addition to their research into the effective delivery and release of anticancer agents, these dual temperature- and pH-responsive nanosystems have also been explored for the release of other therapeutic agents, such as isoniazid (an antitubercular agent) (Xing et al., 2011).

The pH and redox potential appear modified in certain pathological conditions, including in all cancer cells (Meng et al., 2009). Most of the research studies concerning pH and redox dual stimuli-sensitive nanosystems are related to anticancer agents (Table 5.1). A promising paradigm for cancer therapy is attending the specific microenvironment of tumor regions. Therefore, in tumor tissues, both stimuli environments of acidic endosomal pH value (5.0–5.5) and high concentration of GSH (i.e. high reduction environment) in the cytoplasm can be combined to produce dually pH/redox-responsive systems, leading to rapidly and more complete release, and enhanced uptake of chemotherapy drugs in tumor cells under endo/lysosomal pH and/or cytosolic reductive conditions (Wu et al., 2013). Additionally, these nanosystems have been designed in order to increase in vivo stability of nanosystems via disulfide-crosslinking (Cheng et al., 2013). Different authors developed micelles from copolymers that have pH and reduction dual sensitivities, demonstrating an improvement in the uptake of doxorubicin (DOX) in different cancer cell lines, e.g., HepG2, the human liver cancer cell line (Chen et al., 2011b); HeLa, human cervical cancer cells (Chen et al., 2013a), murine breast cancer 4T1 cells (Chen et al., 2016b); and an effective inhibition in the cell growth, compared with single pH or reduction-responsive micelles. These studies were carried out using confocal laser scanning microscopy in order to examine the intracellular release and biodistribution; MTT assays investigated the antitumor activity. These micelles were prepared using different copolymers, including biodegradable ones which are indicated in Table 5.1. The authors demonstrated that a boosted release of DOX from pH/redox-sensitive micelles could be triggered in response to both low pH and high GSH concentration, i.e., to the intracellular acidic and reduction environment of cancer cells, leading to enhanced uptake into the nuclei of cancer cells and higher cytotoxicity in vitro than single control nanosystems. Yoon and collaborators demonstrated a synergistically induced cell apoptosis with the release of doxorubicin with antisense bcl-2 oligodeoxynucleotides from PEG-nanoparticles (Yoon et al., 2013).

Others nanoplatforms have also been used to prepare dual pH- and redox-responsive systems, such as polymersomes (Sun et al., 2014; Zhang et al., 2012), nanoparticles (Remant Bahadur et al., 2012; Shu et al., 2010), nanohydrogels (Pan et al., 2012), and nanocrystals (Chatzipavlidis et al., 2013). The disulfide-crosslinking of micelles and other nanosystems can improve the extracellular stability, maintaining their ability for intracellular drug release (Xu et al., 2009; Li et al., 2009; Zhang et al., 2012; Chen et al., 2011b; Sun et al., 2014; Dai et al., 2011, 2015; Shu et al., 2010). For example, Zhang and collaborators developed dual pH and redox-responsive nanosized polymersomes that encapsulated Cytochrome C, a protein (Zhang et al., 2012). In addition to efficient loading of protein and stability, the authors reported an improvement in apoptosis of MCF-7 cancer cells, compared to free Cytochrome C. The literature also reported in vivo animal studies using dual pH/redox stimuli-responsive nanosystems in order to investigate their therapeutic effect (Chen et al., 2016b; Dai et al., 2011). Using breast tumor-bearing mice, Chen and collaborators demonstrated that

pH/redox-responsive micelles improved the anticancer efficiency of DOX and concluded it as useful to reduce side effects (Chen et al., 2016b).

Taking the benefits related with magnetic field stimulation, magnetic nanosystems have been used associated with other stimuli (e.g., pH), once magnetic nanoparticles can integrate several functionalities such as pH-sensitive moieties in their surface. The dual pH- and magnetic-sensitive nanosystems have the advantage to combine pH-triggered drug release with magnetic-targeting, magnetic chemotherapy, and magnetic imaging (Cheng et al., 2013). As with the other dual-stimuli-responsive nanosystems, these sensitive carriers have also been explored as an attractive strategy to optimize treatments in the area of cancer, as it often results in synergistic effects (e.g., chemotherapy and hyperthermia). Generally, these systems can conjugate three unique features: magnetically controllable, specific targeting, and pH-responsive. It is possible to remotely control the movement of magnetic carrier to achieve the targeted region by the application of an external magnetic field (Zhao et al., 2012), reducing the side effects of anticancer agents. There are studies in the literature that proved the in vitro efficacy of anticancer agents loaded in pH-responsive magnetic nanocarriers (Barick et al., 2012; Zhao et al., 2012). For example, Barick et al. demonstrated that doxorubicin hydrochloride-loaded pH-sensitive charge-conversional peptide mimic shell cross-linked magnetic Fe_3O_4 nanoparticles (a biocompatible carrier) exhibited higher in vitro cytotoxicity activity to HeLa cells than individual treatments of magnetic nanoparticles with external activation magnetic field and anticancer drug-loaded magnetic nanoparticles (Barick et al., 2012). The conjugation of magnetic nanoparticles with some targeting moieties (e.g., arginine-glycine-aspartic acid (RGD) and folate) can improve the specific targeting/recognition of carriers to specific tumor cells (Zhao et al., 2012; Guo et al., 2011). Arginine-glycine-aspartic acid contains small peptides that specifically attach the cell adhesion $\alpha_v\beta_3$ integrin (e.g., U-87 MG cells). This integrin has an important role in tumor angiogenesis, progression, and spread (Xiong et al., 2002). In addition to anticancer agents, other therapeutic agents such as antiinflammatories have also been tested in dual pH and magnetic stimuli-responsive nanosystems (Gan et al., 2011, 2012). Therefore, these nanosystems represent a promising candidate in the formulation of in vivo targeted delivery of therapeutic agents to low pH tissues (e.g., tumors and inflammatory sites).

Concerning the dual temperature- and redox-sensitive systems, each of these stimuli used alone in a responsive nanosystem demonstrated a promising approach for biomedical applications, such as triggered intracellular delivery of encapsulated cargo, mainly for anticancer agents. The tumor microenvironment presents slightly higher temperature than that of normal tissues. In sensitive systems, thermal-materials are designed to undergo a collapse/destruction of system structure and consequently release its payload in response to elevated temperature in tumor or upon externally induced local hyperthermia (Liang and Liu, 2016). As previously mentioned, redox activity is another stimulus that has been widely investigated due to the difference between mildly oxidizing extracellular and

reducing intracellular spaces (Lv et al., 2011). Some studies associated them into the same carrier and demonstrated its efficacy to control drug delivery due to the destruction of nanogels structure, either upon physical stimulus (cooling to room temperature) and/or upon biochemically relevant stimulus (treatment with a reducing agent) (Lv et al., 2011; Tan et al., 2011). However, the cited studies did not perform assays using tumor cell lines nor in vivo testing.

The inefficient cellular uptake and low intracellular drug concentration are the main limitations/challenges for the efficacy of delivery systems including nanocarriers in cancer therapy. In order to overcome such issues, Du et al. and Sankaranarayanan et al. developed dual pH-sensitive polymer nanoparticles which contained two pH-responsive moieties. These polymeric nanoparticles reversed their surface charge in response to the extracellular and intracellular pH gradients through chemical mechanisms, facilitating tumor cell internalization of nanoparticles and intracellular anticancer agent delivery (Du et al., 2011; Sankaranarayanan et al., 2010).

Other dual-stimuli responsive nanoparticles using miscellaneous stimuli included pH/cis diol boronate cross-linked micelles (Li et al., 2012); temperature- and light-sensitive micelles (Feng et al., 2010); redox- and light-responsive micelles (Han et al., 2012); ultrasound- and redox-responsive micelles from a biodegradable block copolymer (Han et al., 2012); and duplex DNA attached to mesoporous silica nanoparticles (Chen et al., 2011a). Most of these miscellaneous dual sensitive nanosystems presented in neither cell culture assays nor in vivo studies.

Light is an attractive external stimulus since it can perform both temporal and spatial controlled drug release (Volodkin et al., 2009). Since ultrasound waves can penetrate much deeper into the body than light, this extracorporeal stimulus has also been intensively explored for the treatment of solid tumors (Kennedy, 2005).

5.2.3.2 Multistimuli responsive nanosystems

Different multistimuli responsive nanosystems have also been developed (Table 5.2) (e.g., nanogels, magnetic mesoporous silica nanoparticle, supramolecular vesicles, micelles), combining in the same nanoformulation three or more stimuli-sensitivities such as temperature/pH/magnetic (Chang et al., 2011), temperature/pH/redox (Qiao et al., 2011), temperature/redox/guest molecule (Wang et al., 2011a), temperature/pH/guest molecule (Cao et al., 2014; Loh et al., 2012), magnetic/pH/redox (Chen et al., 2014; Bilalis et al., 2012) and pH/redox/light (Zhang et al., 2015). Most of these studies also cover both cancer areas, i.e., therapeutic and diagnostic (Qiao et al., 2011; Chang et al., 2011; Cao et al., 2014; Wang et al., 2011a). Despite some studies demonstrating comparable/similar in vitro cytotoxicity activity of multisensitive responsive nanosystem to cell tumor lines as free anticancer agent, they have advantages such as remarkably reduced damage for normal cells (Chang et al., 2011; Cao et al., 2014). Chen and collaborators developed a multifunctional nanographene oxide-based triple

stimuli-responsive system that demonstrated through in vitro cells assays as useful for realizing the theranostics (i.e., simultaneous therapeutic and diagnostic imaging) of cancer in an intelligent, on-demand, and efficient manner ([Chen et al., 2014](#)).

5.3 CONCLUSIONS AND FUTURE PERSPECTIVES

An attractive approach to reach targeted drug delivery is the use of nanosystems that respond to internal or external stimuli. Diverse types of responsive materials and different types of stimuli able to trigger the drug release to a specific site (spacial control) and at the right time (temporal control) have been used for in vitro proofs of concept. The development of such stimuli-responsive nanoplat-forms seems to have promising effects to maximize their goal (being therapeutical or diagnostic agent) and minimize adverse effects through a specific targeting and release of the drug at particular sites, cells, or at molecular level.

Although scientific papers have suggested the great potential of these systems for clinical application, only a few stimuli-responsive nanosystems, namely thermosensitive liposomes and magnetic iron oxide nanoparticles which respond to external stimuli, have reached the clinical phases. This limitation could be explained by various factors, such as ([Mura et al., 2013](#)) the complexity of architectural design or problems related to transferring for large scale production, many available stimuli-responsive systems contain nonbiodegradable or nonbio-compatible materials and unknown toxicologic profiles of these nanosystems.

Two examples of products in clinical stages are liposomes ThermoDox and iron oxide nanoparticles Nanotherm. The liposomes ThermoDox encapsulate an anticancer drug, doxorubicin ([Delcea et al., 2011](#)). With a temperature of 40–45°C, the heat-sensitive liposomes quickly change their structure, generating openings in the liposomes that release doxorubicin. Studies in animal demonstrated that ThermoDox delivered 25 times more doxorubicin into tumors, compared with doxorubicin intravenous infusion, and 5 times more than standard liposomal formulations. This technology should be used in association with heat-based treatments (e.g., radiofrequency thermal ablation, microwave hyperthermia). ThermoDox is in clinical trials for the treatment of breast cancer (Phase II) and hepatocellular carcinoma (Phase III).

The iron oxide nanoparticulate Nanotherm is a new thermotherapy based on biocompatible aminosilane-coated superparamagnetic iron oxide nanoparticles with a diameter approximately of 15 nm ([magforce, 2017](#)). After stereotactic injection of nanoparticles into the tumor, the particles agglomerate immediately in the tumor tissue. They respond to magnetic stimuli, which are of no harm to humans, generating heat within the tumor while sparing the surrounding healthy tissue. They are approved for the treatment of glioblastoma. The association of Nanotherm therapy with percutaneous radiotherapy is feasible and safe in

recurrent glioblastoma. This therapy obtained the European certification (CE mark) for the treatment of brain tumors in 2010.

In order to achieve clinical benefits and impacts of the stimuli-responsive nanosystem, future efforts should be going toward development of biodegradable and noncytotoxic polymers that can efficiently load in the nanosystems, avoid drug elimination from circulation, accumulate the nanosystem preferentially in the targeted site, and appropriately modulate the drug release in response to clinically viable external and/or internal stimuli. Additionally, more investigation concerning the targeting ligands (e.g., peptides, aptamers) attached to sensitive nanoplateforms is also required for pathologically specific delivery and adequate release of therapeutic agents. Despite these limitations, we believed that such stimuli-responsive nanosystems will have an important role in the control of diseases with global impact, such as cancer.

ACKNOWLEDGMENTS

The authors thank to National Funds through FCT—Portuguese Foundation for Science and Technology, within the framework of the Strategic Funding UID/Multi/04546/2013.

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Stimuli sensitive ocular drug delivery systems

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CHAPTER OUTLINE

6.1 Introduction	211
6.2 Anatomical Characteristics of the Eye	212
6.2.1 Barriers to Topical Ocular Drug Delivery	215
6.2.2 Classic Routes of Ophthalmic Administration	215
6.3 Hydrogel as Drug Delivery Systems	217
6.3.1 Stimuli-Sensitive Hydrogels	218
6.3.2 Properties of In Situ Hydrogels: Studies of Stimuli Response	222
6.3.3 Stimuli-Responsive Hydrogels as Ophthalmic Drug Delivery Systems: Drug-Loading Efficiency and Drug Release	229
6.4 Other Pharmaceutical Systems for the Modification of Drug Loading and Release Properties	231
6.5 New Technologies for Determining Ocular Residence Time and Ocular Biodistribution of Hydrogels	242
6.6 Toxicity and Safety of Polymers Used in Stimuli Gel System: In Vitro, Ex Vivo and In vivo Assays	246
6.7 Effect of the Hydrogels on the Corneal Regeneration Capacity	247
6.8 In Vivo Studies in Animals and Effectiveness of Drugs When They are Incorporated in the Hydrogels	248
6.9 Studies of Drug Penetration and Promotion	249
References	251

6.1 INTRODUCTION

Efficient ocular drug delivery remains a challenge for drug delivery researchers, due to the complexity and particularity of the anatomy and physiology of the eye. Conventional eye drops account for 90% of formulations in the market.

Despite the excellent compliance by patients, one of the major pitfalls is the difficulty in maintaining optimal drug concentrations in the eye tissues, caused by the rapid precorneal drug loss and the presence of different barriers for drug absorption; most importantly, the effective drug clearance mechanism from eye surface, caused by dynamic barriers such as choroidal and conjunctival blood flow. The lymphatic clearance, or tear dilution, the static absorption barriers such as the different layers of cornea, sclera, and retina, and the effect of the efflux pumps, are the main reasons that causes a rapid loss of drug of the eye surface and hinders drug diffusion and penetration in the eye tissues. Considering these points, the development of a suitable drug delivery system becomes increasingly important, particularly for delivering substances to the posterior segment of the eye.

To improve ocular drug bioavailability, significant efforts have been made to develop safe and efficient drug delivery systems for ophthalmic administration. Different proposals have been evaluated to overcome the difficulties associated with ophthalmic drug delivery. In last decade, the use of nanotechnology is an emerging field to increase the effectivity of the ocular treatments. In order to understand how this colloidal system works, it is necessary to briefly introduce the main problems associated with ophthalmic administration.

6.2 ANATOMICAL CHARACTERISTICS OF THE EYE

Physiological and anatomical characteristics protect the eye against toxic external effects. Protective eye mechanisms include the specific structure of the cornea, blinking, reflex lachrymation, tear film composition, drainage, and the corneal sensitivity (Molokhia et al., 2013).

The natural anatomical ocular barriers to drug bioavailability have a great impact on pharmacokinetics. There are three main routes for the administering ocular medication: topical, intraocular, and systemic. Although eye drops are 90% of ophthalmic preparations in clinical practice, they have very poor bioavailability and short duration of action.

The main disadvantage associated with systemic administration of ocular therapeutics is its low ocular bioavailability; no more the 2% of the administered dose reaches the anterior segment. Therefore, for ocular diseases related to the anterior segment of the eye, topical administration of medications is the selected route of application (Souza et al., 2014).

The eyes are located within the orbits for ensuring a high level of physical protection. The eye captures visual information and transmits this along the optic nerve to be processed by the visual cortex of the brain. The eye is essentially made of three layers: sclera, uveal tract, and retina (Fig. 6.1). The sclera is the outer fibrous layer, which provides the mechanical stability to protect the eye. Mainly comprising collagen and elastic fiber. The important function of sclera is

to offer resistance to internal and external forces and to provide attachment for the extraocular muscle insertions. The sclera constitutes the back 5/6ths of the eye ball, and transparent cornea provides the forward 1/6th. The episclera is the outermost layer of the sclera and has a rich blood supply. The cornea is a transparent avascular multilayer membrane which has five layers (epithelium, Bowman's membrane stroma, Descemet's membrane, and endothelium). A new layer between the Descemet's membrane and stroma was discovered, named Dua's layer, which is 15 μm thick (Dua et al., 2013).

The lacrimal glands are located behind the superior tarsus at the upper and outer orbital quadrant. They secrete the greatest portion of precorneal tear fluid, which bathes the anterior surface of the eye and drains through the nasolacrimal ducts. The tear film consists of three layers: the outer layer is lipid rich and helps to prevent evaporation of the water content. This layer is composed of cholesterol esters, free sterols, free fatty acids and waxes; the Meibomian, Zeis and Moll glands of the upper eyelids secrete the lipids. The pH of tear fluid oscillates between 7.0 and 7.4. The aqueous components are generated by Krause and Wolfring glands and the mucin is produced by Crypts of Henle, glands of Manz, and Goblet cells. The tear volume in the normal eye is 5–9 μL . Reflex tears are stimulated by irritants and can vary from 3 to 400 $\mu\text{L}/\text{min}$ (Gonzalez Barcia, 2011; Fernández-Ferreiro, 2016).

The epithelium is the more external layer. It is a lipophilic tissue and provides around 90% impediment to hydrophilic drug penetration and only 10% for hydrophobic medications. The epithelial membrane has three types of cells: basal, polygonal, and squamous. New cells are generated from limbal stem cells at the basal layer, where they have column-like shape as they develop and older cells change shape during the process. Another important function of the epithelium is the protection that it offers against ultraviolet radiation. The epithelia surface has high concentration of ascorbic acid and tryptophan, both molecules act as UV radiation scavengers.

The Bowman's membrane forms a transitional layer toward the stroma. It is homogenous, has 8–14 μm thickness, and this membrane does not regenerate if it is damaged. The stroma constitutes the main portion of the corneal thickness; it consists of a hydrophilic gel made of collagen fibrils and mucopolysaccharides, and has almost 80% water. The stroma, due to its hydrophilic nature, is a resistant barrier to lipophilic molecules (Fernández-Ferreiro, 2017).

The innermost layer of the cornea is the endothelium, whose function is to allow the permeation of nutrients and maintain the hydration of the stroma. This is important to maintain transparency of the cornea and to avoid edema (Hillery et al., 2002).

The middle layer of the eye consists of the iris, ciliary body, and choroid. The ciliary body is a ring of tissue that extends from the base of the iris to the choroid. The ciliary muscle is in a contracted state to allow the lens to become convex. The ciliary body is also the area of production of aqueous humor. The iris is a diaphragm located in front of the lens and ciliary body, which separates the

anterior and posterior chamber. The color of the iris depends on the amount of melanin expressed in it. The iris is made up of two types of muscles: dilator and sphincter muscles. The sympathetic nervous system innervates dilator muscles and the parasympathetic nervous system innervates sphincter muscles (Rimpelä et al., 2016).

The eye contains three main chambers: anterior chamber, posterior chamber, and vitreous cavity. Just behind the cornea is the anterior chamber. This structure houses the iris and aqueous humor, a fluid similar to blood plasma. It has less protein and higher concentration of pyruvate, lactate, and ascorbate; and lower levels of glucose and urea than the blood plasma. It is secreted by the ciliary body. Aqueous humor is continuously replenished, leaving the chamber via the trabecular meshwork and is drained through the Schlemm canal. This mechanism maintains the intraocular pressure at 16 mmHg. Pressure increases over 21 mmHg are associated with ocular hypertension. The Schlemm canal connects with the venous system through a network of 25–35 collector channels (Missel, 2002).

The vitreous cavity comprises 80% of the volume of the eye. It is avascular and clear structure consisting of collagen, hyaluronic acid, polysaccharide and vitrosin, but its main component is water (about 98%). Vitreous humor has a pH of approximately 7.5 and a viscosity 2–4 times that of water. Its viscous properties allow it to return to its normal shape when it is compressed (Kummer et al., 2007).

The inner layer of the eye is the retina, which is a network of neurons that process light. It is a multilayer structure, about 0.5 mm thick, lining the posterior inner surface of the eye. Photoreceptor cells are included throughout the retina, consisting of cones and rods. Cone cells are related with color perception, whilst rod cells detect light monochromatically and are sensitive at low light levels. Color perception is concentrated close to the macula. Metabolic maintenance of retina is regulated by the retinal pigmented epithelium (RPE), which is part of the blood–retina barrier (BRB) (Capozzi et al., 2013) (Fig. 6.1).

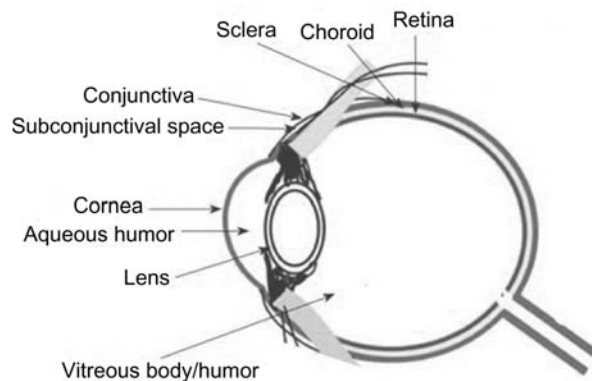


FIGURE 6.1

Anatomic characteristics of the eye.

6.2.1 BARRIERS TO TOPICAL OCULAR DRUG DELIVERY

Physicochemical barriers include characteristics of the drug, such as solubility, lipophilicity, pH of tears, drug–protein interactions, instilled volume drainage, tears production and turnover, tears evaporation, molecular size, osmolality, surface tension, and viscosity (Gonzalez Barcia, 2011).

When the eye is exposed to a hypotonic ophthalmic solution, the corneal epithelium becomes more permeable and water flows into the cornea causing edema. Hypertonic solutions have a dehydrating effect. Both solutions are irritating to the eye and increase the rate of tears. pH is important in drug ionization and for product stability. Administration of solutions that have a surface tension lower than of the tears destabilizes the lacrimal film and disperse the lipid layer into droplets. The oily film reduces the rate of evaporation of the water layer, retaining a greater amount of water. Surfactants are included in ophthalmic preparations to solubilize or disperse drugs. Furthermore, nonionic surfactants are less irritant than cationic and anionic surfactants, although have the capacity to remove the mucous layer and break the tight junctional complexes of the cornea, increasing drug permeability (Fernández Ferreiro et al., 2014).

Viscosifying agents, such as hydroxyl methyl cellulose, poly vinyl alcohol, hydroxypropyl methylcellulose, and polyethylene glycols are used in ophthalmic solutions to prolong the retention time of the drug on the surface of the eye. Natural polymers, such as alginates, veegum, xanthan gum, gelatine, acacia, and tragacanth have more problems because the risk of bacterial and fungal contamination is higher (Kim et al., 2014).

The metabolic barriers include the activity of different enzymes, such as cytochrome P450, monoamino oxidases, acid hydrolases, esterases, aldehyde and ketone reductases, transpeptidases, and acetyltransferase (Nakano et al., 2014).

The biological barriers include stratified corneal epithelium, corneal stroma, and corneal endothelium, sclera and conjunctiva, blood–eye barrier and BRB (Urtti, 2006).

6.2.2 CLASSIC ROUTES OF OPHTHALMIC ADMINISTRATION

6.2.2.1 Topical administration

6.2.2.1.1 Solutions

Topical drug delivery is the most accepted route for administering ophthalmic medication and ophthalmic solutions are the most common topical preparation. They are the easiest to manufacture and are relatively easy to administer to the patient. As disadvantages, solutions are rapidly drained out of the eye. Solubilization problems are very common with these preparations. The volume of the eye drop administered depends on the opening of the dropper and the physicochemical characteristics of the liquid (Loftsson et al., 2005).

6.2.2.1.2 Suspensions

Their use is very frequent for the manufacturing of steroids. Particles of a suspension must be dispersible on shaking by the patient for reaching a uniform dose administration. Technological problems with these preparations are: polymorphic changes during storage and cake formation. The average particle size in most of the ophthalmic suspensions is $<10\ \mu\text{m}$ (Plager et al., 2009).

6.2.2.1.3 Semisolid ophthalmic preparations

Ointments, the classic pharmaceutical form, have been used as an option to decrease drug drainage by the tear flow and increase the corneal residence time. Ointments have the advantage of allowing the incorporation of drugs with poor water solubility, reducing nasolacrimal drainage and minimization of tear dilution. White petrolatum and liquid petrolatum are commonly used as bases for ophthalmic preparations. Water soluble bases such as carbomer and poly(ethylene glycol) are frequently used. Drawbacks with these pharmaceutical forms are: they are more difficult to administer compared to solutions, and the blurring of vision because of the difference in refractive index between nonaqueous ointment base and tears reduces patient compliance (Ali and Lehmussaari, 2006).

Gels are semisolid systems with water soluble bases and are more favorable than ointments for hydrophilic drugs. Polymers such as carbomer, poloxamer, polyvinyl alcohol, and hydroxypropyl methyl cellulose are used for their manufacturing (Zignani et al., 1995).

6.2.2.2 Periocular drug delivery

Periocular delivery includes subconjunctival, subtenon, peribulbar, and retrobulbar routes which place the drug in close proximity to the sclera. They are the preferred routes for treating diseases of the uveal tract and sclera.

6.2.2.2.1 Subconjunctival drug delivery

The subconjunctival route avoids the permeability barrier of the conjunctiva and cornea, and can achieve both anterior and posterior drug level. It is very useful for administering antibiotics as prophylactic agents in cataract surgery.

6.2.2.2.2 Subtenon drug delivery

Subtenon injection is between the sclera and Tenon's capsule. This capsule is a sheet of connective tissue between the globe and the orbit which provides a smooth socket allowing free movement of the globe. This pathway permits a prolonged contact time of the drug with the sclera. Molecules of sizes up to 70,000 Da have shown to permeate through it. Injection via subtenon route is used to administer steroids, local anesthetics, and anticancer drugs (Amrite et al., 2008).

6.2.2.2.3 Intravitreal drug delivery

Intravitreal administration is done by injecting a drug solution, suspension, or intraocular implants into the vitreous cavity. It is the main route to deliver macromolecules to the posterior segment into the eye. A volume between 20 and 100 μL can be injected. Local anesthetic is mandatory with this procedure. An invasive procedure, the intravitreal injection requires the penetration of all layers of the ocular globe and can be associated with complications such as endophthalmitis, retinal detachment, uveitis cataract, and intraocular hemorrhage. The drugs most used by intravitreal route are anti-VEGF molecules such as ranibizumab, bevacizumab, and aflibercept; and corticosteroids such as dexamethasone and antibiotics (Anderson et al., 2010).

6.3 HYDROGEL AS DRUG DELIVERY SYSTEMS

Hydrogels are highly biocompatible systems that have made them interesting tools for the ocular administration of drugs. They are three-dimensional polymeric networks that can capture and retain large amounts of water. However, the so-called stimuli-sensitive, only swell in the presence of some physical or chemical stimuli.

The three-dimensional hydrogel structure is formed by means of cross-linking between the polymer chains. The cross-linking can have a chemical (covalent bonds) or physical nature (electrostatic interactions, bridges of van der Waals forces, hydrogen bonds, hydrophobic interactions). However, some physical hydrogels may undergo cross-linking processes to improve their mechanical properties (Ahmed, 2015; Shetye and Godbole, 2015).

Among the procedures used to cross-link hydrogels, they can be classified in physical (radiation gamma (X-rays, UV)) or chemical procedures, which use cross-linking agents. These agents can be: (1) small multifunctional molecules capable of join the functional groups of the polymer forming the hydrogel; or (2) monomers that polymerize and cross-link the polymer simultaneously. For starting both cross-linking methods, free radicals, temperature, initiators ionic, radiation gamma, or redox agents can be used. This type of procedure is not useful for producing the gelling on-site, in ocular administration.

Hydrogels can be formed from natural, synthetic, or semisynthetic polymers (Seki et al., 2014). Natural polymers can proceed from animal or vegetable sources such as proteins (collagen, gelatine) or polysaccharides (starch, alginate, agarose, hyaluronic acid, and carrageenan). Synthetic polymers can be made through chemical polymerization (poloxamers, PVA, carbopol) and semisynthetic polymers can be obtained from chemical modifications of natural polymers such as cellulose derivatives (Annaka et al., 2011; Kushwaha et al., 2012; Gratieri et al., 2011; Mahlumba et al., 2016).

These systems can present different structures: semicrystalline, in which crystalline and amorphous areas coexist; or amorphous (Ahmed, 2015) aggregates of hydrocolloids (stabilized by hydrogen bonding) (Vashist and Ahmad, 2013).

According to the polymeric composition (Ahmed, 2015), hydrogels can be formed from homopolymers (when the structure is achieved by the repetition of a single monomeric unit) or heteropolymers (formed by different monomers). Copolymeric hydrogels are comprised of two or more different monomer species, with at least one hydrophilic component arranged in a random block or alternating configuration along the chain of the polymer network. Multipolymer interpenetrating polymeric network (IPN) hydrogels are an important class of hydrogels made of two independent cross-linked synthetic and/or natural polymer components, contained in a network form. In semi-IPN hydrogels, one component is a cross-linked polymer and the other component is a noncross-linked polymer (Ahmed, 2015; Deligkaris et al., 2010).

6.3.1 STIMULI-SENSITIVE HYDROGELS

Stimuli-sensitive hydrogels consist of polymeric systems that undergo rapid changes by small perturbations in the environment (Rosiak and Yoshii, 1999). The main feature of these smart hydrogels is their ability to recognize external stimuli and responding to them with changes in some of their properties, mainly size, shape, texture, and mechanical properties.

Due to their capacity of adaptation to the environment, stimuli-sensitive hydrogels are widely used in biomedical applications. However, in this chapter, we will focus on ophthalmic drug delivery systems. For ocular administration, the sensitive hydrogels have the main advantage that, after administration, the system is a low viscose solution that can be administered easily by dropping on the ocular surface or by injection via periocular or intravitrea. Once the solution is injected or instilled shift to a gel for a phase transition in situ, triggered by the conditions of pH (Wu et al., 2007), temperature (Gratieri et al., 2010), and electrolyte concentration (Fernández-Ferreiro et al., 2015) present in the environment (tear film at the eye surface) (Meshram and Thorat, 2015; Ludwig, 2005).

For ocular administration, in situ gelling formulation should comply to certain requirements: show sol-gel phase transition in eye conditions, provide a controlled release of the drug, to be safe (pH and isotonicity in the physiological range, sterile, clear and transparent), adequate rheological properties (viscosity and gel strength), and to be bioadhesive.

Stimuli-sensitive hydrogels are good candidates to ophthalmic drug administration because they can improve the release behavior of the drug and increase the precorneal residency time of the drug increasing the therapeutic effect (Kushwaha et al., 2012; Meshram and Thorat, 2015). In addition to the stimuli-responsiveness, these smart hydrogels must present other features, such as being biodegradable, biocompatible, not contain contaminants/pyrogen-free, nontoxic, low-cost, high capacity of drug loading, and suitable stability profile (Mahlumba et al., 2016).

6.3.1.1 pH-sensitive hydrogels

An extensive review on in situ gelation was carried out by Jain et al. (2016), including some examples of commercial formulations. pH-sensitive hydrogels included different polymeric systems that are able to gel under the pH conditions of the eye surface (7–7.4). The most widely used pH-sensitive polymers are the acrylic derivatives, alone or in combination with other polymers. As an example, polyacrylic acid hydrogels (Carbopol 940) in combination with hydroxypropylmethylcellulose (Methocel E50LV) can spontaneously gel in response to changes in pH after their administration at the target site.

In other cases, the release of the drug is triggered by changes in the pH. Koyamatsu and colleagues (2014) obtained reverse pH-responsive polymer micelles for protein release. The polymer was formed by a diblock copolymer of poly(ethylene glycol) modified with a phospholipid and poly(D,L-lactic-co-glycolide) with terminal carboxyl group. The system shows a pH-dependent release of two model proteins, bovine serum albumin and streptavidin, releasing protein at neutral and basic pH conditions, whereas the proteins remained in the interior at acidic pH.

Another pH-sensitive polymer used in ocular administration is the pseudolatex of cellulose acetate phthalate. This polymer coagulates in contact with the tear fluid due to a change in the pH. Achouri et al. (2012) increased the residency time of a formulation of cellulose acetate phthalate of pilocarpine, in comparison with the free drug solution.

Chiu and coworkers (2008) prepared polymer vesicles of acrylic acid and 1,2-distearoyl-rac-glycerol acrylate copolymers. The vesicles, which were prepared by a water/oil/water multiple emulsion technique, had a membrane with pH-responsive channels. These channels were permeable to hydrophilic solutes at pH 8, due to the ionization of acrylic acid residues, and impermeable at pH 5.

6.3.1.2 Temperature-sensitive systems

The increase or reduction of the temperature can promote the release of the drug from hydrogels due to changes in the solubility of the polymer and in the structure of the polymer network. This temperature change can be produced by external or internal factors. *N*-Isopropylacrylamide (PNIPAAm) is a widely used thermoresponsive polymer with a low critical temperature (LCST) of 32°C. Ninawe et al. (2010) uses PNIPAAm implantable hydrogels for the treatment of age-related macular degeneration, that release the model protein immunoglobuline G in the posterior segment of the eye after intrascleral implantation. Prasannan et al. (2014) reported a copolymer of PNIPAAm and acrylic acid with an LCST of 34°C for controlled release of antiglaucoma drugs. They achieve a sustained release profile of the model drug [3H]-epinephrine.

Some polymers, such as carboxymethylcellulose, hydroxypropylmethylcellulose, chondroitin sulfate, sodium hyaluronate, dextran, alginic acid, and guar gum, have been used as vitreous humor substitutes (Donati et al., 2014; Annaka

et al., 2011). Annaka et al. (2011) prepared an injectable thermoresponsive polymer of poly(ethylene glycol) end-capped with an octadecyl group for long-term vitreous body replacement. This polymer exhibited a rapid gelation, forming a clear gel that completely fills the vitreous cavity with optimal optical, toxicological, and histopathologic characteristics.

Poloxamers (Pluronic) are also regularly used thermoresponsive polymers. They are nonionic triblock copolymers composed of a central hydrophobic chain of polypropylene oxide and two hydrophilic chains of polyethylene oxide. There are several poloxamers with different molecular weights with different properties. Pluronic F-127 is the most commonly used polymer in drug delivery, because it gives colorless and transparent gels at body temperature (Bachhav et al., 2015).

Miller and Donovan (1982) proposed a temperature-sensitive solution of poloxamer to deliver the miotic agent, pilocarpine. They obtained a significant increase in the miotic with a formulation using Pluronic F-127, compared with the aqueous solution of the drug. Poloxamer 407 and 188, and bioadhesive polymer (xanthan gum and alginate) blends proved their suitability for increasing the precorneal contact time and increase the bioavailability of moxifloxacin hydrochloride (Shastri et al., 2010; Achouri et al., 2012).

Pluronic F-127 has the inconvenience that, for in situ gelling, a concentration of 20–25% w/v is required and this concentration causes irritation in the eye. To solve this problem, Shastri et al. (2010) use mixtures of Pluronic F-127 with polymers (carbopol 940, xanthan gum, hydroxy propyl methyl cellulose, and sodium alginate) that increase the viscosity of the system, inducing the gelation at physiological temperature using low concentrations of the poloxamer.

Xyloglucan is a polysaccharide in dilute aqueous solution and, when it is partially degraded by β -galactosidase, exhibits thermally reversible gelation. To change the transition temperature, it is possible to modify the degree of galactose elimination. In addition, when the successful response incorporating pilocarpine hydrochloride formulations containing Xyloglucan or Poloxamer 407 are compared, similar results are obtained, working with concentrations of Xyloglucano 16 times lower than of Poloxamer 407 (Miyazaki et al., 2001; Achouri et al., 2012).

6.3.1.3 Ion-sensitive hydrogel

This section includes polymers that undergo a process of gelation in the presence of the electrolytes presented in tears. One of the polymers used is Gellan gum (Gelrite), which is extracted from *Pseudomonas elodea*. After deacetylation of the polymer, the low-acetyl gellan gum has free carboxylate groups and it is transformed in a viscous gel. In the presence of Na, Ca^{+2} and Mg^{+2} , cations this polymer undergoes sol-to-gel phase transition (Milas and Rinaudo, 1996), allowing an increase in the time of residence in the place of application. Gellan gum hydrogels containing ciprofloxacin hydrochloride were therapeutically efficacious and provided sustained release over an 8-h period in vitro (Balasubramaniam et al., 2003).

drug corneal transport for the treatment of glaucoma. The hydrogel is composed of the ion-sensitive polymer sodium alginate (high glucuronic acid content) showing a pH- and cation-triggered sol-gel transition (Shastri et al., 2010). Pluronic F-127 and a pH-sensitive polymer (chitosan) allows the gellification of the system at temperature above 35°C and pH between 6.9 and 7.0. In addition, the chitosan can enhance the permeation of the drug (Gupta et al., 2007).

Gupta and colleagues (2015a) designed a smart hydrogel made of alginate and chitosan for the administration of levofloxacin. This formulation was able to gel in situ by influence of divalent ions (Ca^{2+}) and pH. The hydrogel presents a drug release of first-order over 12 h. These multisensitive hydrogels increases the permanence time of the drug in the ocular surface, owing to a better resistance to the eye drainage.

6.3.2 PROPERTIES OF IN SITU HYDROGELS: STUDIES OF STIMULI RESPONSE

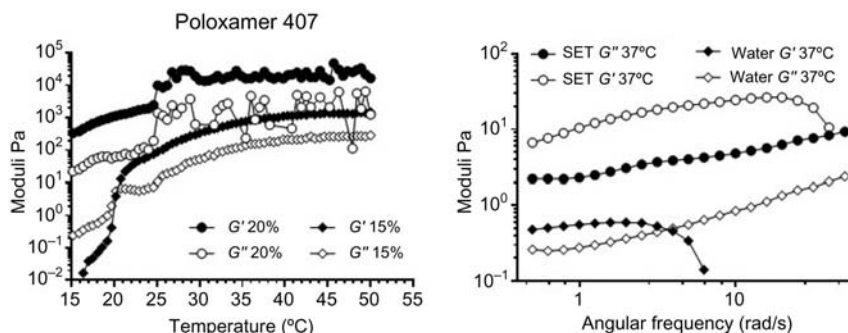
During the development of stimuli-sensitive gels, it is essential to be provided with adequate methodology to characterize their capacity of adaptation to the external stimuli changes. In some cases, the rate of response to medium changes must be slow, but in most cases, a quick response is required, therefore, it will be important to characterize both the magnitude and the rate of response to the stimuli.

The more habitual responses of smart hydrogels to external stimuli, such as temperature, pH, electrochemical, ion or chemicals, are changes in size and shape, viscoelastic properties, or diffusivity, so the main part of the methodology used is based on the measurements of these properties.

6.3.2.1 Rheometry

Rheometry is one of the methods habitually used to characterize the mechanical properties and the cross-linked structure of hydrogels. Rheological techniques may be used to characterize the changes in the viscoelastic properties of smart hydrogels caused by external stimuli, being the oscillatory rheometry most widely used. Oscillatory rheometers induce a sinusoidal shear deformation in the hydrogel samples, measuring the resultant stress response of the material. Normally, the resultant stress response is determined in function of ω , the frequency of oscillation of the shear deformation. The viscoelastic behavior of the material is typically characterized by the storage (elastic) modulus, $G'(\omega)$, and the loss (viscous) modulus, $G''(\omega)$, which respectively characterize the solid-like and fluid-like contributions. The ratio between loss and storage modulus, named the “phase angle” or “ $\tan \delta$ ”, is also a useful parameter for viscoelastic characterization. Changes in both moduli and in $\tan \delta$ can be monitored to characterize the magnitude and rate of response of smart gels to the stimulus.

Fig. 6.3 shows two examples of the changes produced on the viscoelastic properties of a thermosensitive and an ion-sensitive ophthalmic hydrogel, mediated by

**FIGURE 6.3**

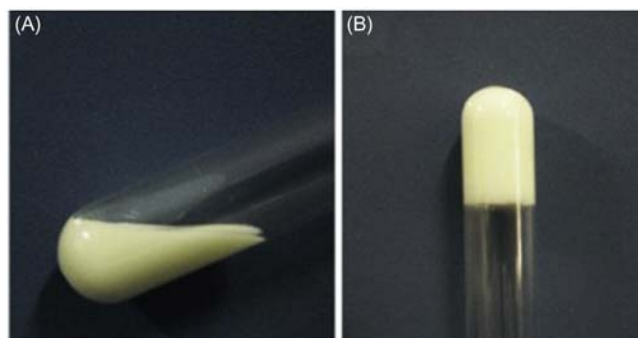
Changes on loss (G'') and storage (G') moduli of thermosensitive (left) and ion-sensitive hydrogels (right) by effect of temperature changes or dilution with simulated eye tears (SET) or water in a proportion 90:10 (v/v) respectively.

changes in the temperature or in the ion concentration of the media, respectively. As can be observed, in both cases the gelation of the systems produces an increase of both the storage $G'(\omega)$ and the loss modulus $G''(\omega)$. Thermosensitive hydrogels (Fig. 6.3 left) obtained by the dispersions of 15% of the triblock copolymer poloxamer 407 in water (data from) at temperatures below 20°C behaves as a solution, showing values of storage modulus lower than loss modulus. At temperatures greater than 20°C or at concentrations higher than 20% of the polymer, the value of $G'(\omega)$ is higher than $G''(\omega)$ and both increase with temperature, suggesting the formation of structured systems caused by the thermally induced gelation of the polymer, which improves their consistence with the temperature.

Fig. 6.3 (right) shows the mechanical behavior of ion-sensitive hydrogels obtained by the dispersion of a mixture of gellan gum/k-carrageenan (proportion of 4:1) at 0.84% (w/v) after their dilution with SET or water. Modulus values of hydrogels dispersed in water behave as a structured material in repose or at low shear deformation values, but lost the structure when moderate or high angular frequencies are applied. Nevertheless, after dilution on simulated tear fluid, the interactions with ions induce the gelation of the dispersion, generating the increase of the modulus and in the value of the single phase angle, $\tan \delta$, as a consequence of the formation of a high structured system (Fernández-Ferreiro et al., 2015). Oscillatory rheometry is a methodology that was used for different authors to characterize the response of a smart hydrogel to changes with temperature or pH (Li et al., 2015), and to determinate the conditions for sol-gel transition.

6.3.2.2 Test tube inversion

One of the most common methods to determine the sol-gel transition conditions of an in situ hydrogel system is the test tube inversion (Fig. 6.4). In this method, typically a tube or vial containing the sample is turned upside-down to observe

**FIGURE 6.4**

Thermal response observation using test tube inversion method of the injectable PECE/Collagen/n-HA hydrogel composite. (A) room temperature and (B) 37°C.

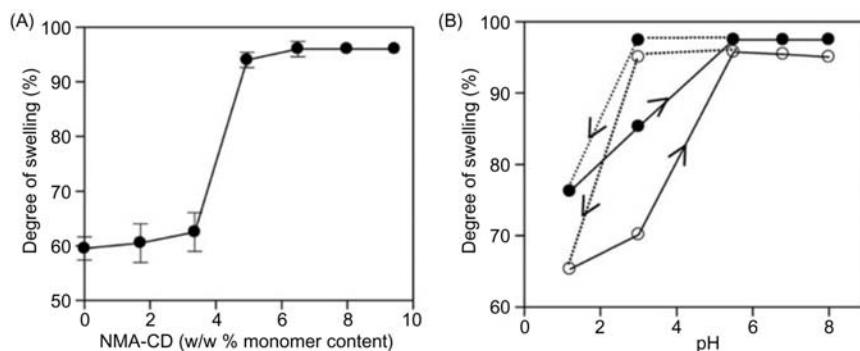
Source: Figure taken from Fu, S., Ni, P., Wang, B., Chu, B., Zheng, L., Luo, F., et al., 2012. *Injectable and thermo-sensitive PEG-PCL-PEG copolymer/collagen/n-HA hydrogel composite for guided bone regeneration. Biomaterials* 33, 4801–4809. doi:10.1016/j.biomaterials.2012.03.040.

from which conditions the sample stops to flow under its own weight. It is assumed that, in the gel state, the sample will not flow due to a yield stress, whereas the sol state will show flow even if the sample is a viscous liquid. Nevertheless, it should be given special care when viscous samples are studied because it is difficult to detect the sol-gel transition and conversely, to misinterpret a gel with a small yield stress to be a sol.

The simplicity of this test makes the method widely used to determine the temperature of gel formation in thermosensitive gels (Fu et al., 2012). In this case, the tube samples are normally immersed into water or an oil bath, increasing the temperature stepwise. After each temperature step, the test tubes are inverted in order to check whether the sample flows or not.

6.3.2.3 Shape and size change monitoring

Another popular methodology used to characterize the capacity of response to stimuli of smart hydrogels is the monitoring of dimension, shape, or weight change produced by the effect of stimuli. Different techniques have been used to measure the magnitude of the changes: (1) manual determination of hydrogel dimensions (direct or under microscopic observation) (Tudor et al., 2016; Ni et al., 2016; García Gonzalez et al., 1993; Siemoneit et al., 2006; Zhao et al., 2015), (2) weight changes monitoring, (3) control of changes using image analysis techniques, (4) universal testing machines, (5) dynamic mechanical analysis (Zhao et al., 2015; Tomić et al., 2010; Barwood et al., 2014), or (6) thermomechanical analysis (Zhao et al., 2015; Adesanya et al., 2014; Tomić et al., 2010; Barwood et al., 2014; Lee et al., 2016).

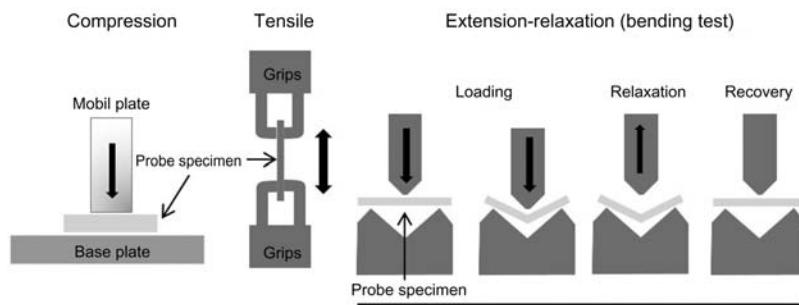
**FIGURE 6.5**

Dependence of degree of swelling of Acrylic/cyclodextrin-based hydrogels on acrylamidomethyl- γ -cyclodextrin ration and pH (Siemoneit et al., 2006) (A: hydrogels prepared with 3 M sodium acrylate and 39 mM BIS on the content in acrylamidomethyl- γ -cyclodextrin B:hydrogels; B: prepared without (*open circle*) or with (*closed circle*) 9.4% of -CD-NMA). A hysteresis cycle was observed for the dependence of the degree of swelling on pH.

One of the simplest and most widely used methods for monitoring shape, dimension, or weight is by manual determination of changes in the function of stimuli (Ni et al., 2016; García Gonzalez et al., 1993). Examples of this methodology are: the use of changes in diameter and in weight of hydrogels to evaluate the multistimuli-responsive capacity to the pH changes of smart hydrogels with ferrocene groups pendant of polymer networks (Ni et al., 2016); or the increases in weight of acrylic/cyclodextrin hydrogels with pH (Fig. 6.5) (Siemoneit et al., 2006).

Moreover, it is possible to measure changes in swelling pressure, hydrogel volume or one-way shape memory behavior using universal testing machines. To develop experiments, a hydrogel sample is typically placed between two plates that exert a minimum pressure on the sample to ensure contact (compression experiments), clamped by two grips (tensile experiments), or to develop extension-relaxation tests (bending test) (Fig. 6.6).

In the studies of response to stimuli (i.e., studies of response to pH, ions, or solvent temperature), the contact of hydrogel with a solvent is habitually needed. In these cases, a special sample holder that allows the contact of the solvent with the hydrogel replaces the lower plate or the bottom grips. Once the stimuli are applied, load cell and extensometers or displacement transducers can register changes in dimensions, pressure or stress versus time. An example of this kind of experiment is the characterization of the thermoresponsible capacity of *N*-isopropylacrylamide, alginate/PNIPAAm ICE hydrogel (Bakarich et al., 2015) or the kinetics of electrically and chemically induced swelling of polymethacrylic acid (PMAA) membranes (Grimshaw et al., 1990).

**FIGURE 6.6**

Scheme of the compression, tensile and bending test using a universal testing machine.

Modification in shapes can be also used to evaluate the stimuli response capacity of hydrogels (Zhao et al., 2015). Adesanya et al. (2014) uses the determination of changes in hydrogel's curvature to characterize the effect of electroactive stimulation on hydrogels based on poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) copolymer, modified incorporating telechelic methacrylic end functionalities. With this aim, hydrogels are placed between two platinum wire electrodes immersed in 0.1 M NaCl solution, using an electrophoresis flat bed. An electric potential difference was applied, and the changes in gel curvature versus time were monitored using digital camera and image analysis software.

Similar methodology was employed by Barwood et al. (2014) to measure the changes in the shape of an acrylate-based thermally activated polymer. This aim uses a customized platform consisting of a hotplate, a glass enclosure, and a temperature probe. The sample was placed inside the enclosure at a controlled temperature and deformed using a preheated steel weight for a specific time. After this time, the probe was cooled, the external force was removed and finally the sample was placed inside the heated glass enclosure. The recovery of the original shape was determined from still photographs captured, at appropriate time intervals, from video recordings.

Lee et al. (2016) also uses changes in shape to characterize carbon nanotube yarn-based glucose sensing artificial muscle. To study the response of the carbon nanotubes to the glucose concentration the mechanical movement driven by the swelling/deswelling properties of the hydrogel was determined. The hydrogel was put in contact with PBS buffers with different glucose concentrations for 20 min and with PBS buffer solution for 30 min after this, to remove the glucose from the sample, determining the rotation angle of the artificial muscle.

6.3.2.4 Spectroscopic analysis

Changes in stimuli-sensitive hydrogels can also be monitored using spectroscopic methods, such as NMR, photon correlation spectroscopy (PCS), IR or UV-Vis spectroscopy (Xia et al., 2015).

In systems where gel-sol transition is accompanied by changes in the transparency, it is possible to use the changes in transmittance or in turbidity to determine the clouding and the gelling point. Zheng et al. (2016) use transmittance changes at 500 nm caused by modification of temperature to determine the clouding points of *N*-isopropylacrylamide thermosensitive hydrogels. This polymer forms a transparent and colorless system at room temperature, but, turned into a white opaque system at temperatures up to 25°C. The cloud temperature obtained from *N*-isopropylacrylamide hydrogels was 32–33°C, and the critical phase transition temperature calculated using transmittance measurements agreed with that obtained by ¹H-NMR and photon correlation spectroscopy.

Dynamic light scattering measurement is also a useful technique, especially when they are intended to characterize micro and nanogels. Sun et al. (2015) used this technique to study the thermo- and pH-sensitivities of (*N*-isopropylacrylamide)/chitosan/Fe₃O₄ microgels (Fig. 6.7). The results indicate that swelling of the PNIPAM/CS/Fe₃O₄ microgels decreased with the increase of the temperature from 28°C to 40°C and increase with increasing pH.

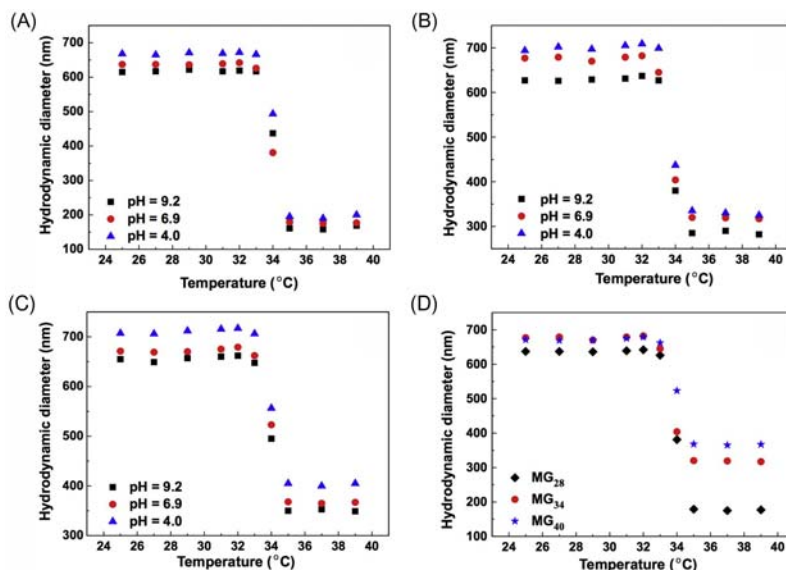
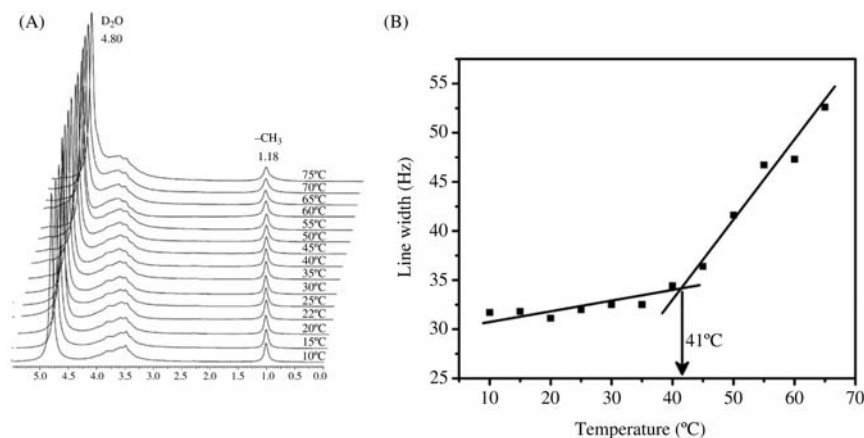


FIGURE 6.7

The effect of temperature and pH on the hydrodynamic diameter of tree different (*N*-isopropylacrylamide)/chitosan/Fe₃O₄ microgels (A: MG28, B: MG34 and C: MG40) and temperature dependence of the hydrodynamic diameter of MG28, MG34 and MG40 at pH = 6.9 (D).

Source: From Sun, M., Zhu, A., Zhang, Q., Ye, M., Liu, Q., 2015. Smart shape-controlled synthesis of poly(*N*-isopropylacrylamide)/chitosan/Fe₃O₄ microgels. *Eur. Polym. J.* 66, 569–576. doi:10.1016/j.eurpolymj.2015.03.023.

**FIGURE 6.8**

Plots of (A) ¹H-NMR spectra of CMG-g-PEPO at different temperatures (10–75°C) and (B) proton line width of methylene peak at different temperatures.

Source: From Gupta, N.R., Torris A.T., Wadgaonkar, A., Rajamohanan, P.P., Ducouret, P.R., Hourdet, G., et al., 2015b. Synthesis and characterization of PEPO grafted carboxymethyl guar and carboxymethyl tamarind as new thermo-associating polymers. *Carbohydr. Polym.* 117, 331–338. doi:10.1016/j.carbpol.2014.09.073.

Gupta et al. (2015b) use a combination of ¹H-NMR and fluorescence spectroscopy to characterize the thermogelification of polymers of poly(ethylene oxide-co-propylene oxide) grafted with polysaccharides (CMG-g-PEPO). Fig. 6.8 shows the characterization of the thermoassociation of the polymers using NMR spectroscopy. Authors use the proton signal of the methylene peak of the polypropylene chain at different temperatures to determine the thermogelification point. The author indicates that the temperature of association obtained from the NMR experiments corresponds very well with the observed by rheological measurements.

Xia et al. (2015) combine UV-Vis spectroscopy, IR analysis, ¹H-NMR and dynamic light scattering measurement to study the thermoinduced phase transition of hydrogels based on oligo(ethylene glycol) methacrylates. Using these techniques identifies a double phase transition of this hydrogel, useful to design smart materials.

6.3.2.5 Techniques used on film characterization

When smart hydrogels are thin films or form surface coatings, special techniques are needed, such as AFM, ellipsometry, profilometry, or confocal microscopy, capable of measuring changes in fine surfaces.

Uppalapati et al. (2015) evaluated the pH-responsive behavior of immobilized poly(4-vinylphenol) (PVP) monolayers by ellipsometry, quartz crystal microbalance (QCM), and surface plasmon resonance (Fig. 6.9). The PVP films exhibited

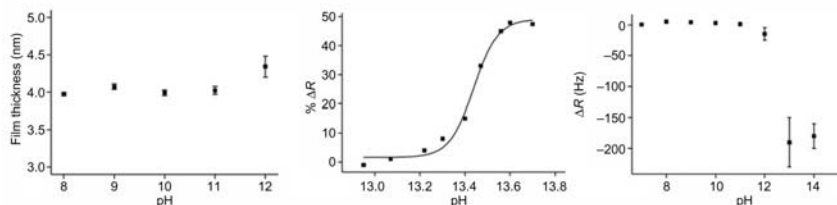


FIGURE 6.9

Thicknesses, Percent change in reflectivity ($\% \Delta R$) and QCM frequency responses (ΔF) of covalently attached PVP films to phosphate buffer of varying pH.

Source: From Uppalapati, S., Kong, N., Norberg, O., Ramström, O., Yan, M., 2015. Ionization of covalent immobilized poly(4-vinylphenol) monolayers measured by ellipsometry, QCM and SPR. *Appl. Surf. Sci.* 343, 166–171. doi:10.1016/j.apsusc.2015.03.014.

pH-response behavior at pH > 12, and no changes were observed between pH 7 and 12.

Finally, Thomas et al. (2015) uses a novel laser temperature-jump technique and a complementary thickness measurement of the polymer layers using AFM in order to characterize the pH-dependent thermoresponsive switching behavior of Poly(*N,N*-dimethylaminoethyl methacrylate) brushes. The laser temperature-jump technique allows for calculating the swelling ratio of the membranes in function of the temperature, observing strong changes in swelling at pH values, down to 7. The increase on the hydration of the poly(*N,N*-dimethylaminoethyl methacrylate) brushes in acidic solutions is also observed by AFM measurements.

6.3.3 STIMULI-RESPONSIVE HYDROGELS AS OPHTHALMIC DRUG DELIVERY SYSTEMS: DRUG-LOADING EFFICIENCY AND DRUG RELEASE

The so-called smart hydrogels as vehicles for ophthalmic applications have gained growing attention because they were expected to provide better features, such as prolonged periods of retention times at the cornea, prevent drug loss from precorneal area, ease of administration, increase the ocular absorption, and reduced frequency of administration. These characteristics support an alternative strategy of using smart biocompatible hydrogels for the treatment of ocular disease over conventional formulations (eye drops, suspension, solution, or emulsion).

Depending on the method used to cause sol-gel phase transition on the ocular surface, several types of system have been developed (UV-induced gelation, pH-triggered systems, temperature-dependent systems, or ion-activated systems). Overall, the stimuli-responsive hydrogels are expected to increase their viscosity upon instillation in the eye, and subsequently, to achieve longer precorneal retention. The main goal of pharmaceutical technology is to find a therapeutically

efficient and sustain drug delivery, safe, and nonirritant formulations with good bioavailability that allow for reducing the frequent administration usual in ophthalmic pathologies. Despite their efficacy, most drugs used in ocular applications have limitations, such as low water solubility, rapid degradation, or undergo leaching from formulation, which leads to a very low ocular bioavailability. The drug delivery based on stimuli-responsive gel can overcome these disadvantages as *in situ* gel can be administered in drop form and immediately after, create a gel from sol phase upon interacting with its specific stimuli (pH, cations, UV, temperature, and so on). There are different approaches and contributions to obtain ocular gel-forming formulations, depending on the drug, polymers, or the specific physicochemical stimuli used to display the sol-phase transition on the corneal surface. It is not our intention to compile all developments in ocular drug delivery by stimuli-responsive hydrogels; however, we would like to show the most interesting and new directions in research on *in situ* forming gels. Particularly, the impact of these kinds of formulations on drug release and to get more insight into those incorporating other pharmaceutical forms to reach the ideal features mentioned previously that should be displayed by an ophthalmic formulation.

Avastin (bevacizumab) is the drug most commonly used in the treatment of corneal neovascularization by subconjunctival injection and topical instillation. Although topically applied, it is effective. Bevacizumab presents as its main drawback a short half-life. Consequently, frequent administration in patients is required to achieve therapeutic clinical effects. *In situ* covalently cross-linked PEG hydrogel systems for the sustained delivery of Avastin were investigated (Yu et al., 2014). A PEG hydrogel was formed *in situ* by thiol-maleimide reaction between 4-arm PEG-Mal and 4-arm PEG-SH. Avastin (bevacizumab injection) (25 mg/mL) was mixed with polymers dispersion at a final concentration of 0.45% w/v. Protein-loading did not modify the sol-gel transition system and the drug release was shown to be sustainable for 14 days, although an initial burst release was observed. The sustained release properties of PEG hydrogels were decreased with increasing the cross-linking density of the hydrogels. The release mechanism was based on the combination of firstly, drug diffusion and, in a second stage, the hydrogel degradation without showing any apparent hydrolysis of degradation of the protein drug.

A thermosensitive hydrogel, based on PEG-PCL-PEG, was developed to deliver diclofenac sodium to the ocular surface (Luo et al., 2016). The sol-gel transition studies demonstrated that the gel polymer solutions (30%) was liquid at 4°C, while it was become an unflowable gel at physiological temperature. The entrapment of diclofenac sodium was achieved at the same proportion of commercial eye drops (0.1% w/v). An effective sustained release of diclofenac sodium in PBS solution was obtained for 7 days; however, the higher loadings of drug favored a slower drug release. Authors justified the differences in the release on the basis of the diclofenac water solubility, among other factors. The favorable ocular tolerability results and the improvement in the bioavailability of the drug suggests that these systems could be a serious alternative to conventional

diclofenac eye drops used for ophthalmic applications. More recently, other thermoresponsive delivery systems based on PEO–PPO–PEO poloxamer) and chitosan were studied (Gratieri et al., 2010) as an alternative tool to eye drops with the aim to retain drugs after topical administration to the eye. In vitro mucoadhesion studies using a materials-testing machine and in vivo studies, performed by the measurements of the retention times in human eyes, confirmed that higher concentrations of chitosan led to the highest mucoadhesive force and clearly affected the gel properties. Unfortunately, in this study, in situ forming gels were not loaded with any drug and subsequently no release data were provided.

A major investigation into this approach saw that loading of fluconazole into poloxamer-chitosan as components of an in situ gel-forming system (Gratieri et al., 2011) resulted in a sustained release of drug, which led to also achieving a great increment in the drug permeated across rabbit cornea, compared to the fluconazole dissolution. The prolonged release of fluconazole would allow better patient compliance in fungal keratitis, since less frequent instillations would be required.

Poly(*N*-isopropylacrylamide)-chitosan (PNIPAAm–CS) has been found useful for administering timolol maleate by in vivo pharmacokinetics studies in rabbits (Cao et al., 2007) when compared with conventional eye drops. Following the instillation of the in situ forming gels and drug solution loaded at the same concentration (0.5%), the therapeutic activity of the sustained release formulation was clearly enhanced, showing a reduction in the intraocular pressure, compared to the solution.

Other examples of thermoresponsive in situ gel-forming that has been successfully investigated, with the aim to retain the drug for prolonged times, are made up of mixtures of polymers such as alginate and hydroxypropylmethylcellulose (Liu et al., 2006). In contrast to the thermoresponsive hydrogels, this system consisted of an ion-activated system in which sol-gel transition takes place in the presence of divalent cations that can be captured from tear fluid. Different release profiles under physiological conditions were obtained, depending on the ratio of both polymers but the release studies for gatifloxacin, an antibacterial agent useful in the treatment of uveitis, suggested a sustained release over an 8 h by a diffusion mechanism for the formulation including 1% alginate and 2% of HPMC.

6.4 OTHER PHARMACEUTICAL SYSTEMS FOR THE MODIFICATION OF DRUG LOADING AND RELEASE PROPERTIES

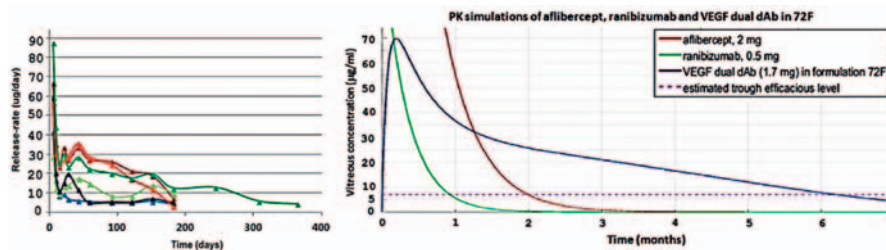
Continuous research on ophthalmic drug forms has been conducted in order to achieve a suitable controlled release of the drug of interest to ocular tissues. This increasing interest comes from the fact that the conventional systems are not able to selectively target the injured tissue or to retain the drug for prolonged times to

achieve sustainable levels in the site of action. It should also be kept in mind, that hydrogels can be successfully used for delivering hydrophilic drugs to the ocular surface or even to the posterior segments of the eye. However, the delivery of hydrophobic agents will surely face some problems, such as the poor loading of drugs or the inappropriate release pattern in the physiological aqueous environment of the eye. All these issues can be improved by the combination of different colloidal carriers and in situ gelling polymers. With the ultimate goal of enhancing ocular drug bioavailability, especially the hydrophobic ones, and the prolongation of the therapeutic effect. Finally, to reduce the frequency of administration, newer drug delivery systems comprising smart hydrogels can incorporate other pharmaceutical forms, such as: (1) colloidal systems such as microparticles, nanoparticles, liposomes, niosomes, polymeric micelles, or dendrimers; (2) implantable systems such as contact lenses, in situ gels or polymeric inserts; and (3) incorporating excipients to enhance drug solubility and therefore to increase its bioavailability; or (4) by using active components into the formulation to enable the longer contact time between the drug of interest and the corneal surface or to increase the permeation of the stability of poorly soluble or unstable drugs. These kinds of excipients include cyclodextrins, chelating agents, or surfactants. These strategies have been developed by modifying hydrogels but have successfully been used before for other biomedical applications, for example, tissue engineering or drug delivery, as excellent reviews have reported (Jeong et al., 2012; Roy and Gupta, 2003; Bajpai et al., 2008; Agrawal et al., 2012; Casolaro et al., 2012; Jung and Chauhan, 2012; Gong et al., 2013).

Among the advantages of microparticulate or nanoparticulate formulations, the stabilization of the drug and its controlled and sustained release in the precorneal surface or in some of the ocular aqueous compartments are some of the most important studies. In the case of topical application, the carrier system would allow a longer time of release by the interaction between the glycoproteins of the cornea, conjunctiva, and the pharmaceutical form, resulting in a reduced precorneal drug loss. Some other ophthalmic pathologies require frequent drug administrations for a prolonged time, such as uveitis, diabetic retinopathy, diabetic macular edema, age-related macular degeneration or cytomegalovirus retinitis. Conventional formulations are not completely successful, due to the difficulty to deliver drug to the posterior segment of the eye and the associated loss of vision. Microparticles and nanoparticles can serve as excellent platforms to provide adequate drug concentrations released in a sustained manner as reservoir to the posterior segment of the eye. Therefore, the challenge still relies on the development of drug formulations able to maintain therapeutic drug concentrations with greater lifetime to minimize the frequency of injections and the systemic side effects caused by the high doses needed to achieve a clinical response. Advanced drug delivery systems based on microparticles have also been developed in order to increase drug bioavailability across ocular membranes, to obtain a sustained drug release or reduce its toxicity. Remembering that the main features of microparticles such as size, sphericity, drug release properties or biocompatibility, these

pharmaceutical drug carriers could be administrated in solution, as eye drops or intraocular injection.

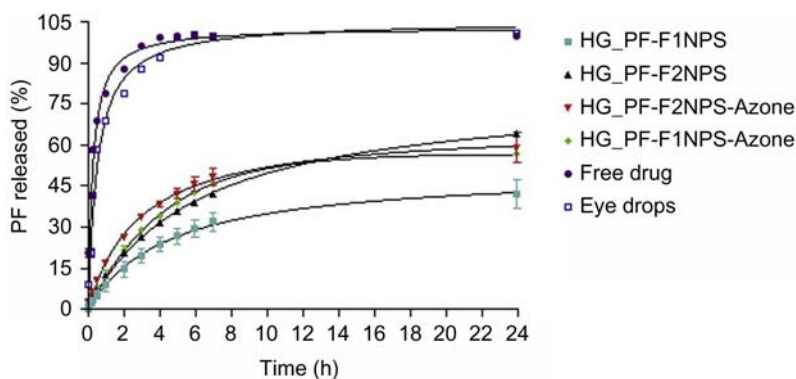
Some other strategies developed in other biopharmaceutical fields may be used with ophthalmic purposes. For example, the development of PLA microparticles prepared by means of thermoreversible gel-method, an alternative to the more conventional double emulsion/solvent evaporation techniques for the encapsulation and delivery of protein drugs (Leo et al., 2006). By this method, the thermoreversible Pluronic F-127 was incorporated during the particle preparation, resulting in high encapsulation efficiency values, a lower burst effect than the homologs emulsion/solvent evaporation samples but a faster complete release of protein at 72 h. These differences were attributable to the protein localization in the core of particles and the presence of pores on the particles surface. In the field of tissue engineering, some interesting approaches have also been carried out based on hydrogel-embedded microparticles for the delivery of different substances with different release kinetics, depending on the applications. Although several attempts have been made to develop polymeric microparticles for ophthalmic applications, in a number of investigations, PLGA microspheres have been used for the ocular delivery of different drugs (Chowdhury and Mitra, 2000; Herrero-Vanrell and Refojo, 2001; Hou et al., 2008; Kompella et al., 2003; Gavini et al., 2004; Amrite and Kompella, 2005; Bin Choy et al., 2008; Chennamaneni et al., 2013; Herrero-Vanrell et al., 2014; Leon et al., 2015; Park et al., 2015). Further approaches based on the development of biodegradable and polymeric microparticles can be found in the literature for the entrapment of different kinds of drugs, such as the selective COX-2 inhibitor and celecoxib, also incorporated into PLGA microparticles (Ayalasomayajula and Kompella, 2004) intended to be administered by via subconjunctival injection. Chitosan as bioadhesive agent has also been proposed as polymer-forming microparticle for topical administration of drugs (Genta et al., 1997; Rassu et al., 2009), combined with polyvinyl pyrrolidone derivatives (Abashzadeh et al., 2011), albumin (Addo et al., 2015) or more recently, microparticles based on chitosan and poly(vinyl alcohol) loading pilocarpine for glaucoma (Cadinoiu et al., 2015). A particularly successful approach was developed by Adamson et al. (2016) where a delivery device based on PolyActive hydrogel was administered (intravitreal injection) to nonhuman primates to evaluate the delivery of anti-vascular endothelial growth factor (VEGF) over a 6-month period. This research clearly demonstrated the preservation and the efficacy of the functional activity of VEGF over 6 months after a single intravitreal injection in laser-induced model of choroidal neovascularization (Fig. 6.10). Despite the numerous examples of research about microparticles for ocular delivery of drugs, hydrogels carrying microparticles and developed for ophthalmic prolonged action of a drug are hardly inexistent and only a few approaches have explored this possibility, as it does occur in other research fields as tissue engineering (Hou et al., 2008; Van Tomme et al., 2005). Accordingly, a microparticle-loaded gel composed of ethylcellulose and hydroxypropyl methyl cellulose as gelling agent were developed to provide an ocular topical delivery of

**FIGURE 6.10**

In vitro release profile of anti-VEGF and its functional activity over 6 months assessed in primate model of wet age-related macular degeneration from microparticulate formulations based on polyethylene glycol-polybutylphthalate (Adamson et al., 2016).

ketorolac. This strategy was intended to serve as a sort of microsp sponge able to delivery ketorolac for 8 h with good properties of bioadhesion that were inversely related to the gelling agent concentration (Kumar, 2013). Another strategy for delivering ciprofloxacin from microparticles dispersed in a gelled system was developed by Bonferoni et al. (2007). In this work, microparticles were prepared using chondroitin sulfate and involved polymers with known mucoadhesive ability, such as hydroxypropylcellulose, hyaluronic acid, and polyacrylic acid (carbopol 934P) in the presence of chitosan, which was absorbed around the particles to confer a more compact coating to the chondroitin microparticles. The relationship between the mucoadhesion, the corneal residence time, and the presence of drug in rabbit tear fluid was clearly demonstrated. This effect was attributed to the viscosifying polymer concentration and the combination and ionic interaction between chondroitin and chitosan.

Nanoparticles as a colloidal drug delivery system can be administered, instilled, incorporated in eye drop solution for being administered topically onto the eye (Nagarwal et al., 2009; Ibrahim et al., 2013; Sabzevari et al., 2013; Rafie et al., 2010; Quinteros et al., 2016), or even formulated in suspension for intraocular injection (Xu et al., 2013; Zhao et al., 2017). Nevertheless, a strategy less explored at this time is hydrogel-embedded nanoparticles for delivering drugs. Nanoparticles made up of poly(lactic-co-glycolic acid) (PLGA) were dispersed into carbomer hydrogel intended to be used for the treatment of edema on the ocular surface (Abrego et al., 2015). Carbomer, as gelling agent in semi-solid vehicles, has been extensively used for ocular applications. In this study, the antiinflammatory agent was successfully encapsulated into PLGA nanoparticles around 300 nm that were fabricated by the solvent displacement technique. In vitro drug release performed using Franz diffusion cells of panopropfen revealed that the nanoparticle-loaded hydrogel displayed a sustained release profile, whereas commercial eye drops resulted in a fast drug release (Fig. 6.11). Among the various kinetic models used to interpret the release of drug, the hyperbola model showed the best fitting to the release curves. This work is a

**FIGURE 6.11**

In vitro release profiles of pranopfen (PF) from several formulation of nanoparticles (PF-F1NPs) and nanoparticles dispersed in Carbomer 934 hydrogels (with and without azone) and the comparison with the drug release from commercial eye drops and free drug solution (Abrego et al., 2015).

good example how the biopharmaceutical properties of a drug can be improved by incorporating nanoparticles in hydrogels.

Poly(lactic-co-glycolic acid) nanoparticles have shown to be an interesting delivery system due to its controlled release properties and degradation to safe products in many biomedical applications. Accordingly, PLGA nanoparticles have also been explored as drug vehicles to enhance drug concentration and retention onto the corneal surface with promising results in vivo studies (Gupta et al., 2013a). Gupta et al. (2013b) have reported the development and characterization of PLGA nanoparticles suspended in chitosan liquid formulation for ocular administration, whereupon contact to physiological pH with can form a gel, which resulted in prolonged release of spafloxacin. Nanocrystals of drug (forskolin) dispersed in the in situ hydrogel-forming (polycarbophil-Poloxamer 407) polymer have been successful in delivering the drug in a controlled manner and reducing the intraocular pressure as in vivo studies showed (Gupta et al., 2010). This strategy allowed for releasing the antiglaucoma drug during 5 h, extending the delivery, compared to the raw drug suspension where it was released almost immediately. Other attempts to enhance the drug permeation and the residence time of the formulations have been based on modifications of in situ pH-sensitive gel as nanoemulsified-triggered polymeric nanoparticulate gel for sustained ocular delivery of acetazolamide (Singh et al., 2014); or more recently, the development of nanogels made up of Poly(*N*-isopropylacrylamide-methacrylic acid-vinylpyrrolidone) [P(NIPAAm-MAA-VP)] encapsulating ciprofloxacin with good antibacterial effects, as therapeutic efficacy was shown in in vivo studies (Davaran et al., 2015).

An effective approach for improving the ocular retention of a drug from a smart hydrogel is the encapsulation of the active agent within a microemulsion system that was dispersed in a gellan gum solution to form a microemulsion in situ electrolyte-triggered gelling system (Gan et al., 2009). In this work, the incorporation of drug was done by the dispersion in the gel solution, with a loading of 2% resulting in an extremely high encapsulation efficiency (approximately 95%). The release studies carried out in artificial tear fluid in vials showed that the best sustained profile was obtained for the formulation, including the major proportion of gellan gum. The instillation of the microemulsion-based formulations in rabbits for the lipophilic drug, cyclosporine, provided therapeutic levels in the cornea at 32 h after administration. The low bioavailability of the immunosuppressant cyclosporine was also the subject of study by a microemulsion approach (Kapoor and Chauhan, 2008). The improvement of the biopharmaceutical properties of the drug was achieved by the formulation of poly(2-hydroxyethylmethacrylate) (*p*-HEMA) hydrogel containing microemulsions or micelles of Brij 97. Although no cytocompatibility of ocular tolerability studies were assayed in this work, the detailed investigation on release kinetics from hydrogels showed a slow release of cyclosporine for about 20 days, compared to drug release obtained only from *p*-HEMA gels. To investigate the effect of polymerization of gels on drug activity, cyclosporine loading of gels was studied by incorporating the drug into the oil phase of microemulsion and also by soaking the gels for several days until equilibrium was reached. Due to the physicochemical characteristics of the drug (hydrophobic) and the surfactant, it was seen that the mechanism controlling the release occurred by diffusion, which explained the slow release profile from gels. In another approach, terbinafine hydrochloride was used as antifungal agent to be administered to rabbits with the aim of enhancing the penetration into the cornea and aqueous humor, thereby achieving high and inhibitive concentrations to treat fungal keratitis. Terbinafine-loaded nanoemulsions were prepared with different compositions, according to a pseudoternary phase diagram, and then incorporated to in situ gel, based on gellan gum. The association between the optimized nanoemulsion and gellan gels was found to display a more delayed drug release rate than nanoemulsion alone. The terbinafine pharmacokinetics in the aqueous humor of rabbits after instillation showed a clear improvement, compared to the oily drug solution, with a delay in $T_{\max}$ and an increase in the AUC of more than two-fold, attributable to the ability of the gelled system to increase the corneal wettability and prolong the droplet residence time on the corneal surface.

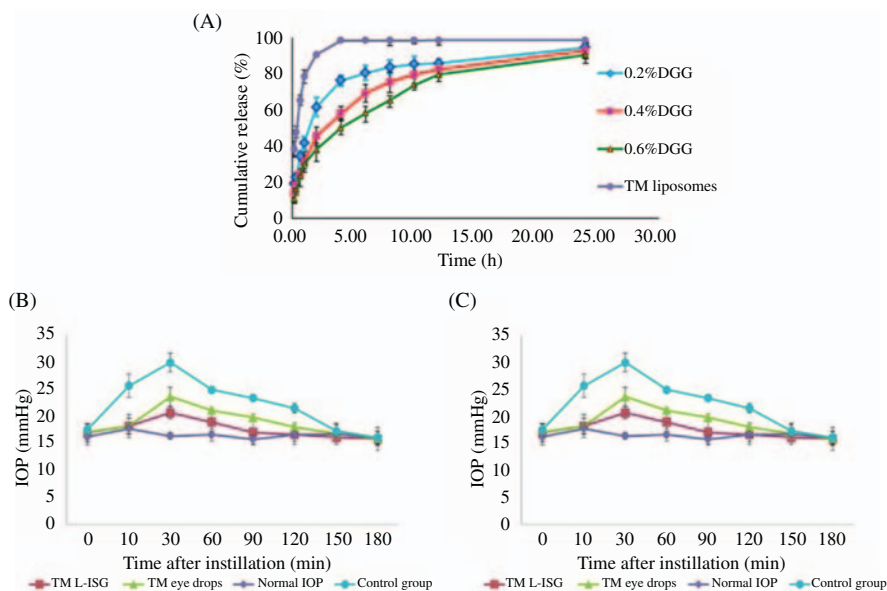
A number of studies attempt to develop the entrapment of drugs into liposomes for ocular administration, always with the main purpose of clarifying the systemic administration of drug to both treat topical pathologies (with liposomes formulated in most cases as eye drops) and targeting the posterior segment of the eye (Hironaka et al., 2009; Hironaka et al., 2011; Bochot and Fattal, 2012; Shafaa et al., 2011; Kaiser et al., 2013; Honda et al., 2013; Tsukamoto et al., 2013; Davis et al., 2014; Lajunen et al., 2014; Vicario-de-la-Torre et al., 2014; Dong et al., 2015). Other researchers have also studied the advantageous features of

liposome-based hydrogels and solid-lipid nanoparticles (Silva et al., 2012) with several biomedical applications (Ruel-Gariépy et al., 2002). However, the design of formulations of liposomes incorporated within in situ gels has not received so much attention. One of the first approaches describing a more advanced ocular delivery system, based on the dispersion of liposomes into a thermosensitive gels, was made by Bochot et al. (1998). A formulation prepared using Poloxamer 407, a copolymer of ethylene oxide and propylene oxide, and PEG as stabilizer for liposomes was characterized in terms of physicochemical features. Although no drug was assayed, this work showed the ability of PEG to sterically stabilize the lipidic bilayer of liposomes and maintain their structure into the gel. An aqueous fluorescent marker, calcein, was loaded within liposomes at an encapsulation efficiency ranging from 0.6% to 4.5%. Release studies confirmed that more retained fluorescent marker inside liposomes was achieved when stabilized liposomes were used, indicating an interaction between the polymer and the phospholipid bilayers and leading to the formation of pores, which resulted in a faster release of calcein.

The prolonged release of drugs to the ocular surface and, subsequently, the prolonging of the time of contact, is essential for delivery systems intended for ophthalmic therapies. Multilamellar liposomes were examined for encapsulating ciprofloxacin and delivering the drug and incorporated in conventional gels made up of poly(vinyl alcohol) and polymethacrylic acid (Budai et al., 2007). The hydrogels were loaded with the same concentration as commercially available product (0.1%), whereas liposomal hydrogel system with ciprofloxacin showed encapsulation efficiency with slight differences ranging from approximately 11%–14%. In vitro release studies revealed a retarded release of ciprofloxacin of liposomes prepared with α -L-DPPC; compared to those liposomes made up of lecithin, it was more prolonged. This effect was attributed to the differences in the composition of the lipid bilayer and, consequently, the effect of the changes in membrane fluidity and permeability on the solubility of the drug.

Further examples of liposomes incorporated within in situ gels for ophthalmic use was reported by Yu et al. (2015). Timolol maleate, as antiglaucoma agent, was encapsulated into liposomes fabricated by the reverse evaporation technique, coupled with pH-gradient methods, and after being incorporated into gellan gum gels. The encapsulation efficiency of the liposomes was $\sim 47\%$ and the release rate of formulations consisting of liposomes dispersed in gellan gum exhibited a slower release than liposomes alone (Fig. 6.12). As other studies have compared, the retention time in the cornea as well as bioavailability was enhanced when liposome-based hydrogels were used.

Vitamin A palmitate was also loaded in liposomes by He et al. (2013). The aim of this work was to increase the corneal penetration and to achieve longer retention times on the corneal surface of a hydrogel-based formulation useful for dry eyes treatment. Cationic liposomes were obtained with excellent encapsulation efficiency of approximately 84%–85%, depending on the formulation, and their subsequent incorporation to poloxamer 407 gels did not affect the gelation

**FIGURE 6.12**

(A) In vitro release of timolol maleate from liposomes incorporated into ion-sensitive deacetylated gellam gum gels (DGG) compared with drug-loaded liposomes and (B), results of intraocular pressure as a function of time of timolol maleate-loaded liposomes dispersed in in situ gellam gum gel in normal and glaucomatous rabbits. Control group was treated with NaCl (Yu et al., 2015).

ability of the gels. Coating liposomes with a chitosan derivative, *N*-trimethylchitosan, used as penetration enhancer, resulted in a more enhanced sustained release of vitamin A from liposomes and better transcorneal permeation than marketed gels, leading to an improvement in the ocular drug bioavailability, as in vivo corneal retention studies demonstrated.

Other studies have also shown that the entrapment of drugs, such as ofloxacin, in liposomes formulated as topically in situ thermosensitive hydrogels, improved the ocular penetration of the drug by providing a better and more sustained release profile (Hosny, 2009). After a detailed study about the factor affecting the encapsulation efficiency of ofloxacin into multilamellar liposomes, an optimized formulation with a maximum EE% of approximately 65% was obtained. Chitosan and β -glycerolphosphate were used as a thermogelling vehicle. Transcorneal permeation curves of liposomal hydrogel, liposomal suspension, and drug suspension revealed significant differences, showing greatly enhanced permeation percentages for liposomal formulations. This was, in part, due to the fact that lipid components of liposomes facilitated the solubilization of the drug and also due to the prolongation of the retention of the formulation at corneal site, provided by chitosan.

Other types of vesicular drug delivery system such as niosomes (nonionic surfactant vesicles, a particular type of liposome) have been demonstrated to also provide a controlled release of some drugs for ophthalmic administration of drugs or to deliver genetic material into the retina (Vyas et al., 1998; Aggarwal et al., 2007; Abdelbary and El-gendy, 2008; Maiti et al., 2011; Abdelkader et al., 2014; Gaafar et al., 2014; Hasan, 2014; Puras et al., 2014; Zubairu et al., 2015; Zeng et al., 2016). Niosome-based hydrogels are other novel approaches carried out for the ocular delivery of different drugs. Systems comprising chitosan or carbopol (Aggarwal and Kaur, 2005) or carbopol/methyl cellulose (Kapadia et al., 2009) hydrogels served as gelling vehicle for timolol and acyclovir-loaded niosomes, respectively. Both kinds of formulations demonstrated better control of drug release and in vivo behavior than niosome suspension. In situ gel-forming systems shown overcome the disadvantages associated with the instillation of colloidal suspension alone. More recently, in vivo studies using niosomes of 2–3 μm , dispersed in chitosan, have shown to increase the residence time of the formulation in rabbit eyes, compared to the timolol solution, although no data about technological features of formulations were provided (Kaur et al., 2010). The development of voriconazole-loaded niosomes suspended in an in situ gelling vehicle composed of sodium carboxymethylcellulose and sodium alginate was characterized and tested in in vivo studies (Shukr, 2016), showing an increased bioavailability in rabbits.

Promising results were obtained by Abu Hashim et al. (2014), where nonionic surfactant vesicles containing atenolol were prepared in different cholesterol/Span 60 ratios. Another study reported that niosome-based hydrogels efficiently increased the stability of the drug (atenolol) (Abu Hashim et al., 2014). The maximum entrapment efficiency of the niosomal dispersion (80%) was coincident with the 2:1 molar ratio of Span 60:cholesterol. This fact was attributed to the stabilizing effect of bilayers by cholesterol when used at certain concentrations. In this work, several polymers were tested as gel systems (carbopol 934P, hydroxypropylmethylcellulose, chitosan, and sodium alginate). According to the release profiles, niosomes dispersed in carbopol gel have exhibited the slower and sustained in vitro release of atenolol.

Increasing the residence time has always been a particularly challenging issue in ocular delivery. With this aim in mind, Lavanya et al. (2014) have examined the encapsulation of linezolid, a broad spectrum antibiotic, in niosome-based in situ hydrogels also prepared with carbopol (971P) and hydroxypropylmethylcellulose in different ratios. All formulations displayed excellent entrapment efficiency, whereas an effective control of linezolid release in simulated media was obtained for 24 h, although no extremely high differences were observed among different formulations of niosomal in situ forming gels.

More recently, fluconazole-loaded niosomes were incorporated into poloxamer 407 and chitosan gel vehicles (Fetih, 2016). In this work, the encapsulation efficiency was found to be greatly affected by the composition on surfactant:cholesterol ratio. Variations of cholesterol and Span 60 (as surfactant) concentration

resulted in maximum entrapment efficiency up to 85%. Interestingly, poloxamer-containing niosomes exhibited a more sustained release when compared to drug solution. The decrease in the percentage of drug released was directly related to the cholesterol content of niosomes. The incorporation of niosomes to gel vehicles delayed even longer the fluconazole release, compared to the niosomal suspensions. The release kinetics were determined to be in agreement with the diffusion model. Overall, the niosome-based hydrogels prepared with poloxamer exhibited better properties and drug transcorneal permeation than those based on chitosan alone (Fig. 6.13).

The administration of drug entrapped in polymeric micelles to the eye has been also studied. For example, the use of Soluplus (polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol copolymer) prepared at certain concentrations (1–2 mM) displayed in situ gelling ability, depending on the temperature (Alvarez-Rivera et al., 2016). In vitro studies on bovine corneal permeability showed an increase on the concentration of the antioxidant alpha-lipoic acid in the cornea; the permeation of the drug was found to be notably augmented when formulated in polymeric nanomicelles without irritation, according to HET-CAM test. A good comparison of the ability for delivering the antifungal agent itraconazole using polymeric micelles associated with in situ gel (carbopol), or the commercial eye drop and pure drug suspension, was made by Jaiswal et al. (2015). The ex vivo transcorneal permeation of the drug using pluronics micelles was superior to the suspension/eye drops form of the drug. The final optimized

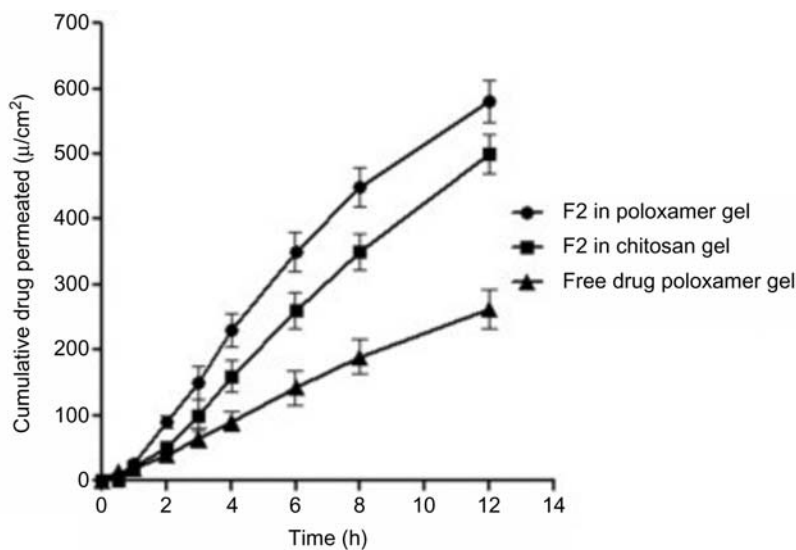


FIGURE 6.13

Permeation profiles of fluconazole across excised rabbit cornea from poloxamer gel and niosomal formulation in poloxamer and chitosan gels, here named as F2 (Fetih, 2016).

formulation of micelles was able to sustain the release of itraconazole for 8 h, a similar pattern obtained for polymeric micelles dispersed in carbopol gels and significantly higher than the permeation profiles of eye drops and drug suspension.

In order to improve the biopharmaceutical profile of drugs for the ocular application, cyclodextrins have been found to overcome some important issues, such as increasing drug solubility (by complexation with hydrophobic drugs), reducing its toxicity, or acting as promotion enhancers across biological membranes like the cornea or even, retaining the drug at the precorneal site. The use of cyclodextrins for ocular drug delivery has been extensively reviewed (Lofsson and Järvinen, 1999; Lofsson and Stefánsson, 2002). Concerning ophthalmic administration, some approaches have been done later using cyclodextrin-based formulations. More precisely, most of them were included in eye drop solutions or in contact lenses, mainly to increase the bioavailability of different drug across the cornea or to obtain sustained levels of drugs at corneal surface (García-Fernández et al., 2013; Moya-Ortega et al., 2013).

More scarcely, a number of studies have been carried out to investigate the effect of in situ gelling formulation on the prolonged release of drug-loaded cyclodextrins. Accordingly, Pawar et al. (2013) designed a temperature-sensitive gelling system made of pluronics and sodium alginate, to evaluate the release properties of hydroxypropyl- β -cyclodextrin (HP- β -CD) and voriconazole inclusion complexes for ophthalmic drug delivery. Voriconazole permeation studies performed using Franz diffusion cells and excised goat cornea have shown, although with some differences on the permeability behavior of each kind of membrane, all formulation achieved a sustained release for several hours, good stability, and inhibition of fungal growth (*Candida albicans* and *Aspergillus fumigatus*). Previously, also using HP- β -CD to obtain complexes with the epithelial growth factor (rhEGF), an attempt was made with the purpose of delivering the growth factor from a thermosensitive gels based on poloxamers 188 and 407. Different formulations preserved the physical stability at 4°C of the polypeptide and provided a prolonged release, compared to control, and longer corneal contact time of the drug in rabbits (Kim et al., 2002). Fernández-Ferreiro et al. (2014) designed, formulated, and evaluated fluconazole inclusion complexes with hydroxypropyl- β -cyclodextrin (HPBCD) and sulfobutylether- β -cyclodextrin (SBECD) incorporated to ion-sensitive gel, based on gellan gum and k-carrageenan. HPBCD-fluconazole complexes were found to be less cytotoxic than those prepared with SBECD, as kinetic curves of cell survival rate showed. The stability constant for HPBCD was also higher when this cyclodextrin was used. The incorporation of cyclodextrin into the gel allowed for increasing the drug concentration from 0.2% to 1%; the release studies from the hydrogels containing the free and the complexed drug indicated that gels comprising drug-cyclodextrin complexes retained the drug better (4 h) than the free drug into the gels (1 h). The study showed clearly that cyclodextrin-containing gels are safe vehicles providing a fast and more prolonged drug release and, therefore, a good strategy to improve the bioavailability of ophthalmic antifungal formulations.

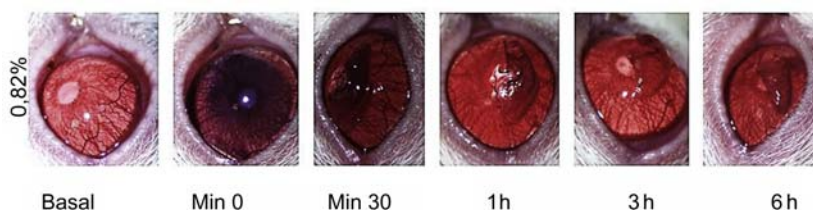
Different strategies have been carried out to design a variety of drug delivery systems based on stimuli-responsive or smart gels, as discussed in this section. This scenario still presents some challenging tasks for drug delivery technology. Among the factors that should be considered for the ultimate approval of a drug delivery system for ophthalmic applications are: the biocompatibility and nonirritant effect of the pharmaceutical device, the inconvenience caused to patients, sterilization and preservation of ophthalmic in situ hydrogels, or finally, the in vivo studies and the institution approval. Only after these key factors are solved, an advanced drug delivery system for ocular usage can be therapeutically acceptable. The development of carriers that exhibit good ocular behavior and which enables the active substance to the anterior or posterior segment of the eye is becoming increasingly important in the treatment of ocular diseases; to facilitate drug efficacy and to improve the sustained release without causing irritation. Above all, the delivery systems developed must be acceptable from the compliance point of view of patients, who are, in fact, the end-users of any successful research in the field of controlled drug delivery.

6.5 NEW TECHNOLOGIES FOR DETERMINING OCULAR RESIDENCE TIME AND OCULAR BIODISTRIBUTION OF HYDROGELS

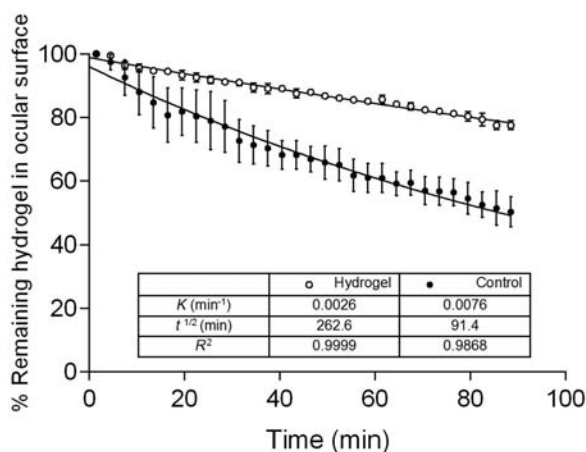
The study of corneal surface permanence of ophthalmic formulations has always been a challenge (Tomlinson and Khanal, 2005). Over the years, different methodologies have been described as ex vivo techniques developed to provide perfusion systems that simulate ocular clearance (Liu and Wang, 2009), in vitro methods in which the 3D impression creates irrigated interparebral spaces (Bajgrowicz et al., 2015), and even mathematical models which estimate the effect of the viscosity of the formulations on the nasolacrimal drainage (Zhu and Chauhan, 2008).

The described methods try to simulate the effect of the formulation on the ocular surface, with other methods of direct measurement in vivo. The use of visible light to examine ocular processes can be considered the oldest form of ophthalmic imaging (Eter, 2009), and it can be applied for direct observation of the permanence of ophthalmic formulations stained with frequently used dyes in the eye, such as fluorescein sodium, bengal rose, methylene blue, alcian blue, trypan blue, lysamine indocyanine green, proflavin, mercurochrome, or argirole (Cortes and Cajiales, 2007; Joshi et al., 1997) (Fig. 6.14).

The application of fluorescein sodium and derivatives thereof (Meadows et al., 2002; Mochizuki et al., 2009) have been used to characterize the biopermanence of different formulations containing hyaluronic acid (Mochizuki et al., 2008), and substances such as methylene blue and derivatives have also been used to characterize the biopermanence of ion-sensitive hydrogels (Liu et al.,

**FIGURE 6.14**

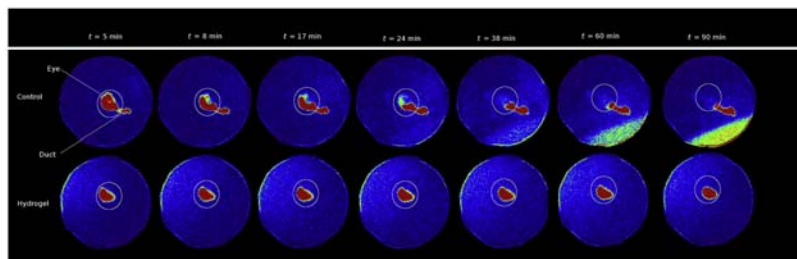
Macrophotography of the right eye of a Sprague-Dawley rat after the instillation of 7.5 μL of the ion-sensitive hydrogel containing Trypan Blue (Fernández-Ferreiro et al., 2015).

**FIGURE 6.15**

Dynamic study with anesthetized animal. Kinetic profile of hydrogel (*open circles*) and control solution (*close circles*) during 1.5 h postinstillation (Fernández-Ferreiro and Silva-Rodriguez, 2017).

2010; Gourav and Arushi, 2012; Fernández-Ferreiro et al., 2015) Another, more complex, imaging technique based on the use of radiopharmaceuticals is scintigraphy. Initially, radiopharmaceuticals, such as $^{99\text{m}}\text{Tc}$ in ophthalmology, have been used for the diagnosis and characterization of nasolacrimal duct obstructions. However, the technique has been subsequently focusing on its application for the determination of residence time of different ophthalmic products (Fernández-Ferreiro and Silva-Rodriguez, 2017; Detorakis et al., 2014; Kotina et al., 2015) (Figs. 6.15 and 6.16).

It has been used for the comparison of ocular permanence times between artificial tears (Snibson et al., 1992; Snibson et al., 1990), to characterize thermosensitive hydrogels (Wei et al., 2002), sensitive ions (Greaves et al., 1990;

**FIGURE 6.16**

Shows the scintigraphy image. In the upper images (control eye) we observed that the signal decreases in the corneal surface, moving toward the nasolacrimal duct, while in the bottom images signal obtained in the eye with hydrogel remains unchanged (Fernández-Ferreiro and Silva-Rodriguez, 2017).

Rupenthal et al., 2011; Kashikar and Gonjari, 2014), antiglaucomatous eye drops with PVA and gellan gum (Greaves et al., 1992; Meseguer et al., 1996), gels with carbomers (Wilson et al., 1998), chytosan (Felt et al., 1999; Gupta et al., 2009), liposomal ophthalmic agents (Fitzgerald et al., 1987), and even to determine the effect that ultrasound can have on ocular drug penetration (“[Pharmacokinetics of radiotracers in the ocular tissues exposed to infrasound and ultrasound phonophoreses],” 2006).

Positron emission tomography, or PET scanning, is also based on the use of radiopharmaceuticals; it has a higher resolution and ability to integrate three-dimensional images than scintigraphy. By now, this methodology has not yet been widely used in the field of ophthalmology. So far, there has been only one work that determines the time of ocular biopermanence of a chytosan gel labeled with ^{124}I (Dangl et al., 2009; Schmetterer et al., 2010; Kuntner et al., 2011). Conversely, Fernández-Ferreiro et al. (2017) have shown that the use of small-animal PET and ^{18}F radiotracers in ocular pharmacokinetics of ophthalmic formulations is feasible and it could be a safe method for future ocular pharmacokinetic studies in humans. This tool has less transportation time than scintigraphy, and probably ends up overshadowing it, since it has a high potential for the performance of ocular pharmacokinetic studies, being already used in intravitreal studies for its greater precision and versatility (Christoforidis et al., 2011, 2012).

However, nuclear magnetic resonance is another imaging tool used in ophthalmology, with different purposes such as the study of drug release by subconjunctival, intravitreal, intrascleral injection; periocular injections; or the study of ocular iontophoresis (Li et al., 2004, 2008; Kim et al., 2005; Alikacem et al., 2000) (Fig. 6.17).

Most of the contrast agents used in MRI are hydrophilic filler compounds which, when characterizing the time of ocular permanence of hydrogels on the

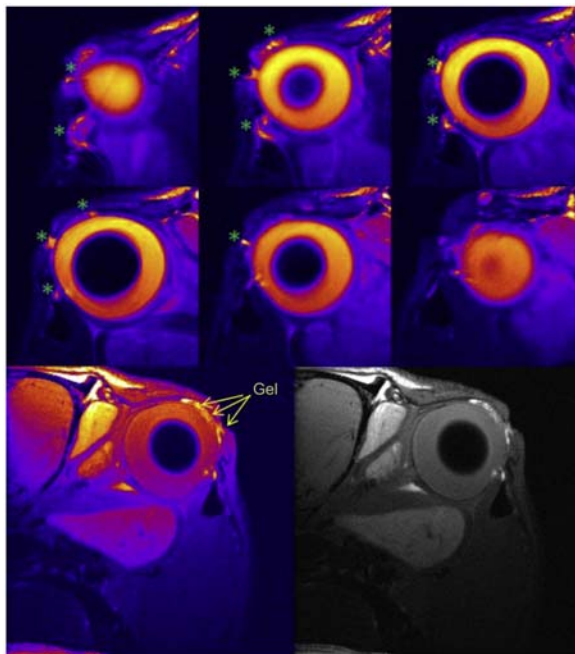


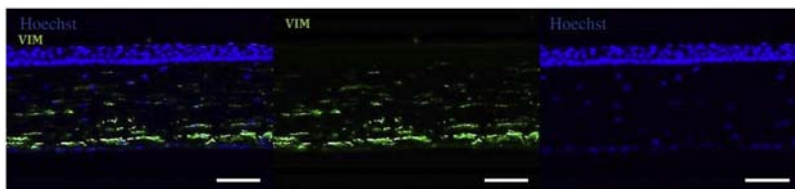
FIGURE 6.17

RMI images of the rat eye 2 h after instillation of ion-sensitive hydrogel indicate the hyperintense signals caused by hydrogel (Fernández-Ferreiro et al., 2015).

ocular surface, can be problematic because they can establish specific interactions with the eye, and therefore this could interfere with ocular clearance and with the determined pharmacokinetics (Fernández-Ferreiro, 2015). Thus far, the specific contrast provided by the hydrogel itself (water and biopolymers) is used only in a study of determination of ocular biopermanence (Fernández-Ferreiro and Silva-Rodríguez, 2017) (Fig. 6.18).

Another imaging techniques that do not require radioactive labeling or contrast for use is computed tomography (CT). Its use is based on measuring the corneal thickness before and after administration of the topical formulation at different times and has already been used for comparative studies in artificial tears (Wang et al., 2006; Kaya et al., 2015). CT has also been used in other ocular studies, but as a tool for anatomical support in the interpretation of images obtained by scintigraphy (At'kova et al., 2014).

The techniques previously described are useful in the study of the permanence of ocular gels on the corneal surface and easily translatable to humans, because they are noninvasive and safe techniques, their high cost being the main drawback.

**FIGURE 6.18**

Microphotography of a rat cornea section after 3 months of instillation of ion-sensitive hydrogel. Hoechst allow us to explore the nucleus distribution along the corneal layers, and vimentin was used to observe the distribution and orientation of stromal keratocytes. Scale bar 75 μm (Fernández-Ferreiro et al., 2015).

6.6 TOXICITY AND SAFETY OF POLYMERS USED IN STIMULI GEL SYSTEM: IN VITRO, EX VIVO AND IN VIVO ASSAYS

Ocular tolerance is an essential parameter in the development of ophthalmic preparations; they should not cause irritation or lead to any kind of toxicity (Dumitriu, 2001). Ocular tolerance tests have shown that hydrogels are generally better tolerated than ointments by patients, mainly due to their rheological properties (Shastri et al., 2010). However, it should be taken into account that the eye is a very sensitive organ to many substances and ocular toxicity tests must be obligatory to evaluate the possible associated risks (Jiménez and Kuhn, 2009; Fernández-Ferreiro et al., 2016a). Historically, toxicity tests were performed only on animals, the Draize test being the industry standard (Draize et al., 1944).

At present, legislation in most countries has adapted to try to reduce animal testing and promote alternative techniques. These techniques include ex vivo testing in deceased animal tissue, computer models, and in vitro assays based on cell culture models (Wilson et al., 2015).

In vivo eye safety studies of ophthalmic hydrogels, performed primarily on rats and rabbits, have shown that these are not harmful to the eye. Accordingly, components of hydrogels, such as polyacrylic acid (carbopol C 934p), hydroxypropylmethylcellulose (Dubey and Prabhu, 2014; Singh et al., 2010), ion-sensitive polymers such as gellan gum or kappa carrageenan (Fernández-Ferreiro et al., 2015; Balasubramaniam et al., 2003), thermoreversible polymers such as Pluronic (Cholkar et al., 2013; Chen et al., 2012) or pH-sensitive such as chitosan (de Salamanca et al., 2006; Contreras-Ruiz et al., 2010; Easty et al., 2006) have been tested; all of which are harmless to the ocular surface. However, it should be noted that the use of high concentrations of polymers can cause ocular irritations. A strategy to reduce it is the combination at low concentrations of several polymers with synergistic gelation mechanisms (Shastri et al., 2010).

As for ocular safety studies in vitro, cytotoxicity studies on primary cell lines and cultures of epithelial cells, stromal keratocytes, and conjunctival fibroblasts

should be highlighted, where compounds such as chitosan or alginate show no toxicity (Alonso and Sánchez, 2003; de Salamanca et al., 2006). Polymers used in ion-sensitive smart hydrogels, such as gellan gum and kappa carrageenan (Fernández-Ferreiro et al., 2015); or in thermosensitive ones like Pluronic (Lin et al., 2009), have also been tested, rendering them harmless to cells. Other *ex vivo* studies have also been performed with biopolymers, with the Het-Cam test being the most repeated (Fernández-Ferreiro et al., 2014). Studies with Pluronic (Kanoujia et al., 2014), carbopol (Pathak et al., 2013), gellan carrageenan kappa gum (Fernández-Ferreiro et al., 2015), or xanthan, show the absence of irritation.

6.7 EFFECT OF THE HYDROGELS ON THE CORNEAL REGENERATION CAPACITY

Infections, inflammations, and abrasive eye damage can cause scars on the corneal surface and trigger damage to vision. Controlling and promoting the healing of corneal wounds has proven to be a major challenge (Tint, Wolfson Centre for Stem Cells, Tissue Engineering and Modelling (STEM), School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham and Rose, Division of Ophthalmology and Visual Sciences, Eye, ENT Centre, Queens Medical Centre, University of Nottingham, 2009). Corneal wound-healing is a multifactorial process involving cell death, migration, proliferation, differentiation, and remodeling of the extracellular matrix (Ljubimov and Saghizadeh, 2015).

Currently, therapeutic agents capable of supporting the healing process are varied and nonspecific. These agents can generally be divided into growth factors, cytokines, proteases, cationic peptides, antioxidants, proteins, and antiinflammatories (Ashby and Garret, 2014). The polymers used in ophthalmic hydrogels are being studied for their properties in corneal regeneration (Ågren, 2016; Kirchhof et al., 2015; Grinstaff, 2007). Accordingly, collagen, one of the most abundant proteins in the human cornea (Michelacci, 2003), is being used as a hydrogel to act as a regenerator (Fagerholm et al., 2014), somewhat similar to the gelatin hydrogel that has been used as a corneal fibroblast transporter (Mimura et al., 2008). Alginate is another of the polymers used in the manufacture of hydrogels and has shown regenerative properties of the corneal surface (Lee and Mooney, 2012; Liang et al., 2011; Wright et al., 2012; Fernández-Ferreiro et al., 2015), as well as chitosan (Yao and Mao, 2002; Du et al., 2008). Conversely, many of the components that are part of the hydrogels are also used in the elaboration of artificial tears, being cellulosic derivatives (Garrett et al., 2007), heparin (Denk and Knorr, 1999), and hyaluronic acid (Moreira et al., 2013); some used because of their regenerating capabilities of the corneal surface.

6.8 IN VIVO STUDIES IN ANIMALS AND EFFECTIVENESS OF DRUGS WHEN THEY ARE INCORPORATED IN THE HYDROGELS

To improve the binding between a drug and the hydrogel matrix, and thus prolong the duration of drug release, both physical and chemical strategies can be used (Hoare and Kohane, 2008). The use of poloxamers as polymers in the development of ophthalmic gel–hydrogels sensitive to stimuli has been used to incorporate various drugs such as doxycycline (He et al., 2011), betaxolol (Bachhav et al., 2015), ciprofloxacin (Cho et al., 2003), or fluconazole (Fernández-Ferreiro et al., 2014). Examples of drugs that have been incorporated in these hydrogels and whose effectiveness has been evaluated in vivo are shown in Table 6.1.

Table 6.1 Examples of Ophthalmic Stimuli-Sensitive Hydrogels With Drugs

Polymers	Ophthalmic Drugs	Reference
Temperature-sensitive polymers	rhEGF	Kim et al. (2002)
	Dorzolamide	Barse et al. (2016)
	Indomethacin	Chetoni et al. (2000)
	Tolmetin	Bucolo and Spadaro (1997)
	Dexamethasone	Gao et al. (2010)
	Brimonidine	Ibrahim et al. (2013), Patel and Shah (2012), Patil and Tagalpallewar (2012)
	Puerarin	Qi et al. (2007)
	Timolol	El-Kamel (2002)
	Pilocarpine	Miller and Donovan (1982), Desai and Blanchard (2000)
	Betaxolol	Huang et al. (2016)
pH-sensitive polymers	Moxifloxacin	Shastri et al. (2010)
	Timolol	Aggarwal and Kaur (2005), Gupta (2010)
	Pilocarpine	Lin and Sung (2000)
	Betaxolol	Plager et al. (2009)
	Fluconazole	Lihong et al. (2014)
Ion-sensitive polymers	Gatifloxacin	Kanoujia et al. (2014)
	Ketothiophene	Zhu et al. (2015)
	Indomethacin	Balasubramaniam et al. (2003)
	Brimonidine	Geethalakshmi et al. (2012)
	Timolol	Nelson et al. (1996)
	Levofloxacin	Saher et al. (2016)
	Gatifloxacin	Dojjad et al. (2006)
	Perfloxacin	Sultana et al. (2006)

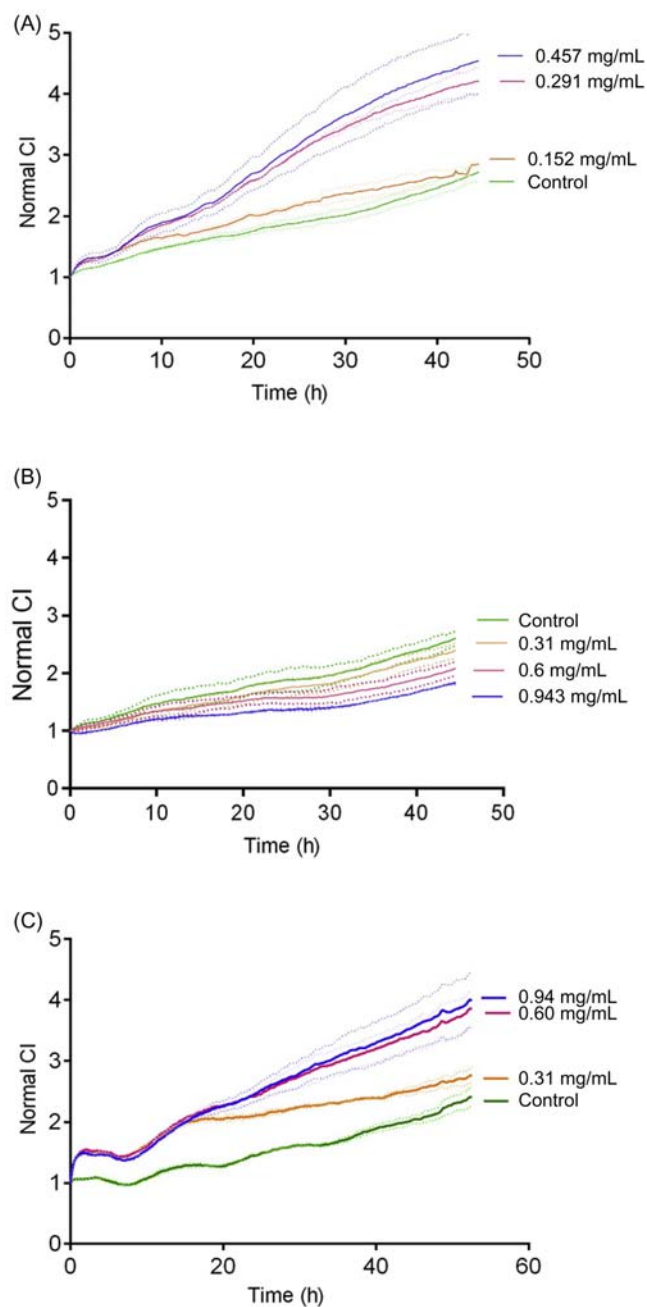
In all cases, longer and sustained drug releases are achieved. Such releases correspond to the observed *in vivo* effects, where the response is improved, compared to solution eye drops containing the drug in the absence of polymers. There have been several responses, of antiglaucomatous type (Barse et al., 2016; Huang et al., 2016; Ibrahim et al., 2013; Patel and Shah, 2012; Patil and Tagalpallewar, 2012; El-Kamel, 2002; Aggarwal and Kaur, 2005; Gupta, 2010; Plager et al., 2009; Geethalakshmi et al., 2012; Nelson et al., 1996), antiinflammatory (Chetoni et al., 2000; Gao et al., 2010; Zhu et al., 2015; Balasubramaniam et al., 2003) and antiinfective (Shastri et al., 2010; Lihong et al., 2014; Kanoujia et al., 2014; Saher et al., 2016; Doijad et al., 2006; Sultana et al., 2006) than with conventional eye drops, without ocular adverse effects after administration. Many of the hydrogels are already being used in the clinic, such as those containing Timolol (Timoptix-XE, Regel depot technology), Interleukin-2 (Cytorin), Azithromycin (Azasite), Pilocarpine (Pilopine HS), Lidocaine (Akten) or Ganciclovir (Virgan) (Pallavi and Gondkar, 2015).

6.9 STUDIES OF DRUG PENETRATION AND PROMOTION

Topical ophthalmic drug transmittance into the aqueous humor is severely conditioned by multiple causes, the cornea being one of the main limiting factors. The degree of drug transmittance through it is conditioned by physiological factors, such as precorneal clearance and nasolacrimal drainage; physicochemical factors, such as molecular weight of the drug and lipophilicity; and factors associated with the formulation itself (Manjusha and Majumdar, 2001).

There are many compounds that are known to increase the penetration of drugs through the cornea, such as surfactants used in ocular formulations, benzalkonium chloride or sodium lauryl sulfate, however these usually have counteracting ocular toxicity (Droy-Lefaix et al., 2013). The challenge in ophthalmic formulation is to avoid eye barriers without causing permanent damage to the tissue (Fernández-Ferreiro et al., 2016a; Fernández-Ferreiro et al., 2016b), which is why noncytotoxic compounds such as biopolymers are currently being studied (Fig. 6.19). In addition to increasing contact time of the drug in the ocular surface, they also promote its transmittance through it (Ng et al., 2010; Zambito and Di, 2011). Many of the polymers used in compounding have been studied as inhibitors of efflux pumps by multiple mechanisms of action (Gaikwad and Bhatia, 2013; Gokulgandhi et al., 2011).

Chitosan has been shown to be able to increase the permeation of antiinfectives (Di Colo et al., 2004; Di Colo, 2004), and its mechanism of action may be due to a decrease in gap junctions between cells (Kaur et al., 2010). Others, such as poloxamers, have been used as permeation promoters for their ability to inhibit efflux pumps (Barse et al., 2016; Bucolo and Spadaro, 1997; Batrakova et al., 2001), as well as ion-sensitive polymers such as gellan gum (Gupta et al., 2010;

**FIGURE 6.19**

Ocular cytotoxicity studies of different polysaccharides in primary corneal human keratocytes obtained using real-time xCELLigence impedance analysis: (A) Kappa carrageenan, (B) gellan gum, and (C) alginate.

Gaikwad and Bhatia, 2013; Sayed et al., 2015) or the pH-sensitive as the carbopol (Wu et al., 2007). Other compounds, such as the cellulosic derivatives (Vulovic et al., 1990) or hyaluronic acid derivatives, also improve the corneal permeation of some drugs studied (Wang et al., 2006).

Alternatively, the development of new ophthalmic formulations, such as nanosomes and nanoemulsions incorporating polymers such as alginate, pluronic or carbopol (Kanoujia et al., 2014; Pathak et al., 2013; Zubairu et al., 2015) have also favored the transmittance of active principles through the corneal surface.

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Stimuli-sensitive nanomaterials for antimicrobial drug delivery

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CHAPTER OUTLINE

7.1 Introduction	271
7.2 Physicochemical Behavior of nanoparticles on Drug Property Improvement	273
7.2.1 Influence of Particle–Particle Interaction at Nanoscale to Antibacterial Activity	274
7.3 Mechanism of Action of Antimicrobial Nanoparticles.....	276
7.4 Type of Antimicrobial Nanoparticles.....	278
7.5 Effect of Stimuli on Drug Delivery.....	279
7.5.1 Effect of Temperature and pH on Antimicrobial Nanoparticles	280
7.5.2 Effect of Light on Antimicrobial Nanoparticle.....	283
7.5.3 Effect of the Enzyme on the Antimicrobial Nanoparticle.....	291
7.5.4 Effect of Magnet on Antimicrobial Nanoparticle	293
7.6 Conclusions and Future Prospects.....	296
Acknowledgment.....	296
References	297

7.1 INTRODUCTION

Antibiotic resistance, an emerging global concern to healthcare, has become life-threatening and traumatic phenomenon in the world. This has compelled researchers worldwide to focus on developing new devices for delivery of antibiotics and/or new antibiotics with neo-mechanistic approaches to the prevention of resistant and effective therapeutic treatment. Generally, antimicrobial resistance (AMR) (Zolfaghari et al., 2009) is defined by the growth of microorganisms, such as bacteria, fungi or protozoans, in the presence of antimicrobial compounds that are believed to inhibit their growth. As per the literature, for a chemical agent to become a drug entity, it must produce their desired pharmacological action and move across various impediments viz. penetration of intestinal barrier, and sustain metabolic reaction, before reaching the desired site of action. However, it is

commonly observed that many drugs can cause cell death or induce a significant change in cell behavior and toxicity to normal cells, thereby undermining their use. Even though the therapeutic efficacy of these drugs has been well established, inefficient delivery systems could result in an inadequate therapeutic index and local or systemic adverse effects, including cutaneous irritation, peeling, scaling, and gut flora reduction. Therefore, in the current scenario, it is of interest for the concerned group of researchers to design and develop application-oriented materials with a distinct mechanism toward killing microorganisms, such as bacteria, in place of the traditional mechanisms. Furthermore, although the recent advances in nanotechnology (Feynman, 1991) have unraveled new avenues with immense potential to prevent the catastrophic consequences (Huh et al., 2011) of antibiotic resistance and antibiotic-producing toxicity, researchers still find many concerns about the understanding of nanotechnology and control of matters at nano scale (Prasad, 2012). Nanomaterials at the molecular and/or atomic level produce unique and well defined physicochemical, pharmacodynamic, and pharmacokinetic properties induced by the effects of ultra-small and controllable quantum size effect, shape (spheres, rods, wires, planes, stars, cages, multipods) (Perrault and Chan 2009), surface-by-volume ratio, functionalizable structure and surface charge, and unusual electrodynamic interaction with biological systems. The relevant precedent literature on this topic observed that these unique and exclusive properties of nanomaterials make them suitable for antimicrobial applications. These physical and chemical properties (Burda et al., 2005), which could be induced properly based on required applications to facilitate the administration of antimicrobial drugs, are the pivotal parameters to overcome some of the limitations in traditional antimicrobial therapeutics. Moreover, their potent antimicrobial efficacy is due to the large ratio of surface area to volume, which has provided them with an advantage over their bacterial-resistant chemical counterparts. The applications of nanotechnology in medicine (Zhang et al., 2008) have been extensively explored in various medical areas, especially in the field of drug delivery over last few decades, including gene therapy (Panyam and Labhasetwar, 2012; Giljohann et al., 2010), fluorescent-based bio-imaging agents or biological markers (Sharma et al., 2006), magnetic resonance imaging (MRI) contrast enhancers (Kopelman et al., 2005), DNA-templated probes (Hilliard et al., 2002), biosensors (De et al., 2008; Underwood and Van Eps, 2012; Salata, 2004), hyperthermia-aided tumor eradication, tissue engineering (Sun and Matthew, 2000), and antibacterial and antiviral agents (Zhang et al., 2010).

The formation of nanoparticle-based drugs through physical adsorption, encapsulation and/or chemical modification can improve the clinical parameters (pharmacokinetics and therapeutic index) of the drugs, in comparison to their free drug counterparts. Generally, these methods improve the serum solubility, the lifetime, sustainability, and availability of the single or multiple therapeutic agents, preferentially to the particular tissues and cells of interest (Davis et al., 2008). Spurred by the recent progress in materials science for drug delivery, stimuli-responsive carriers that transport a drug in spatial-, temporal-, and dosage-controlled fashion

have tended to receive more substantive attention from academia and industry to become one of the precise and potential delivery systems. This concept of a smart carrier for drug delivery involves designing and constructing a nanostructure (or microstructure) with therapeutic drug-loading capability, expected to protect the drug entities from degradation by enzymes in the living organism, to enhance the solubility and bioavailability of insoluble drugs, to extend the circulation half-life, and to enhance its permeability and accumulation at the site of interest. In particular, the designed smart nanocarriers need to be responsive to a specific stimulus in order to release or activate the required therapeutic entity only when it is desired. Therefore, the design and development of nanoscale stimuli-responsive systems that are proficient to control drug biodistribution in response to specific stimuli, either exogenous (variations in temperature, magnetic field, ultrasound intensity, light, electric pulses, and mechanical forces) or endogenous (changes in pH, enzyme concentration, or redox gradients), are the key advances in the field of stimuli-responsive nanomaterials. The spatiotemporally-controlled biodistribution of nanoparticles at the atomic and/or molecular level is enhanced by light following mainly reactive oxygen species (ROS)-mediated mechanism, pH changing the electrostatic interaction and external magnetic field possessing ability to separate the different microorganism using the magnetic nanoparticle. Accordingly, stimuli-responsive nano-based components support improving the shelf-life of drugs, site-specific activity, water solubility, biocompatibility, toxicological profile, sustained drug release profile, and bioavailability of antibiotics at the site of action over a period of time. Consequently, in the current situation, bioactive nanomaterials with high efficiency and less toxicity possessed the ability to be smart therapeutics, which could be delivered by stimuli-responsive devices made by nanomaterials as one of the most able carriers for transporting drugs into infected areas: the elusive “magic bullet.” This chapter will cover recent studies in the development of stimuli-sensitive nanomaterials for antimicrobial drug delivery in the presence of light, pH, magnetism, enzymes, and other endogenous or exogenous agents that can activate the nanostructure to release the drug molecules at the target site, avoiding obstacles and cytotoxicity en route or both.

7.2 PHYSICOCHEMICAL BEHAVIOR OF NANOPARTICLES ON DRUG PROPERTY IMPROVEMENT

Nanomaterials often have unique physical and chemical properties in comparison with bulk materials at the cellular, atomic, and molecular levels (Emerich and Thanos, 2006). Generally, the bulk materials have constant physical and chemical properties regardless of size, whereas the properties of the same at the nanoscale are the function of their sizes, as the presence of atoms at the surface area of a material is increased. The prime parameters of nanoparticles are their unique high surface-to-volume ratio, different shapes (including aspect ratios where

appropriate), sizes, and the morphological sub-structure of the substances. The ability to create the three-dimensional multi-component structures of nanoparticles enables the delivery of hydrophobic, poorly water-soluble molecules, and to selectively target to a preferred site in the living body, overcoming various biological obstacles. For bulk materials, those with sizes larger than one micron, the presence of atoms at the surface is insignificant in relation to the number of atoms in the bulk of the material. The behavior of nanoparticles is assumed to rely on its size at some point in between the angstrom level and the micrometer scale. Instead of classic theory, complex quantum mechanical models are appropriate to predict the evolution of such behaviors.

The physicochemical properties, including size, shape, coating, chemical modification of the pure particles or mixture with other nanoparticles in various ratios and solvent affect greatly their medicinal properties, such as antibacterial activity (Gatoo et al., 2014). In addition, the physicochemical properties of nanomaterials, such as crystallinity, hydrophobicity, surface instability, electronic states, surface roughness, and radius of curvature also contribute in different ways to protein binding and thus enhancing their bioactivity.

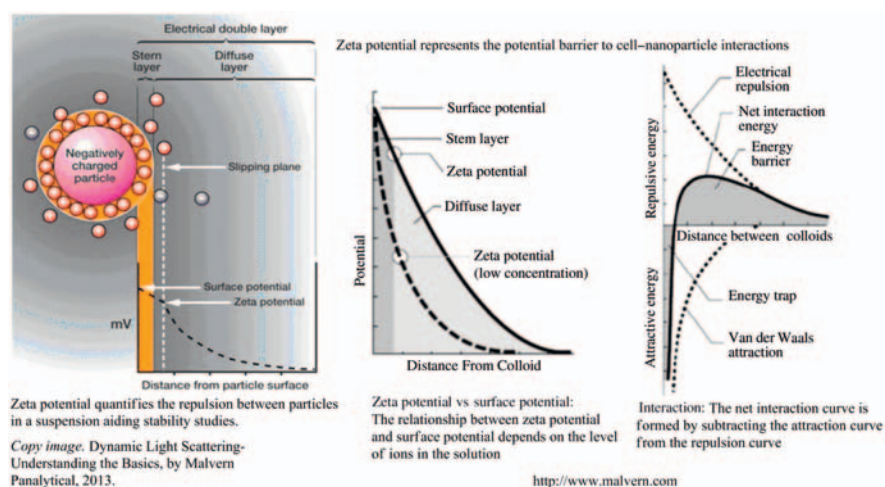
7.2.1 INFLUENCE OF PARTICLE—PARTICLE INTERACTION AT NANOSCALE TO ANTIBACTERIAL ACTIVITY

One of the most important properties of the nanoparticle is their interaction in a fluid system, particularly with a biological medium, and the bacterial surface area, including the surrounding environment. Generally, particle motion in the solution system is determined by the particle—particle interactions. However in the macroscale, particle—particle interactions are characterized by collision and particle contacts, which are commonly understood by elastic and inelastic transfer of momentum (Crowe et al., 1998). In literature, particle-fluid flow interactions have been studied extensively in terms of drag and torque coefficient. The most important external fields are the force fields related to mass, like gravitation and centrifugal forces. The different interactions of particles at macro and nanoscale are discussed in Table 7.1.

In the nanoscale, particle—particle potential interactions are either dominated by DLVO, van der Waals forces, Brownian and electrostatic interactions, or covalent interactions as an additional long range force (Table 7.1). Additionally, the potential interactions with a fluid flow on the nanoscale are determined by electrochemical double layers (EDL), which isolate the particles' surface from the surrounding fluid. Particle aggregation, which is ubiquitous to the nanoparticle, is determined by the interparticle interaction and depends on the viscosity and polarizability of the fluid. Consequently, by the modification of the surface layer, surface charge, surface energy, and solvation (the tendency of a colloid to coagulate) can be enhanced or hindered. This nanoparticle—nanoparticle interaction is of key importance to describing physical and chemical processes and the temporal evolution of free nanoparticles. The interaction forces, either attractive or repulsive,

Table 7.1 Different Interactions of Particles at Macro and Nanoscale

	Particle–Particle Interaction	Particle–Fluid Flow Interaction	External and Internal Fields
Macroscale	Collision, contact forces	Drag, buoyancy, Magnus forces	Gravitation and centrifugation forces
Nanoscale	Derjaguin, Landau, Vervy, and Overbeek (DLVO)-forces and sinter necks	Electrochemical double layer	Brownian motion, electrical and magnetic

**FIGURE 7.1**

Zeta potential as the potential barrier to cell-nanoparticle interaction.

crucially determine the fate of individual and collective nanoparticles. These interactions among nanoparticles, resulting in aggregates and/or agglomerates, may influence their behavior (Table 7.1).

The stability of nanoparticles in solution is predominantly governed by one of the two mechanisms, namely electrostatic or steric repulsion. The electrostatic potential at the (EDL) surrounding a nanoparticle in solution is known as the zeta potential. The nanoparticles with a zeta potential between -10 and $+10$ mV are considered approximately neutral, while nanoparticles with zeta potentials of greater than $+30$ mV or less than -30 mV are considered strongly cationic and strongly anionic, respectively. Since most cellular membranes are negatively charged, zeta potential can facilitate the nanoparticle with a cationic charge to permeate membranes displaying more toxicity associated with cell wall disruption. Conversely, nanoparticles with negative zeta potential (Fig. 7.1) experienced minimal electrostatic interactions with negative bacterial surface charges; the

weak interactions of these nanoparticles could be the explanation for their requirement in relatively high concentrations to produce an antibacterial effect (Honary and Zahir, 2013). It is worth mentioning that the stimuli can affect the surface charges and thereby the stability of the nanoparticles. Consequently, the zeta potential of the nanoparticles can also be affected by the stimulating agents (Freitas and Müller, 1998).

7.3 MECHANISM OF ACTION OF ANTIMICROBIAL NANOPARTICLES

Nanomaterials as antibacterial agents are complementary to traditional antibiotics, with the unique modes of action and/or targeting different cellular parts in a unique way with a spatial and temporal dimensions, compared with existing antibiotics (Mondal and Bera, 2014). The efficacy of these nanoparticles varies with their characteristic size, shape, concentration, and, moreover, the atomic abundance on the surface of nanomaterials; these physical changes at nanostate of the material are directly related to the properties of such materials. As the size of the particle decreases, the percentage of atoms on the surface increases, relative to the total atoms of material in the bulk, amplifying the physical and chemical activities. Accordingly, the higher population of the nanoparticles on the surface facilitates antimicrobial activity with negligible toxicity, compared with small molecule-based antimicrobial agents.

The accountability of the mechanistic action of nanoparticles is difficult, as it varies depending on the type of material and type of diseases. Inorganic metal or metal oxide-derived nanoparticles can interact with cell membrane of the bacteria by electrostatic interaction. This damages the bacterial membrane and induce toxic oxidative stress on the bacteria by the formation of cellular radical and non-radical oxygen species through partial reduction of oxygen, such as hydroxyl radical ($\cdot\text{OH}$), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2). These ROS damage DNA by attacking the membrane, made of polyunsaturated phospholipids, in bacteria and many other macromolecules, blocking the functions of the bacterial cell. In this context, light as a promising external stimulating agent could sensitize a particular class of compounds to transform the triplet oxygen ($^3\text{O}_2$) present in and around tissues into ROS in the close proximity of the diseased cells, leading to death, oxidation of organic compounds, and *Escherichia coli* cells to carbon dioxide. The oxidation of bacterial cell components, such as lipids and DNA, might therefore result in subsequent cell death (Fig. 7.2) (Nathan et al., 2013). Another family of antibacterial nanomaterials, reactive nitrogen species (RNS), acts together with ROS to disrupt the cell membranes. RNS, derived from nitric oxide (NO) and superoxide anion (O_2^-) (Kutner and Friedman, 2013), is involved in multiple mechanisms of action to directly damage the proteins that contain transition metal centers, such as hemoglobin, myoglobin, and cytochrome C by oxidizing ferrous heme into its corresponding ferric forms.

In addition to metal/metal oxide, a special classes of organic compounds, such as phthalocyanines (PC), phenothiazine, porphyrin dyes referred as photosensitizers (which are triggered by light in the presences of oxygen) act as promising agents in photodynamic therapy (PDT) via the formation of two types of species collectively referred as ROS/RNS (Konig et al., 2000; Niedre et al., 2005; Bonnett et al., 1993). These photosensitizers, in conjugation with silver, quantum dots, iron oxide, and gold-based hybrid nanoparticles, proved as potential entities with enhanced antimicrobial and anticancer activity in the presence of suitable wavelength of light (Masilela and Nyokong, 2012). In addition, the use of nanoparticles as potential vehicles for photosensitizers became one of the intrigued materials for PDT (Bechet et al., 2008). In the case of polymeric organic nanoparticles, their mechanisms of action are assumed that either the cationic groups are able to disrupt the bacterial cell membrane with some hydrophobic chains of certain lengths to penetrate and burst the bacterial cell membrane, or high levels of positive charges of nanoparticles, a function of pH, are capable of conferring antimicrobial properties irrespective of hydrophobic chain length. Thus, it is believed that the cell death is due to an ion exchange mechanism between the bacterial cell membrane and the charged surface of the nanoparticles (Fig. 7.2). The antibacterial effect of polycations is dependent on the ability of multiple charges for attaching and interacting with the cell membrane. These findings suggest the possibility of engineering a variety of polymer-based positively charged surfaces to create a wide variety of contact-killing materials (Lichter and Rubner, 2009). As an example, the antibacterial mode of action of polysaccharide

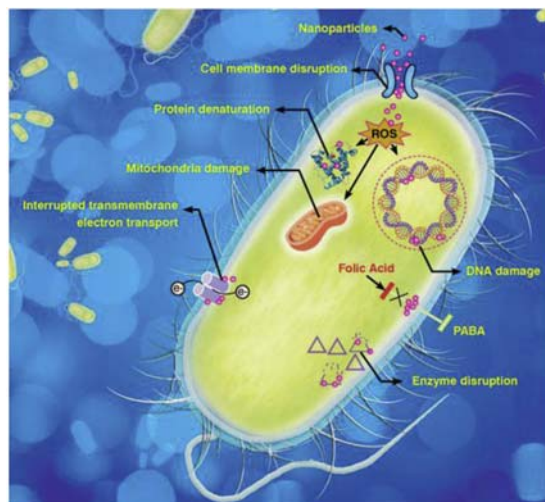


FIGURE 7.2

Probable mechanisms of nano-antibacterials.

Source: From Nanowerk Spotlight, Sep. 10, 2013.

chitosan is due to the interaction of lipopolysaccharides with chitosan, resulting in the destabilization of membrane proteins and membrane lysis, leading to cell death (Rabea et al., 2003a). These surface charges of the polymeric organic nanoparticles could be tuned by changing the pH as a stimulus of the environment.

7.4 TYPE OF ANTIMICROBIAL NANOPARTICLES

The emergence of nanoparticle-based technology became prominent with the development of a new generation of promising broad-spectrum antimicrobial nanoparticle agents, due to their extraordinary physiochemical and functionalization properties to unravel the limitations experienced by traditional antimicrobial agents. The literature mainly includes the inorganic, organic, and hybrid antimicrobial nanoparticle agents. To the best of our knowledge, however, metals and metal oxides of zinc, silver, TiO₂, and copper in their bulk form (Loomba and Scarabelli, 2013) are active antimicrobial agents, but their corresponding nanoparticles exhibit potent antibacterial effect at nano dimensions (Seil and Webster, 2012). One of the metal nanoparticles widely used as an effective antimicrobial agent against bacteria, fungi, and viruses is silver nanoparticles, which show superior antimicrobial effect against various pathogens, in vitro and in vivo, to those equivalents of larger diameter bulk (Kemp et al., 2009). Titanium dioxide (TiO₂) nanomaterials, in addition to the important antimicrobial activities toward Gram-positive and Gram-negative bacteria are also effective against most resistant microorganisms, including *Bacillus*. Broad-spectrum bactericidal, ZnO-based nanoparticles (Sirelkhatim et al., 2015) exhibit a wide range of antimicrobial activity against various Gram-negative and Gram-positive microorganisms.

Although Fe₃O₄ and Au in their bulk form are inert and found to lack antimicrobial properties, at nanoscale Fe₃O₄ demonstrates antiadherent properties and significantly reduces both Gram-negative and Gram-positive bacterial colonization (Anghel et al., 2014). Similarly, Au-derived nanoparticles and nanorods have been reported as bactericidal when they were appropriately functionalized for photothermally enhanced PDT and found to bind to different antibiotics, such as ampicillin, vancomycin, antibacterial enzyme lysozyme, and penicillin (Norman et al., 2008). Pt-based nanomaterial particles of 1–3 nm size were bactericidal to *Pseudomonas aeruginosa* cells, whereas those of 4–21 nm size exhibited bacterio-compatible properties like the antimicrobial activity of CuO, ZnO, MgO, and MgF₂ (Chen et al., 2010; Ren et al., 2009; Baek et al., 2011). It is common that the inorganic nanomaterials as antibacterial agents have been found in medicinal fields, but their toxicity and less bioavailability are pushing the development of organic nanoparticles forward (Li et al., 2012), which would have higher biodegradability, biocompatibility and bioavailability, chances of surface modifications, and control for targeted drug delivery and release to eradicate affected cells while sparing normal cells.

The loading of therapeutics into nanoparticles by different means, such as physical adsorption, encapsulation or chemical conjugation improved their pharmacokinetics and therapeutic index. In addition to these benefits, nanoparticle-assisted drug delivery has been recognized as more advantageous in different aspects, including serum solubility, lifetime, and distribution to the tissues and cells of interest of the drug candidates. In another examples, polymeric nanoparticles eradicate microorganisms either by releasing antibiotics, antimicrobial peptides, and antimicrobial agents or by contact-killing cationic surfaces, such as quaternary ammonium compounds (Waschinski and Tiller, 2005), alkyl pyridiniums, or quaternary phosphonium. Poly- ϵ -lysine is a cationic homopeptide of L-lysine, which is effective against Gram-positive and Gram-negative bacteria. It also displays activity against spores of *Bacillus coagulans*, *Bacillus stearothermophilus*, and *Bacillus subtilis* (Hiraki, 1995). Polyimidazole-based derivatives are another family of antimicrobial polymers, consisting of aromatic/heterocyclic groups (Anderson and Long, 2010). Polymeric nano-sized antimicrobial agents, which are nonvolatile and chemically stable, are known to have long-term antimicrobial activity (Kenawy et al., 2007). Similarly, lipid nanoparticles are attractive for their biocompatibility, versatility, and their ability to target biofilm infections. Polycationic chitosan nanoparticles have broad-spectrum antibacterial, antiviral, and antifungal activity (Lee et al., 2014). Chitosan nanoparticles display considerable antibacterial activity, which depends on several factors, including pH and solvent (Rabea et al., 2003b).

7.5 EFFECT OF STIMULI ON DRUG DELIVERY

In order to produce their desired pharmacological action, drugs or pro-drugs must cross multiple biological obstacles *viz.* intestinal barrier, metabolic reaction, etc. before reaching the desired site of action. Oral drugs require optimum stability in the region of the gastrointestinal (GI) tract and to penetrate intestinal epithelium easily for achieving high systemic bioavailability (Alonso, 2004). Similarly, drug delivery through skin, nasal, and pulmonary routes requires efficient transport of drugs across the epithelium. While most of the low molecular weight drugs (<900 Da) are delivered through oral administration, for drugs comprising peptides, proteins, large molecules, and polynucleotides, intravenous (IV) administration remains the most direct and efficient route to deliver drugs. An additional barrier is the blood–brain barrier (BBB), which restricts the diffusion of large or hydrophilic molecules into the cerebrospinal fluid and is a major obstacle for the treatment of most central nervous system and brain disorders. Multiple nanoparticle-based strategies, including liposome, nanosphere, and cationic albumin nanoparticles are under development to deliver drugs across the BBB (Silva, 2008).

Even after successful administration, many drugs can cause cell death or induce a significant change in cell behavior, toxicity to normal cells thereby undermining their use. The design of unique nanoscale stimuli-responsive systems that are able to control drug biodistribution in response to specific stimuli, either exogenous (variations in temperature, magnetic field, ultrasound intensity, light, or electric pulses) or endogenous (changes in pH, enzyme concentration or redox gradients), are the key advances in the field of stimuli-responsive nanomaterials (Mura et al., 2013). To further improve the therapeutic efficacy of antimicrobial nanoparticles, researchers are sincerely involved in exploring stimuli-responsive nanoparticles that are supposed to remain inactive until they are triggered by cues found in the microenvironment of infection sites.

7.5.1 EFFECT OF TEMPERATURE AND PH ON ANTIMICROBIAL NANOPARTICLES

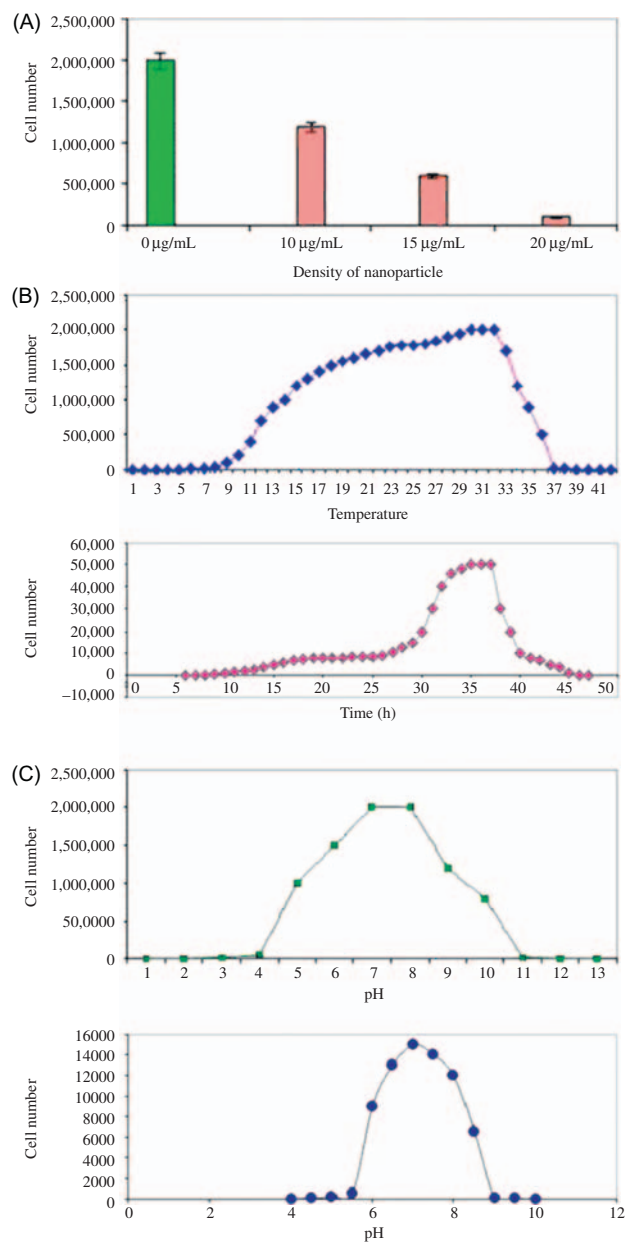
The temperature and pH of the environment have a great impact on the efficacy of antimicrobial nanoparticle agents. As per literature precedents, the pH of the medium plays an important role in controlling the shape and size of nanoparticles (Bae et al., 2007). In the biological system, the pH gradient along the GI tract (Kararli, 1995) and the acidic environment of human skin (Schmid-Wendtner and Korting, 2006) affects site-specific antibiotic delivery. At the intracellular level, nanoparticles have been formulated to respond to the acidic pH inside the endolysosomal compartments for triggered drug release (Simões et al., 2001; Imbuluzqueta et al., 2010; Chu et al., 1990). In general, bacterial infections are characterized by very low pH values because of anaerobic fermentation and subsequent inflammation. In this regard, systemic antibiotic therapy was achieved by localized acidity-promoted surface charge switching at the sites of interactions with the negatively charged bacterial cell wall; it led to increasing nanoparticle uptake in both Gram-positive and Gram-negative bacteria. For example, tri-block copolymer nanoparticles composed of poly(lactic-co-glycolic acid) (PLGA), poly-L-histidine, and poly(ethylene glycol) (PEG) have been reported for acid-responsive antibiotic delivery (Radovic-Moreno et al., 2012). These nanoparticles maintained a negative charge at neutral pH; however, when they are exposed to an acidic pH, the protonation of the imidazole groups switched the surface charge to a positive one, resulting in enhanced bacterial binding and improved antibacterial efficacy.

Conversely, the effects of calcination temperature on the medicinal properties of nanoparticles are also well elaborated in the literature. Generally, the antibacterial properties of nanoparticles decrease with increasing the calcination temperature, which directly affects the surface morphology, crystallinity, optical properties, and size of nanoparticles. With an increase in temperature, the grain size of nanoparticles increases due to aggregation at a higher temperature through interparticle electrostatic interactions. Recently, Pourali et al. (2013) reported the effect of

temperature on the antimicrobial activity of biosynthesized silver nanoparticles from *Bacillus* sp. and *Acinetobacter schindleri*, capped with proteins. The antibacterial assays of the samples were carried out before and after heat treatment at 100 and 300°C, which reflects that high temperatures reduced the antimicrobial properties of thermal-treated silver nanoparticles due to loss of the capping proteins and their antiagglomeration tendency. The antibacterial assays of ZnO nanoparticles dispersed in glycerol using ammonium citrate as the dispersant, were evaluated by [Saliani et al. \(2015\)](#), through measuring the growth of *E. coli* and *Staphylococcus aureus* at different concentrations of ZnO-NPs as well as at different incubation temperatures (25–42°C) and pH levels (4–10 for *E. coli* and 5–10 for *S. aureus*). The results showed that, with increasing ZnO nanofluid concentration, the inhibitory effect increases against *E. coli* and *S. aureus* and the highest activity with the maximum toxicity was observed at pH = 4 and pH = 5 for *E. coli* and *S. aureus*, respectively. With the rise in temperature, the toxicity of ZnO nanofluid increased and the highest antibacterial activity was observed at 42°C. [Kuthati et al. \(2015\)](#) described the synthesis of stable silver-immobilized IBN-4 nanoparticles with pH sensitive hydrazine, although it was nonreleasing at pH 7.5. The pH that triggers silver release from IBN-4 nanoparticles was tested against *Staphylococcus epidermidis*, *S. aureus*, and *E. coli*; the activity was more severe against Gram-negative *E. coli* cells than *B. subtilis* cells.

The antibacterial activity of biosynthesized face-centered cubic (fcc) crystalline silver nanoparticles using *Bacillus brevis* by the extracellular method, was evaluated against *E. coli* by [Chitra and Annadurai \(2014\)](#), with various concentrations (10, 20, 30, 40, and 50 µL) and different pH (5, 7.2, and 9). The smallest nanoparticles synthesized using pH 9 showed more antibacterial activity than large particles, which were synthesized using pH 7.2 and 5. The polysaccharides ([Lin et al., 2009](#)), heparin, and chitosan as base-sensitive nanoparticles for treating gastric pathogens, such as *Helicobacter pylori*, are self-assembled to form nanoparticles at pH 1.2–2.5. However, upon contact with *H. pylori* at the gastric epithelium with physiological pH, the chitosan deprotonated, causing nanoparticle disassembly and release of drugs for bacteria killing. [Salehi et al. \(2014\)](#) studied the antibacterial effects of cadmium oxide nanoparticles on *S. aureus* at the concentrations of 10, 15, and 20 µg/mL at the variation of pH and temperature. Enhancement of antimicrobial activity was observed with an increase in nanoparticle concentration, and thereby reduction in bacterial growth rate. The minimum temperature required for *S. aureus* bacteria growth is 8°C, the maximum temperature is 45°C and the optimum temperature is between 35 and 37°C. With the double effect phenomenon of both nanoparticle and temperature conversions, a significant deterioration ($P < 0.05$) emerged in the number of bacteria cells. It is found that the minimum required pH for the growth of *S. aureus* bacteria is 4.5 and the maximum is 9.3, while the optimum pH is between 7 and 7.5 ([Fig. 7.3](#)).

A pH-responsive copolymer P(MMA-co-MAA) hydrogel was synthesized from methyl methacrylate (MMA) and methacrylic acid (MAA) as monomers and assembled to Ag-nanoparticles ([Qing-Bo et al., 2014](#)). The P(MMA-co-MAA)/

**FIGURE 7.3**

(A) The inhibitory effect of cadmium oxide nanoparticles on *S. aureus* bacteria (B) the temperature effect on *S. aureus* in the presence of cadmium oxide and in broth (C) the pH effect on *S. aureus* in the presence of cadmium oxide and in broth.

silver nanocomposite hydrogels revealed higher antibacterial efficacy against both *S. aureus* and *B. subtilis* bacteria than the pure P(MMA-co-MAA) counterparts. Two new biopolymer nanoparticles, chitosan-co-PNIPAAm, and alginate-co-PNIPAAm (Hill and Gomes, 2014) were also obtained via self-assembly and top-down approach using poly-*N*-isopropyl acrylamide (PNIPAAm) with glutaraldehyde or calcium chloride cross-linking. The chitosan-co-PNIPAAm nanoparticles at the same pH and temperature showed higher bacterial inhibitors in delivering a natural antimicrobial cinnamon bark extract (CBE), a hydrophobic antibacterial, with controlled release against food-borne pathogens at lower ($P < 0.05$) concentration than free CBE and faster release of CBE over time as well as slightly higher entrapment efficiency. The alginate-co-PNIPAAm nanoparticles were significantly smaller and exhibited a slow and gradual release over a long time period.

The chitosan/ γ -PGA nanoparticles incorporated into the alginate–gelatin hydrogels were developed as an efficient carrier for an antibiotic drug, amoxicillin delivery (Chang et al., 2010). The hydrogels are pH-sensitive, leading to protection of the nanoparticles from the destruction of amoxicillin, which interact specifically with the intercellular spaces or with the cell cytoplasm, the site of *H. pylori* infection by gastric acid. The amoxicillin-loaded nanoparticles had a particle size of 149.6 (6.3 nm) and a zeta potential of 18.9 (3.1 mV), while their amoxicillin loading efficiency was 23.5 (2.7%); the stability of the amoxicillin-loaded nanoparticles at different pH values was similar to those of unloaded nanoparticles.

7.5.2 EFFECT OF LIGHT ON ANTIMICROBIAL NANOPARTICLE

Light-promoted treatments of a wide variety of diseases have been well-known for a long period of time, known as PDT. In this context, Niels Finsen was awarded Nobel Prize in 1903 in recognition of his contribution to the treatment of diseases, especially lupus *vulgaris*, with concentrated light radiation, whereby he has opened a new avenue for medical science (Finsen, 1901). Synergistic with nanoparticlar pharmacodynamic and pharmacokinetics properties, light can influence the antibacterial property with high impact. Although the photoactive behavior of inorganic compounds is well known, organic molecules also show photoactive properties. For example, porphyrins and PC as photosensitizer in PDT (Shakiba et al., 2015; Yang et al., 2013; Yavuz et al., 2009; Schumers et al., 2010) produced ROS, in particular, $^1\text{O}_2$ on exposure to light energy in the presence of triplet oxygen (Ochsner, 1997). This is because of the their extended conjugated electronic structure, large molar absorption extinction constant, and adequate fluorescent quantum yields, which make them suitable for PDT study. The enhanced activities of PDT of nanoparticles are observed due to combining effect of both with improved delivery of the photosensitizer to the target microorganism and an enhanced activity of the photosensitizer. Applications of PDT for the classic antiviral, antifungal, antiprotozoal drugs (Carvalho et al., 2009) and

inactivation of disease-causing microbes in water are one of the interesting and attractive additional approaches (Carvalho et al., 2007). Perni et al. (2009) described light-activated antimicrobial polysiloxane nanoparticles made from a simple swell–encapsulation–shrink method using methylene blue (MB) and nanogold. The antimicrobial activity against Methicillin-resistant staphylococcus aureus (MRSA) and *E. coli* with up to a 3.5 log10 reduction in the viable count when it was exposed for 5 min to laser light irradiation under low power 660 nm and short pulses (10 s) at the catheter entry site. The activity is due to the light-induced production of ROS and other ROS by the MB. Interestingly, the presence of 2 nM gold nanoparticles significantly enhanced the ability of the MB to kill bacteria. Chong et al. (2012) reported water-soluble red-emissive conjugated polymer nanoparticles containing fluorene and boron-dipyrromethene repeat units in the backbones of cationic fluorinated hydroxytelechelic polybutadiene (PBF) in the presence of negatively charged disodium salt 3,3'-dithiodipropionic acid with the size of about 100 nm. Upon photoexcitation with white light (400–800 nm), PBF-nanoparticles can sensitize the oxygen molecule to readily produce ROS for rapidly killing neighboring bacteria and cancer cells. At a light intensity of $45 \text{ mW}^3/\text{cm}^2$ ($108 \text{ J}/\text{cm}^2$) and $90 \text{ mW}^3/\text{cm}^2$ ($216 \text{ J}/\text{cm}^2$), 60% cell killing and 90% bacterial reduction was observed, respectively. Liu and Yang (2003) reported that application of photoactivated ZnO nanoparticle for 10 min incubation with $1 \times 10^{-3} \text{ M}$ and $173 \text{ kJ}/\text{m}^2$ illumination dose reduced Gram-positive *Listeria* and Gram-negative *E. coli* to 3 and 4.7 log10, respectively, although the *Listeria* was more resistant in the presence of light than in the dark. Furthermore, the Gram-positive *B. subtilis* in the presence of visible light was much more sensitive to ZnO nanoparticles than Gram-negative *E. coli* (Fig. 7.4). PLGA nanoparticles loaded with the photosensitizer MB for in vitro evaluations against *Enterococcus faecalis* were experimented by Pagonis et al. (2010). After incubation of colloidal gold particles with PLGA complex for 2.5, 5, and 10 min, and treatment with red light of 665 nm ($60 \text{ J}/\text{cm}^2$), the synergism of MB-based nanoparticles and light showed the effects in the reduction of the colony-forming units (CFUs) in

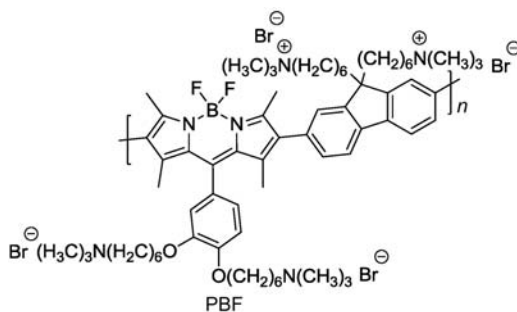


FIGURE 7.4

Fluorene and boron-dipyrromethene conjugate.

planktonic phase and root canals, and it was significantly lower than controls and MB-loaded nanoparticles without light.

The platinum-loaded titania (TiO_2/Pt) photocatalyst has superior antimicrobial properties against soil-borne pathogenic microorganisms. The antibacterial property of the TiO_2 –Pt against nosocomially infected pathogens *S. aureus*, and *Acinetobacter baumannii*, and exotoxin-producing *Streptococcus pyogenes* was reported by Tseng (Tseng et al., 2013). These nanoparticles under visible light irradiation demonstrate the enhanced bactericidal property against human pathogens (~ 2 log reduction), compared to several other available photocatalysts ST01, P-25, C200, BA-PW25 etc. (~ 0 –1 log reduction under visible light). Anehosur et al. (2012) observed that 3 wt% of TiO_2 in TiO_2 nanoparticles with polymethyl methacrylate denture-based resin shows antimicrobial activity against *S. aureus* under visible light. Titanium dioxide with PMMA contributes greater improved denture hygiene than solar energy/light.

Under UV light irradiation ($k < 390$ nm), the antibacterial activity of Ag– $\text{TiO}_2/\text{Ag/a-TiO}_2$ nanocomposite thin film (Akhavan, 2009) against *E. coli* bacteria was 5.1 times stronger than amorphous titanium oxide (a- TiO_2) in the dark. Photo-antibacterial activity of the nanocomposite film exposed in the presence of the solar light was 1.35 and 6.90 times better than the activity of the Ag/a- TiO_2 and a- TiO_2 , respectively, due to release of Ag^+ ion. The main mechanism for silver ion releasing was the interdiffusion of water and silver nanoparticles through pores of the TiO_2 layer; the durability of nanocomposite film was at least 11 times higher than the Ag/a- TiO_2 film.

Visible light-induced photo inactivation of *E. coli* bacteria of carbon nanotube (CNT)-doped TiO_2 thin films was found to be optimum for 20 wt% CNT content (Akhavan et al., 2010). The visible light-induced photo inactivation of the bacteria increased from MWNTs to TiO_2 , to $\text{TiO}_2/\text{MWNTs}$ annealed at 650°C in which the bacteria could even slightly breed on the MWNTs. In addition, the $\text{TiO}_2/\text{MWNTs}$ annealed at 400°C showed a highly improved antibacterial activity than at 100°C because, at 400°C , carbonaceous bonds form at the heterojunction arrays, whereas there is no activity at 100°C .

The core-shell structured $\text{In}_2\text{O}_3@/\text{CaIn}_2\text{O}_4$ substrate has shown superior visible light-induced bactericidal activities in comparison to several other visible light-responsive photocatalysts, but less than TiO_2 . The high performance is enhanced by more easily photoexcited electron transfer between the interfaces of In_2O_3 and CaIn_2O_4 to reduce the electron–hole recombination at the time of photocatalysis. However, in comparison to TiO_2 -based photocatalysts, $\text{In}_2\text{O}_3@/\text{CaIn}_2\text{O}_4$ -based treatments inhibit significant cell death and tissue damages, leading to a superior biocompatibility (Chang et al., 2012). The higher biocompatibility of $\text{In}_2\text{O}_3@/\text{CaIn}_2\text{O}_4$ was experienced by less fluctuation in platelet and white blood cell (WBC) counts during the treatment of mice with $\text{In}_2\text{O}_3@/\text{CaIn}_2\text{O}_4$.

Under ultraviolet A (UV-A) irradiation, the bactericidal effect of TiO_2 photocatalyst on antibiotic-resistant and antibiotic-sensitive bacteria (Tsai et al., 2010) *S. aureus* (MRSA/MSSA), *A. baumannii* (MDRAB/MDSAB), *E. faecalis*

(VRE/VSE), *E. coli* and the bacteriophage MS2 showed that MSSA and MRSA were equally susceptible, and the susceptibility of MDRAB was doubled that of MDSAB ($P < 0.05$). The susceptibility of VSE was 2.4 times that of VRE ($P < 0.05$). In the presence of UV-A, TiO₂ effectively reduces the number of antibiotic-resistant microbes in the CFU in a suspension by 1–3 logs.

The hybrid microspheres (PMS and TiO₂-HMS) made by the TiO₂ nanoparticles and polystyrene were investigated against *E. coli* under the low-intensity UV light and in the dark (Jiang and Zeng, 2010). The percentage of killing by PMS in the dark and under UV light of *E. coli* is around 5.7% and 10.5%, respectively. Surprisingly, after only 2 h, the hybrid nanoparticle killed more than 95% of the bacteria under UV light; even in the dark, more than 50% of the bacteria are killed.

Kong et al. (2010) developed TiO₂/poly[2-(*tert*-butylamino)ethyl methacrylate co-ethylene glycol dimethacrylate core-shell nanoparticles as surface-initiated photopolymerization using titania as an initiator. The higher photocatalytic antibacterial activity of this nanocomposite is observed, compared to that of pristine TiO₂ nanoparticles, due to the combined antibacterial activities of light-driven anti-infective TiO₂ core and a biocidal polymer shell. Even in the dark conditions, these nanoparticles exhibited high antimicrobial efficiency (~95%) against Gram-positive *S. aureus*. Under UV irradiation, the TiO₂/biocidal polymer showed improved inhibition of bacterial growth against Gram-negative *E. coli* and Gram-positive *S. aureus* in comparison to TiO₂ nanoparticles. The photocatalytic and antibacterial property of silver-impregnated TiO₂/nylon-6 nanocomposite mats was tested against *E. coli* (Pant et al., 2011). The result showed good photocatalytic and antimicrobial properties (even in the absence of UV light), excellent recyclability, and stability for potential applications in environmental remediation. The mechanism involves photoexcited electrons transferring from the surface of the Ag NPs to the conduction band of TiO₂, which is trapped by O₂ molecules and other species (e.g., O₂^{•-}, [•]OOH, [•]OH) to oxidize the bacterial organic species.

The minimum bactericidal concentration of the covalently coupled toluidine blue O (TBO)—tiopronin gold conjugate for *S. aureus* was at least four times lower than that of free TBO. Under both 632.8 nm laser and white light, the activity of the gold conjugates significantly exceeded than TBO (Gil-Tomás et al., 2007). For example, at 1 mM concentration under white light for 30 min, the conjugate showed a reduction in CFU of 4.5 log₁₀, compared with 0.5 log₁₀ for TBO at the same concentration. Regardless of light, the minimum bactericidal concentration (MBC) of the conjugate was 0.5 mM, compared to 2 mM for TBO.

Light-activated polymethylmethacrylate doped with porphyrin and silver nanoparticles were tested against different bacteria and exhibited weak antimicrobial response against Gram-negative bacteria (Table 7.2) (Lyutakov et al., 2014). Antimicrobial properties were realized through light-controllable silver release and production of active oxygen from porphyrin. Table 7.2 provides the antimicrobial properties (to (1) *P. aeruginosa* and (2) *S. aureus*) of different coatings

Table 7.2 Antibacterial Activity of Light-Activated Polymethylmethacrylate Doped with Porphyrin and Silver Nanoparticles; Antimicrobial Impact is Designated as Follow: (–) None; (±) Weak; (+) Apparent

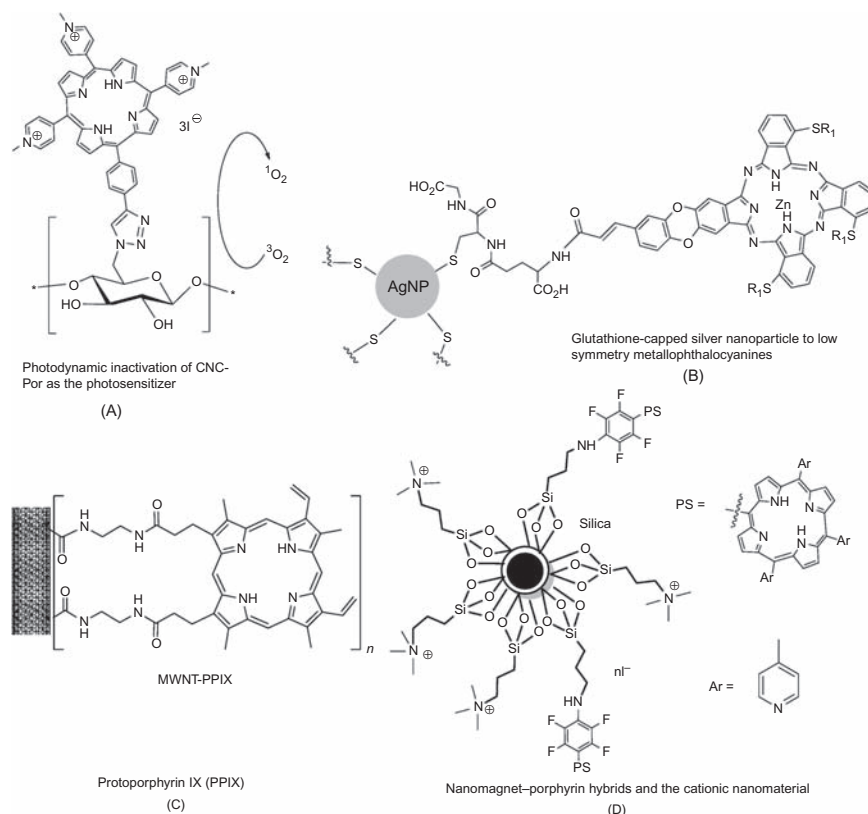
	Under Illumination			Without Illumination		
<i>P. aeruginosa</i>						
Time (min)	30	60	90	30	60	90
AgNO ₃	+	+	+	+	+	+
AgNPs	±	±	+	+	+	+
TPP	–	–	±	–	–	–
AgNPs + TPP	±	+	+	–	–	–
<i>Paradoxurus aureus</i>						
Time (min)	30	60	90	30	60	90
AgNO ₃	+/	±	+/	±	+/	±
AgNPs	–	–	–	–	–	–
TPP	–	±	+	–	–	–
AgNPs + TPP	+	+	+	–	–	±

“in solution” after different times of treatment without/with illumination. It is also observed that silver nanostructure-coated TiO₂, carbon-doped TiO₂ [TiO₂(C)] and nitrogen-doped TiO₂ [TiO₂(N)] thin films contain a high visible-light responsive antibacterial property (Wong et al., 2010). The synergistic antibacterial assay resulted in approximately 5 log reductions of surviving bacteria of *E. coli*, *S. pyogenes*, *S. aureus*, and *A. baumannii*. After photocatalysis (1.2×10^3 lux, 10 min), surviving *E. coli* (CFU) on respective substrates was shown $P < 0.01$, compared with respective Ag/TiO₂ and Ag/TiO₂ (C) groups.

A rod-shaped covalently linked porphyrin-cellulose based nanocrystal (Fig. 7.5A) with an average length of 100–400 nm was developed by Ghiladi and groups (2011). Initially, the covalent conjugation with cellulose materials improved the lifetime or antibiotic activities while conversely, it simultaneously reduces the penetration of the biocidal agent into the environment and aggregation of the nanomaterials. The efficacy of photocatalytic inactivation of *Mycobacterium smegmatis* and *S. aureus* is excellent in contrast to *E. coli*.

Rapulenyane et al. (2013) prepared a hybrid of glutathione-capped silver nanoparticles (GSH-AgNPs) and zinc phthalocyanine to produce nanoconjugate (Fig. 7.5B) showing the antimicrobial activity against *E. coli*, even in the absence of light. These nanoparticles also proved to be potential bactericides in reducing light toxicity in the range of 5–10 mM.

A porphyrin-CNT conjugate MWNT-PPIX conjugates (Fig. 7.5C) as nanocomposite films for the immobilization of antimicrobial agents were synthesized by Mondal and coworkers (2010) from acid-functionalized multi-walled carbon nanotubes (MWNTs) and protoporphyrin IX (PPIX) as an active antimicrobial

**FIGURE 7.5**

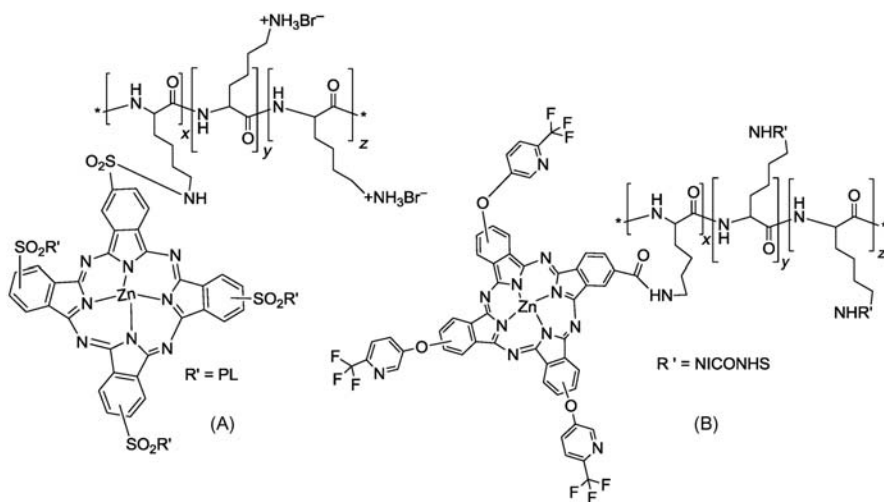
Porphyrin-cellulose based nanocrystal (A), Zn-phthalocyanine and glutathione-capped Ag-nanoparticles (GSH-AgNPs) (B), MWNT-PPIX conjugates (C), and nanomagnet-porphyrin hybrids (D).

agent for Gram-positive *S. aureus* cells in small quantities and in very short time scale. In continuation, [Mondal and coworkers \(2012\)](#) also exploited the effectiveness of MWNT-PPIX conjugate as a photosensitizer for antiviral activity against Influenza A virus-infected mammalian cells. The MWNT-PPIX showed excellent efficacy against influenza viruses with slight or no chance of developing resistance to the treatment ([Fig. 7.5](#)).

To fight against microbial contamination of aqueous contents, [Carvalho et al. \(2010\)](#) developed a cationic nanomagnet-porphyrin hybrid ([Fig. 7.5D](#)) as effective stable hybrid nanomaterial, causing a total photo-inactivation of *E. coli*, T4-like phage and *E. faecalis* on exposure to white light of 21.6, 43.2, and 14.4 J/cm², respectively, at 20 μM. Accordingly, these materials could find application as novel photosensitizers for water or waste water disinfection ([Fig. 7.5](#)).

Table 7.3 *S. aureus* Photo Inhibition at Fluence Rate of 39.6 mW/C m² for 10 min Exposure in Light at MIC₅₀ Conc. for (a) and (b) in pH 7.4

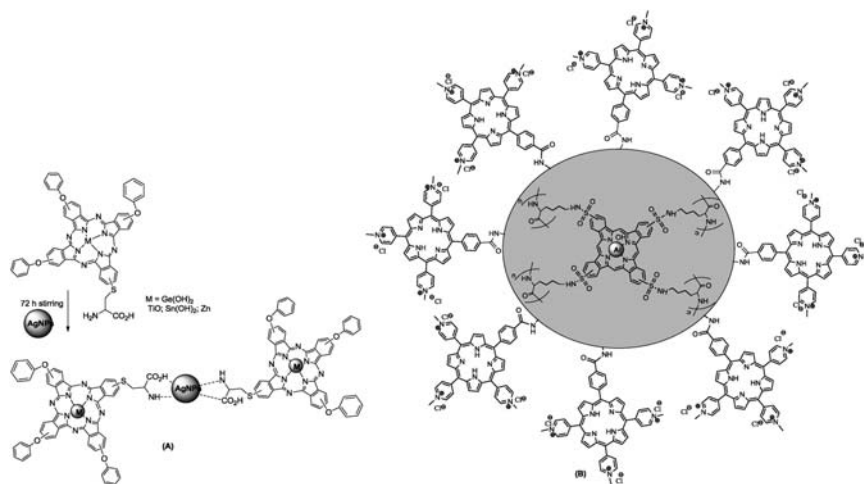
Compound	MIC ₅₀ (μM) Dark	MIC ₅₀ (μM) Light	MIC ₅₀ (μM) with AuNPs	MIC ₅₀ (μM) with AgNPs
(a)	0.75	0.187	0.046	0.0058
(b)	0.375	0.046	<0.046	<0.0058

**FIGURE 7.6**

Zn-phthalocyanines conjugate.

Whereas [Nykong et al. \(2012\)](#) explored the effectiveness of PC as PDT candidate with efficient absorption coefficient ($\mu > 105 \text{ M}^{-1}/\text{cm}$) in the range of 600–800 nm. In this literature, the Zn-phthalocyanine (ZnPC)- ϵ -polylysine (ϵ -PL) conjugates as photodynamic sensitizers are synthesized for the inactivation of *S. aureus* through a defense mechanism. The MIC of the conjugates against *S. aureus* exhibited that the presence of Ag-nanoparticles reduces the expression of the microorganism for both porphyrin conjugates (a) and (b). In addition, it was also observed that Ag-nanoparticle conjugated to (b) is more effective photosensitizer compared to (a) in the PDT against *S. aureus* at 39.6 mW/cm² ([Table 7.3](#)). It was proposed that nanoconjugate (b) might have proper adhesion because of the presence of polylysine in the cell membrane that is sufficient for light-activated destruction of the bacterium ([Fig. 7.6](#)).

Recently, the same research group, reported ([Masilela et al., 2013a](#)) metallophthalocyanines (MPCs) with the core metal atoms Sn, Cu, Zn, and Ti with the PC ring ([Fig. 7.7](#)). The monocysteiny l phthalocyanines (MCsPc) (OH)₂GeMCsPc,

**FIGURE 7.7**

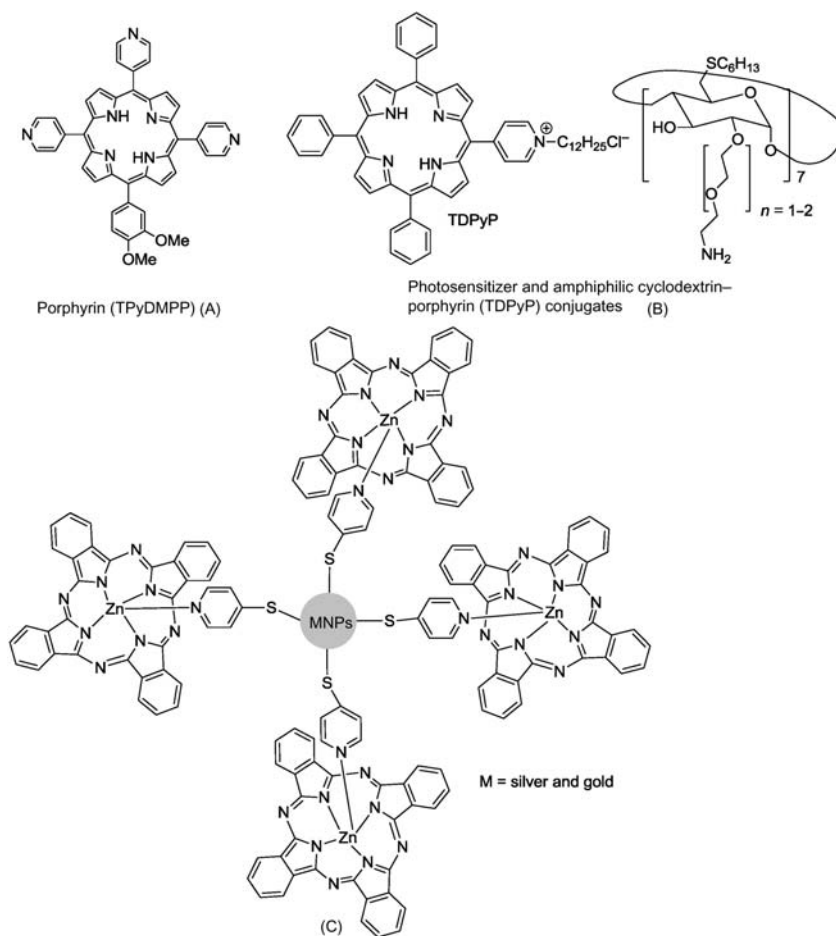
Phthalocyanine-based nanoparticle as photosensitizer (A) and polymer-assisted tetrasulfonato-aluminum phthalocyanine-porphyrin conjugated nanoparticles (B).

SnMCsPc, ZnMCsPc, and OTiMCPc, (monocarboxy phthalocyanine), were synthesized comprising carboxylic acid and an amino group for the coordination with the Ag and Au-nanoparticles. The antibacterial activity of the (OH)₂GeMCsPC showed maximum in the presence and the absence of AgNPs. Whereas, the antibacterial property of the AgNPs showed in an increasing order as follows: cubic < triangular < spherical, even in the absence of MPCs. The antibacterial activities of the MPCs exhibited as SnMCsPC < OTiMCsPC < ZnMCsPC < (OH)₂GeMCsPC in both absence and presence of AgNPs.

The catpolyacrylamide nanoparticles with size 95 ± 10 nm act as delivery materials in PDT for cancer and for photodynamic antimicrobial chemotherapy, as described by Kuruppuarachchi et al. (2011). Polymer-templated tetrasulfonato-aluminum phthalocyanine-porphyrin conjugated nanoparticles exhibited absorbance signals at 424 nm (soret band) and 680 nm (Q-band) for porphyrin and PC, respectively.

In 2013, Creanga et al. (2013) presented the formation of ovoid, disk-shaped, and spherical Ag-porphyrin as colloidal nanoparticles (Fig. 7.8A) with sizes of 65–120 and 30–49 nm as antimicrobial and photosensitizers in the field of PDT and a photovoltaic cell. Ferro et al. (2009) have designed the usage of cationic amphiphilic cyclodextrins as nanoaggregates (Fig. 7.8B) in PDT and antimicrobial agents for methicillin-resistant *S. aureus* (MRSA) and *E. coli*. During experimentation, it is revealed that the conjugation of porphyrin unit increases the photobactericidal activity.

In this area, Masilela reported that bis-(1,6-hexanedithiol) silicon phthalocyanine and Zn-phthalocyanine conjugated to Ag- and Au-nanoparticles improved the photo-physicochemical and antimicrobial properties for both complexes. In this report, the

**FIGURE 7.8**

Some photosensitizers for antimicrobial activity.

Si-phthalocyanine derivative produced better bioactivity in contrast to the zinc phthalocyanine counterpart against different pathogens with light. The zinc phthalocyanine conjugate showed the maximum singlet and triplet oxygen quantum yield in the presence of Au-nanoparticles (Fig. 7.8C) (Masilela et al., 2013b).

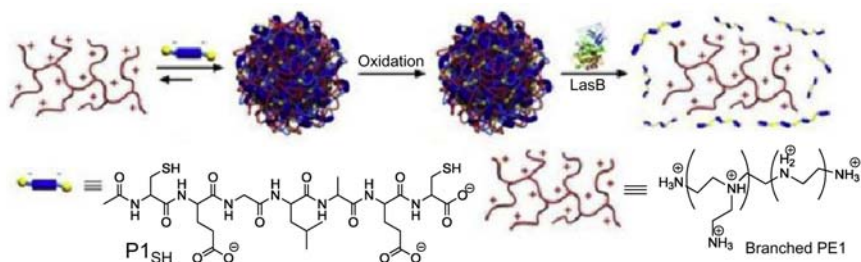
7.5.3 EFFECT OF THE ENZYME ON THE ANTIMICROBIAL NANOPARTICLE

Because of increasing concern for resistant microbial strains, new antimicrobial strategies are required to go beyond the development of new antibiotics, which are limited due to high production cost. In spite of this, the re-evaluation of old

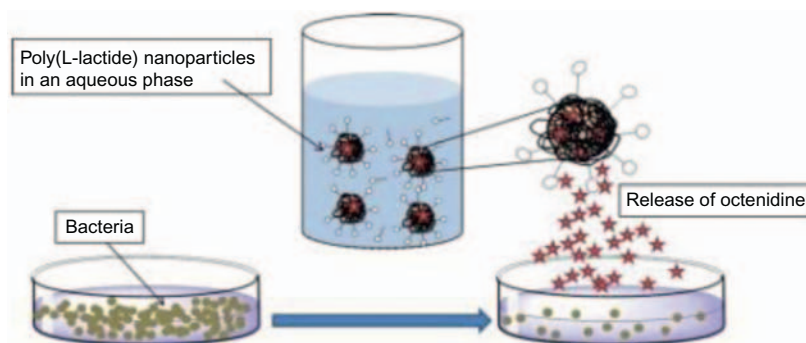
drug candidates that have been left due to their inactivation by enzymes and adverse effects, could maintain the pool of antibiotics in the market. To that end, the introduction of nonconventional delivery strategies of developing enzyme-responsive nanoparticles that will selectively deliver antimicrobial agents in the presence of the pathogen, will eradicate their infections. It can be a very attractive alternative to improve the medical profile of current antimicrobials by this strategy that can deliver antimicrobials at the site of infection to reduce off-target effects, such as toxicity or the emergence of reservoirs of microbial resistance genes in nonpathogenic strains, such as commensal bacteria. Accordingly, in addition to pH gradient, bacterial enzymatic activities, including those of secreted toxins, have also been used to trigger the release of antimicrobial agents to inhibit the growth of the target bacteria (Xiong et al., 2012). Xiong reported a lipase-sensitive polymer-bound triple-layered nanogel (TLN), a bacterial lipase-sensitive poly(ϵ -caprolactone) (PCL) interlayer between the cross-linked polyphosphoester core and the shell of PEG (Fig. 7.1). The antibiotics are protected inside the polyphosphoester core and showed a sustained-release due to the compacted PCL molecular fence, eliminating potential adverse side effects because of premature drug leakage or nonspecific drug release. However, the PCL fence of TLN is subject to degradation by the activity of bacterial lipases, which are abundant in microbial flora, since these enzymes are involved in bacterial lipid metabolism. Using *S. aureus* as the model bacterium and vancomycin as the model antimicrobial, they demonstrated that the TLN released almost all the encapsulated vancomycin within 24 h only in the presence of *S. aureus*, significantly inhibiting *S. aureus* growth. The TLN further delivered the drug into bacteria-infected cells and efficiently released the drug to kill intracellular bacteria. Insua et al. (2016) presented an enzyme-responsive polyion complex (PIC) nanoparticle, which was prepared from antimicrobial poly(ethylene imine) and an anionic enzyme-responsive peptide. These enzyme-responsive PIC nanoparticles are able to selectively deliver the antimicrobial peptides in the presence of *P. aeruginosa elastase* without being affected by other endogenous nonpathogenic strains of these bacteria and can be a powerful tool against AMR (Fig. 7.9).

Thamphiwatana et al. (2014) reported the absorption of chitosan-modified gold nanoparticles (AuChi) onto the surface of liposome and the resulting AuChiliposomes rapidly released the encapsulated doxycycline upon exposure to either purified PLA2 enzyme or PLA2 secreted by *H. pylori* bacteria in culture inhibiting its growth effectively. Overall, the conjugate was referred to as smart “on-demand” antibiotic delivery; the more enzymes or bacteria present at the infection site, the more drugs will be delivered to treat the infected cells. This liposomal delivery technique holds broad application potential for tissue microenvironment-responsive drug delivery.

Grit et al. (2014) also explored the enzyme-triggered release of the antimicrobial agent octenidine out of poly(L-lactide)-based nanoparticles (PLLA-NPs) and its in vitro antibacterial activities in the presence of Gram positive and Gram negative bacteria. In antimicrobial assays, the PLLA nanoparticles showed a greater

**FIGURE 7.9**

Degradation of PIC nanoparticles by LasB and subsequent poly(ethylene imine) (PEI) release.

**FIGURE 7.10**

Enzyme-triggered release of the antimicrobial agent octenidine out of poly(L-lactide)-based nanoparticles.

ability to inhibit the growth of *S. aureus* compared to *E. coli*, demonstrating the viability of this approach for potential future antibacterial therapy (Fig. 7.10).

7.5.4 EFFECT OF MAGNET ON ANTIMICROBIAL NANOPARTICLE

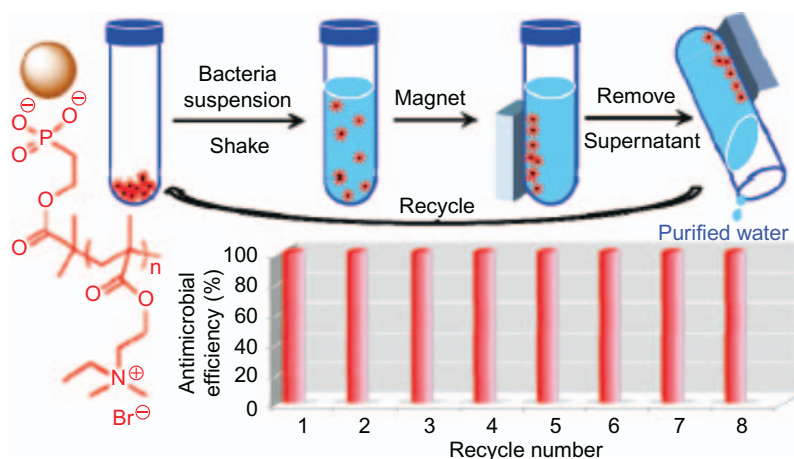
Magnetic nanoparticles are important candidates for various medical applications, such as MRI (Laurent et al., 2008), site-specific drug delivery (Dobson, 2006), labeling and sorting of biological species (Gupta et al., 2007), and therapy (Barakat, 2009), even in food and drug administration (Bulte et al., 1999). Because of their unique characteristics, such as response to external magnetic force and high surface-to-volume ratio, their superparamagnetic properties, high biocompatibility, and lack of toxicity to humans, it is of interest to explore the use of magnetic nanoparticles in combination with various substrates, such as bio-molecules and nanomaterials to generate new antimicrobial agents that combine

antibacterial, antiviral, and antifungal properties. These antibacterial nanomaterials could be highly efficient biocides that could be easily recycled via application of an external magnetic field. Due to the degradable properties of magnetite (Fe_3O_4), it is easily useful in bioseparation and catalytic processes. The magnetic effect of superparamagnetic iron oxide nanoparticles (Sayari and Hamoudi, 2001) causes local hyperthermia in the presence of a magnetic field or, alternatively, by coating with other nanomaterials, such as Ag and Au and their magnetic effect can be utilized to penetrate and destroy biofilms.

The high surface energy and aggregation property of magnetite nanoparticles makes them less suitable for the biomedical application. Consequently, these are frequently coated with an organic or inorganic layer, such as PEG and silica-containing organic materials (Darwish et al., 2015). In the form of a core-shell (silica/PEG shell) structure, many methods are executed to prepare magnetic nanoparticles coated with a polymer, such as polyethylene glycol (PEG) and silica-containing organic material, such as aminopropyltriethoxysilane (APTS) (Yi et al., 2006), tetraethoxysilane (TEOS) (Yoon et al., 2006) which enhances hydrophilicity and improves biocompatibility (Stöber et al., 1968). Their biological effect on Gram-negative *E. coli* and Gram-positive *S. aureus* shows that most of the modified magnetite nanoparticles had a significant effect on *S. aureus* and not on *E. coli*, whereas PEG-magnetite nanoparticles displayed no significant effect on the growth rate of either bacteria (Moghimi et al., 2001). TEOS-magnetite showed a stronger effect only toward *S. aureus*. APTS-magnetite nanoparticles display a degree of antimicrobial activity, allowing for their use in bioapplications, such as drug nanocarriers, where bacterial growth is undesirable (Huang et al., 2014).

Arakha et al. (2015) investigated the role of interaction pattern at the iron oxide nanoparticle (IONP)-bacteria interface affecting the antimicrobial propensity of IONP. Positively charged chitosan molecule coating was used to reverse the surface potential of n-IONP, i.e., positive surface potential IONP (p-IONP). The BacLight fluorescence assay, bacterial growth kinetics, and CFU studies indicated that n-IONP ($<50\ \mu\text{M}$) has insignificant antimicrobial activity against *B. subtilis* and *E. coli*, although coating with chitosan molecule resulted in a significant increase in the antimicrobial propensity of IONP. Additionally, the assay to study ROS indicated relatively higher ROS production upon p-IONP treatment of the bacteria.

Iron oxide is not antibacterial in its bulk form but may exhibit antibacterial properties against *E. coli*, as nanoparticles, after coating with quaternary ammonium (Fig. 7.11) (Dong et al., 2011). Zhuang et al. (2012) reported bacitracin-conjugated iron oxide (Fe_3O_4) nanoparticles with higher antimicrobial activity against both Gram-positive and Gram negative organisms with lower doses, in comparison with bacitracin peptide. Their antibacterial effect and magnetism devised the conjugated bacitracin magnetic nanoparticles to be a potential application in magnet-targeting biomedical applications. The synthesis of multimodal nanoparticles with magnetic core and silver shell showed very significant antibacterial activities

**FIGURE 7.11**

Polymeric quaternary ammonium salt-coated iron oxide derived antibacterial nanoparticles.

against several bacterial strains (Prucek et al., 2011). They also prepared HDP LL37-conjugated magnetic nickel nanoparticles coated with a nonlayer biofilm of polyacrylic acid. Recently, Ismail et al. (2015) demonstrated the antibacterial property of magnetic iron oxide (α - Fe_2O_3) nanoparticles of size 50–110 nm, synthesized by pulsed laser ablation of iron target in dimethylformamide and sodium dodecyl sulfate solutions against Gram-positive *S. aureus* and Gram-negative *E. coli*, *P. aeruginosa*, and *S. marcescens*, resulting a noteworthy inhibition on both bacterial strains, depending on the preparation conditions. The synthesized magnetic nanoparticles were used to rapidly capture *S. aureus* bacteria under the magnetic field effect. Jeong et al. (2015) developed magnetic iron oxide (Fe_3O_4) nanoparticles (NPs) coated with catechol-conjugated poly(vinylpyrrolidone) sulfobetaines (C-PVPS), which is negatively charged and encapsulated by poly(3,4-ethylenedioxythiophene) (PEDOT) to achieve Fe_3O_4 @C-PVPS:PEDOT nanoparticles that could be NIR-irradiated photothermal agents. Photothermal heating show 100% antibacterial efficiency during three cycles against *S. aureus* and *E. coli* in almost, and rapid, effective killing of 99% Gram-positive and Gram-negative bacteria within 5 min of near-infrared (NIR) light exposure. The core-shell nanoparticles (Fe_3O_4 @C-PVPS: PEDOT) exhibit stability, and due to paramagnetic nature they rapidly convert photothermal heat sufficient for use as NIR-irradiated antibacterial photothermal sterilizing agents. Liu et al. (2012) prepared the one-pot synthesis of Ag- Fe_3O_4 nanocomposites in the presence of poly(vinylpyrrolidone) (PVP) at moderate temperature and pressure and the synthesis through the redox reaction between Ag_2O and $\text{Fe}(\text{OH})_2$ without an additional reducing agent. The nanocomposites displayed good antibacterial activity against

E. coli; even magnetic AgNPs exhibits excellent deactivation of bacteria and could be easily gathered and recovered from aqueous solution by externally applying magnetic field.

7.6 CONCLUSIONS AND FUTURE PROSPECTS

The specific internal (biological/pathological) or external agent-stimulated drug releases are advantageous, particularly for the drugs that are required to be released at biorhythm-dependent rates, in the close vicinity of tissues or cellular organelles, or highly hydrolysable and toxic in nature. Conversely, the inclusion of nanotechnology in the devices of drug delivery system (DDS) could improve the pharmacokinetic and pharmacodynamic properties, as they have unique and well defined physicochemical properties such as ultra-small and controllable quantum size effect, shape, surface-to-volume ratio, surface charge, functionalizable structure, and unique electrodynamic interactions to overcome the global concern of antibiotic resistance of bacteria. To that end, a promising and potential alternative to targeted drug delivery, the design of nanocarriers for antimicrobial delivery triggered by exogenous or endogenous stimuli, may represent a “smart” or “intelligent” DDS that could impart the temporal control to drug release. These advanced stimuli-triggered DDSs have been increasingly used to promote physiological specificity and on-demand therapeutic efficacy of antibiotic drugs to undermine the antibacterial resistance in bacteria than the traditional methods of delivering antibiotics. Accordingly, the specific stimuli-responsive nanomaterials for antimicrobial drug delivery can resist other stimulating agents, such as high temperature, pH, and enzymatic hydrolysis, and also sustain the sterilization process in which normal antibiotics are inactivated. Accordingly, stimuli-triggered nanocarrier components help in improving antibiotics shelf-life, site-specific activity, and drug properties by decreasing the lipophilic character and toxic factors while increasing the solubility of the antibiotics in water, cost-effectiveness, patient compliance, bioavailability of antibiotics at the site of action, and sustainable release of drug over a period of time. These unique and smart drug carriers could find broad applicability for transportation of different drug molecule. It could further inspire to design an elegant and faithful programmable drugs carrier with the ability to avoid premature drug release during preparation and administration for several treatments, such as cancer, diabetes, and other metabolic diseases.

ACKNOWLEDGMENT

This work was supported by the grant from the WOS-A project (SR/WOS-A/CS-63/2013 (G)), Department of Science and Technology, New Delhi, Government of India.

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Stimuli-responsive micelles: A nanoplatform for therapeutic and diagnostic applications

8

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CHAPTER OUTLINE

8.1 Introduction	304
8.2 Properties of Polymeric Micelles	304
8.2.1 Size	305
8.2.2 Critical Micelle Concentration and Micelle Stability	305
8.2.3 Ability to Avoid Uptake by the Mononuclear Phagocytic System	305
8.2.4 Core-shell Structure	306
8.3 Structural Aspects of Micelles and Micelle-Forming Amphiphiles	307
8.3.1 Micelle Shape and Morphology	307
8.3.2 Amphiphilic Block Copolymers Used in Fabrication of Micelles	308
8.4 Micelles in Drug Delivery Applications	309
8.5 Micelles in Targeted Drug Delivery	311
8.6 Stimuli-Responsive Micelles	312
8.6.1 pH-Responsive Micelles	313
8.6.2 Thermoresponsive Micelles	318
8.6.3 Photoresponsive Micelles	323
8.6.4 Miscellaneous	329
8.7 Diagnostic Applications	330
8.7.1 Nuclear Imaging	331
8.7.2 Magnetic Resonance Imaging	333
8.7.3 X-Ray Computed Tomography	334
8.7.4 Fluorescence-Based Imaging	335
8.8 Conclusion	335
References	336

8.1 INTRODUCTION

First introduced to the drug delivery armamentarium by Helmut Ringsdorf in early 1980s, polymeric micelles are self-assembled, supramolecular nanoconstructs composed of amphiphilic copolymers (usually block copolymers). Identical to small molecular weight surfactants, amphiphilic copolymers in aqueous solvent, at a given temperature, above a certain concentration known as critical micelle concentration (CMC), form aggregates called micelles. This self-assembly is a thermodynamically driven and reversible phenomenon. At concentrations below the CMC, the amphiphilic species exist in molecularly dissolved form (unimers), whereas above CMC, multimolecular micelles are in thermodynamic equilibrium with unimers (Karayianni and Pispas, 2016). The hydrophobic portion of the amphiphile orients to form a core and the hydrophilic part orients towards an external aqueous phase, forming the corona of micelles. Micelles composed of amphiphilic di- or tri-block copolymers in aqueous medium, are typically in the size range of 10 to 100 nm (Biswas et al., 2013).

As is well known, self-assembly of amphiphilic polymers in micelles arises from the need to minimize energetically unfavorable interactions. Water is a thermodynamically favorable solvent for the hydrophilic block, while it acts as a precipitant for the hydrophobic block of an amphiphile in aqueous medium. This dichotomy drives the aggregation of hydrophobic part of several unimers, so the resultant micelle consists of a swollen hydrophobic core surrounded by a flexible corona of hydrophilic blocks. Forces involved in micellization are the attractive forces between hydrophobic blocks leading them to aggregate as the core and repulsive forces between hydrophilic blocks forming the corona of micelles (Karayianni and Pispas, 2016). The repulsive forces in the corona limits further growth of the micelle. Interactions between the hydrophilic corona-forming block of the amphiphile and the external aqueous medium is responsible for the stabilization of micelles (Riess, 2003). Depending on the specific properties of the amphiphile, an average figure, known as the aggregation number, denotes the number of polymer chains in a micelle.

8.2 PROPERTIES OF POLYMERIC MICELLES

The structural features of polymeric micelles impart certain specific properties to them, an understanding of which is essential to appreciate their utility as drug delivery vehicles. Accordingly, besides certain obvious advantages such as the ability to solubilize hydrophobic drugs in the lipophilic core, spontaneous formation, and drug encapsulation by simple physical mixing, the following characteristics of polymeric micelles are especially relevant:

8.2.1 SIZE

Polymeric micelles possess extraordinarily small size and, owing to the fact that the number of monomers forming a micelle is governed by thermodynamic considerations, they are formed within a narrow size distribution. The size of micelles is critical since it can influence their biodistribution, especially following parenteral administration. Accordingly, while very small micelles with size 5~10 nm or less are eliminated through glomerular filtration (Kabanov et al., 2002), slightly larger ones (less than ~35 nm) may undergo accumulation in the kidney; micelles with a size range of 75 to 115 nm are reported to be lodged in the liver (Yue et al., 2013), and still larger micelles (~145 nm) tend to be taken up primarily by spleen and lung. Polymeric micelles with a size less than 100 nm tend to show a higher extent of accumulation at tumor sites (Yue et al., 2013).

8.2.2 CRITICAL MICELLE CONCENTRATION AND MICELLE STABILITY

The CMC of amphiphilic block copolymers is at least tenfold lower than classic low molecular weight surfactants (Karayianni and Pispas, 2016). This is of paramount significance from drug delivery stand point, as polymeric micelles maintain their core-shell structure even after dilution with blood in human body because of their low critical micellar concentrations (1–30 mg/L) (Nishiyama and Kataoka, 2006).

8.2.3 ABILITY TO AVOID UPTAKE BY THE MONONUCLEAR PHAGOCYtic SYSTEM

Intravenously injected nanoparticles undergo rapid clearance from systemic circulation by phagocytic cells of mononuclear phagocytic system (MPS). This sequence is initiated by adsorption of plasma proteins known as “opsonins” on the nanoparticle surface. Opsonization helps in recognizing nanoparticles by cell surface receptors present on phagocytic cells of MPS, followed by receptor-mediated phagocytic uptake. Mononuclear phagocytic system mediated rapid clearance is one of the major obstacles in successful delivery of therapeutics to their site of action. Longer blood circulation of drug-carrying nanosystems is necessary to elicit therapeutic advantages such as enhanced permeation and retention (EPR)-mediated passive targeting, which is especially relied on for cancer targeting. The circulation half-life of nanoparticles mainly depends upon physicochemical characteristics, such as particle size, shape, surface charge, and surface chemistry (Amoozgar and Yeo, 2012). Hydrophobic nanoparticles with a charged surface undergo significant opsonization and therefore have a shorter half-life, in comparison to hydrophilic nanoparticles with neutral surface (Sheng et al., 2009). Cationic micelles are more susceptible to phagocytosis, leading to shorter blood circulation time in comparison to neutral or anionic micelles. Nanoparticle

MPS-mediated clearance from blood circulation can be successfully evaded by modification of nanoparticle surfaces with long-chain neutral hydrophilic molecules, which form a cloud on the nanoparticle surface and thereby prevent adsorption of opsonins, a phenomenon termed “stealth”. Polyethylene glycol (PEG, molecular weight 2 KDa–5 KDa) is most commonly employed by either covalent attachment on nanoparticle-forming polymer/lipids or physical association with nanoparticle surface to render it hydrophilic. Stealthing of nanoparticles improves the circulation half-life from less than an hour to more than 40 hours (Moghimi et al., 2001). In the case of polymeric micelles, the hydrophilic corona imparts an inherent stealthing property, rendering them less prone to phagocytic uptake by the MPS, resulting in prolonged circulation and a higher opportunity for EPR-mediated passive targeting and therefore, high therapeutic efficiency (Movassaghian et al., 2015).

8.2.4 CORE-SHELL STRUCTURE

Polymeric micelles are phase-divided into inner core and outer corona. This enables separation of their functionalities, while both phases also serve in unison to accomplish the objective of drug delivery (Fig. 8.1). The hydrophobic core serves as drug reservoir, protects drug cargo from surrounding environment during circulation and prevents premature drug release. Physicochemical properties of the core dictate drug loading and drug release characteristics and thus play an important role in determining pharmacological activity. In contrast, the hydrophilic corona can be harnessed to tailor biodistribution, pharmacokinetics, and interaction with biocomponents. The corona of polymeric micelles can be functionalized for actively targeting drug molecules to the tissue of interest by attaching

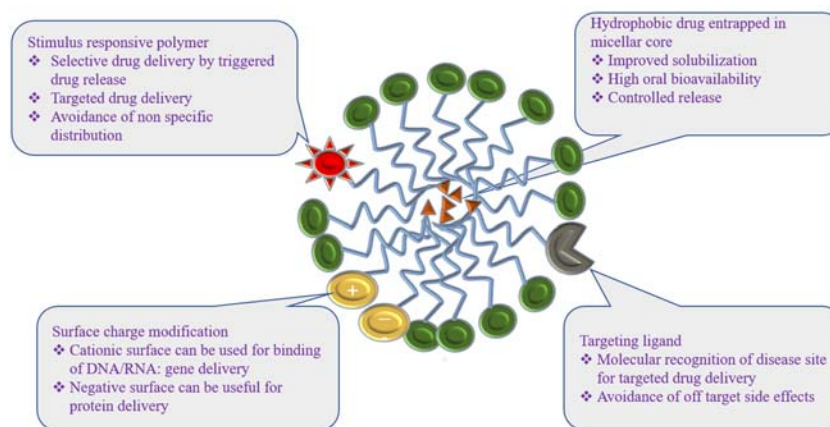


FIGURE 8.1

Salient features of micelles and their applications in drug delivery.

ligands specific for the target tissue/cells, thus facilitating molecular recognition and subsequent interaction of micelles with the target cells.

It is noteworthy that several of these important physicochemical properties such as size, size distribution, surface charge, and surface properties can be easily modified by the suitable choice of either or both components of the amphiphilic block copolymer. Other essential features, such as biocompatibility and ready elimination or biodegradation, can also be built into the amphiphile, given the current ability to synthesize such materials.

8.3 STRUCTURAL ASPECTS OF MICELLES AND MICELLE-FORMING AMPHIPHILES

8.3.1 MICELLE SHAPE AND MORPHOLOGY

The morphology of micelles depends upon the relative length and positions of hydrophobic and hydrophilic parts of the amphiphilic polymer. Although a variety of architectures including linear di-block, triblock, or even nonlinear graft or star-like amphiphiles have been synthesized and studied for micellization, most studies employ classic di-block copolymers, the two blocks being hydrophilic and hydrophobic in nature. In the case of the di-block linear copolymers, a dimensionless expression, namely the packing parameter (P), is used to define relative size of the hydrophobic part of the amphiphilic polymer and resultant shape of micelles.

$$P = V/al$$

where

V is the volume of hydrophobic block,

l is the length of hydrophobic block, and

a is the surface area of hydrophobic block at hydrophobic-hydrophilic interface.

Accordingly, the packing parameter expresses the ratio of molecular volume of hydrophobic chain to the volume actually occupied in the micellar assembly, therefore dictating probable morphology of the micelles. As a general rule, an amphiphile with $P \leq 1/3$ aggregates to form spherical micelles, values of $1/3 < P \leq 1/2$ result in cylindrical micelles, whereas vesicles or polymerosomes are formed when $1/2 < P \leq 1$ (Karayianni and Pispas, 2016). The relative length of hydrophilic and hydrophobic blocks further determines the shape of spherical micelles. Hairy/starlike, also known as core-shell, micelles are characterized by a small dense core and large corona, formed when the hydrophilic block is larger than hydrophobic block. Whereas crew-cut micelles, with a large dense core and short stretched corona, are formed if the relative size of hydrophilic block is smaller than hydrophobic block.

8.3.2 AMPHIPHILIC BLOCK COPOLYMERS USED IN FABRICATION OF MICELLES

The property of amphiphilic block copolymers to self-assemble in a variety of morphological structures, such as spherical micelles, cylindrical micelles, lamellae, vesicles, and bi-continuous structures is an important feature from a drug delivery standpoint. Developments in synthetic chemistry allow the synthesis of block copolymers with well-defined architecture in terms of molecular weight, composition, and spatial arrangement. Amphiphilic block copolymers are classified as linear block copolymer, graft copolymer, dendritic polymer, and star-like polymers, depending upon their architecture of attachment/position of hydrophilic and hydrophobic blocks in the main chain of polymer. The block copolymers of major interest in micelle formation belong to the class of linear and graft polymers.

The hydrophilic blocks commonly used are poly(ethylene oxide) (PEO), also known as poly(ethylene glycol) (PEG), comprising of the monomer unit $-\text{CH}_2-\text{CH}_2-\text{O}-$ with hydroxyl or methoxy end groups. Poly(ethylene glycol) blocks with terminal amine groups are used when chemical conjugation of ligands or drug molecules are desirable. Poly(ethylene glycol) blocks are typically used in lengths ranging from 1 to 15 kDa ([Torchilin, 2006](#)). The major advantages of PEG as a hydrophilic block are nontoxicity, nonimmunogenicity, ability to provide stealthing and ease of approval from regulatory agencies.

Other hydrophilic block-forming units are poly(*N*-isopropyl acrylamide), poly(*N*-vinyl pyrrolidone), poly(alkylacrylic acid), poly(2-ethyl-2oxazoline), and poly(vinyl alcohol). Poly(*N*-isopropyl acrylamide) and poly(alkylacrylic acid) impart temperature and pH sensitivity respectively and are therefore included as the hydrophilic block for preparation of thermoresponsive and pH responsive micelles.

The hydrophobic polymer blocks employed most often include poly(propylene oxide), poly(butylene oxide), poly(methyl methacrylate) and poly(styrene). Di and tri block copolymers composed of poly(ethylene glycol)-poly(propylene oxide) (PEG-PPO, Pluronics®) and poly(ethylene glycol)-poly(butylene oxide) (PEG-PBO) are most widely explored for micelle formation. Poly(ethylene glycol)-poly(styrene) di-block and PEG-polystyrene-PEG triblock copolymer-based amphiphiles have also been the subject of detailed studies ([Bhargava et al., 2006](#)). Amphiphiles based on hydrophobic acrylates, alkyl polycarbonates, biodegradable hydrophobic blocks including poly(L-amino acids) such as poly(aspartic acid), polyesters such as polylactide, lactide/glycolide copolymers, polycaprolactone, phospholipids and long chain fatty acids (e.g. myristic acid, stearic acid); each of these by themselves or in combination with one another have all been reported ([Kumagai et al., 2007](#), [Allen et al., 2000](#), [Somekawa et al., 2015](#), [Dubertret et al., 2002](#), [Varshosaz et al., 2012](#)). Naturally occurring polymeric materials, such as chitosan have been modified via the attachment of various moieties, rendering them capable of forming micelles ([Woraphatphadung et al., 2016](#)).

The choice of optimal hydrophobic block for fabrication of micelle-forming amphiphilic block copolymers mainly depends on the desired thermodynamic and kinetic stability of resulting micelles. Molecular interaction within micelles defines thermodynamic stability, whereas the kinetic stability is determined by the rate of disassembly of hydrophobic blocks in the core (Huang et al., 2012). The disassembly of micellar core in turn depends on the length of hydrophobic block and physical state of the core, e.g., existence below or above the glass transition temperature (T_g) (Rapoport, 2007). Studies based on the core formed of polystyrene block ($T_g \sim 100^\circ\text{C}$) demonstrated that drug release from the core was insensitive to conformational changes in the corona (Chung et al., 2000).

In order to avoid accumulation of micelle-forming polymers in the body, the unimers (amphiphilic polymer) should be composed of either biodegradable components or should undergo renal excretion (molecular mass cut off 40 kDa) (Attia et al., 2011).

8.4 MICELLES IN DRUG DELIVERY APPLICATIONS

An account of the advantages of micelles in various drug delivery applications that can be envisaged or have been employed is presented in Fig. 8.2.

The oral route is always preferred as a portal for drug introduction into the body because of its obvious convenience and high degree of patient compliance. However, in the case of certain molecules, issues such as low oral bioavailability of drug owing to low aqueous solubility, low gastrointestinal permeability, or

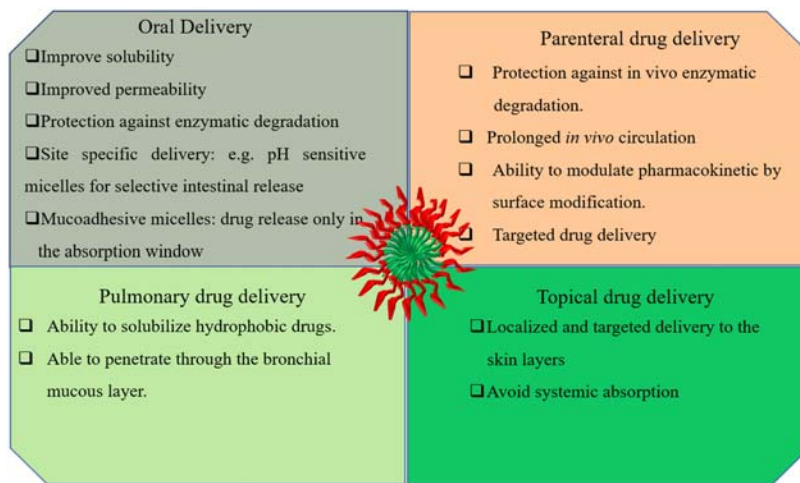


FIGURE 8.2

Advantages of polymeric micelles in various drug delivery applications.

instability in gastrointestinal fluids preclude the use of this route. Polymeric micelles as nanovehicles have the potential to overcome some of these road-blocks. Micelles, through their ability to solubilize hydrophobic agents, may simply be used to deliver water insoluble drugs to the gastrointestinal tract with increased solubility. Furthermore, drug release can be triggered to occur in a more favorable intestinal environment by the use of smart pH-sensitive polymeric micelles (Yang et al., 2016).

Accordingly, pH-sensitive poly(ethylene glycol) – poly(2-methyl-2-carboxyl-propylene carbonate) – vitamin E (mPEG113 – PCC32 – VE4) micelles have been designed with vitamin E serving a dual role of increasing the volume of the hydrophobic core and exerting an inhibitory effect on cellular P-gp efflux pumps. Such a system was used to demonstrate the usefulness for delivery of tacrolimus, a drug with limited amenability for oral delivery due to poor aqueous solubility and owing to it being a potent substrate of P-gp. The outcome of in vivo studies in rats revealed a 6-fold increment in the oral bioavailability of tacrolimus when administered as micelles, than that of tacrolimus solution in rats (Wang et al., 2015). Uptake of the drug across the gut mucosa may require the drug to be released from the micelle. The oral route is also believed to allow access to the drug through the intestinal Peyer's patches and lymphatic system in the form of intact micelles. Accordingly, targeted tumor delivery of docetaxel, when administered orally in micelles embedded in an acid-resistant hydrogel, was achieved. The sequence of events was postulated as: firstly, the micelles passing through the stomach under the umbrella of hydrogel, diffusion of the micelles from the hydrogel in intestinal tract; absorption by Peyer's patches, subsequent long circulation in blood followed by passive accumulation in the tumor tissue; and finally, release of docetaxel in the tumor microenvironment (Wang et al., 2014).

Selective colonic delivery after oral administration has been investigated through micelles composed of azobenzene-linked poly(ethylene glycol)-*b*-poly(styrene) (PEG-*N* = *N*-PS) amphiphilic copolymer. This polymer assembles into a micellar structure in water. Exposure to azoreductase, an enzyme elaborated by the rich bacterial population typically residing in the colon, results in cleavage of the azo-based copolymer junction, and disruption of the micellar assembly and consequent release of drug incorporated within the micellar core (Rao and Khan, 2013).

Topical application of drugs allows targeted delivery directly into the skin layers for localized diseases and dermatological conditions. A major concern during topical delivery of drugs is that the systemic absorption leads to undesirable effects. In this context, application of drug embedded within polymeric micelles may be a successful option to achieve the desirable status of retention in the stratum corneum, viable epidermis, and upper dermal layers. Systematic studies on methoxy-poly(ethylene glycol)-poly-(hexylsubstituted lactides) (MPEG-hexPLA) di-block copolymeric micelles, loaded with tacrolimus for the treatment of psoriasis in one study (Laptev et al., 2014) and antifungal agent econazole nitrate in another study (Bachhav et al., 2011) have affirmed the ability of micelles to

selectively target drug into the skin layers without the risk of systemic absorption, as is the case in the use of classic skin penetration enhancers.

Most literature using polymeric micelles for drug delivery applications, including the use of the stimuli-responsive micelles, is centered around the administration of these vehicles via the parenteral and more specifically via the intravenous route. Moreover, most of the studies are intended to address the problem of inefficient delivery of cytotoxic agents to cancerous cells by means of various targeting approaches. Other applications that also demand parenteral delivery include gene delivery, delivery of diagnostic agents, and the so-called theranostic (therapy plus diagnosis) uses.

8.5 MICELLES IN TARGETED DRUG DELIVERY

Targeting therapeutic agents to their site of action is highly advantageous and desirable, especially in the case of treatments such as cancer chemotherapy. Selective accumulation of therapeutic agents at the target site improves therapeutic efficacy and minimizes off-target side effects. The approaches available for drug targeting include passive targeting, active targeting, and combinations thereof.

Size-dependent biodistribution and longer blood circulation have been the basis of passive targeting of nanosystems. Anatomical and pathophysiological conditions are key considerations in passively targeted micellar drug delivery systems. Hyper vascularization and poorly developed blood vessels consisting of gaps (fenestrations) in the endothelial lining are characteristic of solid tumors. Furthermore, tumor tissues secrete various factors *viz.* nitric oxide, prostaglandins, bradykinin, basic fibroblast growth factor, and vascular permeability factor or vascular endothelial growth factor (VEGF), causing hyperpermeability of tumor microvasculature (Kedar et al., 2010). The effective pore size in the vasculature of most tumors ranges from 200–600 nm (Sarin, 2010). This, coupled with poor lymphatic drainage, allows particles below ~200 nm to permeate and remain in the tumor interstitium. This phenomenon, known as enhanced permeation and retention (EPR) effect, remains the basis of passive targeting in cancer. Fenestrations are also present in the endothelium of certain healthy tissues including glands, mucosal lining of GIT, and kidney, with a size of ~60 nm (Bououdina, 2014). Discontinuous endothelium, with gaps of 50~100 nm, also exist in the liver and spleen (Bououdina, 2014).

Active targeting conversely employs disease/organ/tissue-specific interaction with the nanosystem, including antigen-antibody and ligand-receptor interactions. Several receptors have been identified to be overexpressed by tumor cells. Similarly, tumor-specific antigens have been recognized and characterized. Incorporation of a ligand specific to such a receptor or use of an antibody against the antigen on the surface of a nanosystem, greatly improves its potential to

interact with tumor tissue; this is the basis of active targeting. In the case of polymeric micelles, specific interaction between targeting ligands attached on micellar corona with receptor/antigen expressed on the cell surface can cause selective uptake of drug-carrying cargo in the target tissues. Such interactions are also advantageous in cytoplasmic delivery by receptor-mediated endocytosis, followed by drug release in cytoplasm.

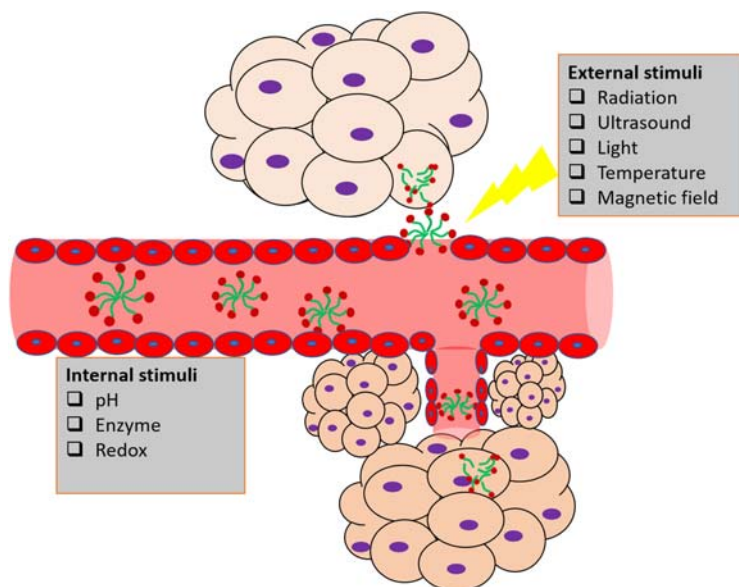
Although active targeting by employing ligand-receptor interaction is interesting due to their specificity, the major limitation remains in terms of exclusivity of the presence of receptor at target site. Accordingly, the receptor, although relatively less in number, if present on nontarget cells, deems them prone to the drug effects, compromising the whole purpose of targeted drug delivery. Another approach towards drug targeting is using micelles engineered to respond to a variety of external triggers, such as light, magnetic field, temperature; or to internal triggers, such as pH or enzymes, and subsequently release the drug at the site of action. The resultant stimuli-responsive micellar nanosystem is discussed in detail in the following sections.

8.6 STIMULI-RESPONSIVE MICELLES

Micelles composed of amphiphilic block copolymer designed with the ability to respond to external/internal physical or chemical stimuli are attractive to drug targeting. An ideal targeted system must be able to avoid drug release and minimize interaction with nontarget tissue while in circulation. However, once at the target tissue, it must interact and release the drug molecules actively. This implies that the drug release must be governed by factors specific to the target site. Stimuli-responsive micelles are extremely useful in this sense; the micelles change their physicochemical properties to control the drug release rate and interaction with cells in response to the surrounding environmental conditions such as pH, temperature, enzymes; or externally applied triggers, such as radiation and ultrasound. These triggers may also be classified depending on their nature as physical, chemical, or biological (Fig. 8.3).

Design of a stimulus-responsive micellar nanosystem requires synthesis of smart polymers that possess functionalities which respond to the trigger/stimulus. Furthermore, specificity of the response to the trigger is important to avoid non-specific drug release, thus minimizing off-target effects. A well designed stimulus-responsive micellar system should exhibit certain basic quality attributes:

1. nanoscale size (50–200 nm) upon encapsulation of therapeutic/diagnostic agents and a narrow size distribution;
2. possess the ability to entrap high pay load of drug;
3. have prolonged blood circulation time to enable higher drug accumulation at the target site;

**FIGURE 8.3**

Schematic representation of targeted drug delivery using stimuli-responsive micelles.

4. have ability to reach target pathological site by active or passive mechanism;
5. must retain the capacity to respond to the external or internal stimulus which in turn, must be specific to the target site.

Clearly, successful development of stimulus responsive micelles needs an interdisciplinary approach and requires expertise from diverse areas including chemical, physical, pharmaceutical, and pharmacological sciences. The following sections deal with a variety of stimuli-responsive micelles categorized by the triggers used for spatial and temporal release. Amongst various triggers possible, the present discussion includes pH, temperature, and radiation, since these are the most widely explored for polymeric micelle-based drug delivery, and a few miscellaneous triggers which have been less frequently employed. A comprehensive review of various approaches as encountered in literature for designing stimuli-responsive polymers and the advantages and limitations thereof have also been presented.

8.6.1 pH-RESPONSIVE MICELLES

The concept of using pH as a means of cellular delivery possibly originates from the observation that certain enveloped viruses developed strategies to make use of the acidification of the endosomal lumen to infect the host cells (Miyauchi et al., 2011). Although the human body maintains a steady pH of 7.4 in case of most fluid compartments, nevertheless, both at the macro (e.g., various segments of the

gastrointestinal tract) and at the micro levels (e.g. lysosome), variations do exist. Several pathological conditions also contribute to this natural variation in pH. For example, the intra-tumoral environment has a more acidic pH because of the lactic acid production, owing to the under-perfused and hypoxic environment within the tumor. Similarly, pH within inflamed tissue is also found to be lowered, as in the case of joints affected by rheumatoid arthritis (Farr et al., 1985). Nanocarriers also encounter a cascade of declining pH values along their journey from tissue interstitium to cytoplasm (early endosome > late endosome > lysosome). In the case of the above situations, a pH or proton concentration-triggered drug release can serve as an effective way of enabling higher drug accumulation at the disease site.

pH-responsive micellar systems therefore incorporate a design wherein the system is stable at physiological pH, but responds to a mild change, usually a decrease in pH. In the case of tumor targeting, following an increased accumulation of the micellar nanovehicle in the tumor attributed to the EPR effect, the release of drug is now triggered due to lowering of pH in the tissue interstitium, or within a cytoplasmic compartment such as the lysosome, depending on the pH at which the micelle forming polymer responds. The drug release may be elicited in either of two ways:

- (1) Release of drug by cleavage of an acid labile bond used to covalently anchor it to the micelles forming amphiphilic block copolymer;
- (2) Destabilization of micellar structure, which, in turn, may be due to the change in hydrophobic/hydrophilic balance, shape or size of the micelles.

A plethora of pH-sensitive materials have been synthesized and evaluated for pH-triggered drug release (Yang et al., 2016, Jones et al., 2003, Yang et al., 2010b). The strategies used in designing pH-responsive polymeric micelles can mainly be grouped into two, based on the approaches described above:

- (1) **A prodrug like approach**, wherein the micelle forming polymers are pH-insensitive, but the drug is covalently linked to the hydrophobic block of the micelle forming amphiphile through linkages that degrade in acidic environment. Examples of such acid labile linkers include hydrazone, acetal, benzoic imine, and ortho-ester. The acid labile linkers may be inserted onto the main polymer chain, side chains, or at the end of the core-forming block. Such a strategy is exemplified by micelles composed of poly(ethylene glycol)-poly(aspartate), where adriamycin was conjugated with hydrophobic poly(aspartate) segment of the amphiphile through acid labile hydrazone bonds (Bae et al., 2005). Micelles obtained with this polymer, were found to hold the drug under physiological conditions (pH 7.4) and release occurred in the acidic microenvironment of endosomes and lysosomes. In vitro evaluation in murine colon adenocarcinoma 26 (C26) cells and human small cell lung cancer cell line, SBC-3, revealed intracellular pH-triggered drug release capability and tumor-infiltrating permeability of the micelles. More significantly, in vivo biodistribution studies revealed the long circulating

nature of the micelles, resulting in greater accumulation at the tumor site, whereas in the case of other organs including heart and kidneys, the micelles failed to undergo retention and were evacuated out, with the drug payload safely bound within (Bae et al., 2005).

- (2) **Change in pH as a means of destabilizing micellar structure** leading to drug release has also been actively explored. The design of micelles is based on the inclusion of titrable groups, such as tertiary amino or carboxylic acid, which results in gain or loss of charge, depending on the pH of surrounding medium. Typically, a hydrophobic segment of the amphiphile contains ionizable groups, the ionization of which results in a change in the hydrophobic/lipophilic balance of the amphiphile, which in turn results in breakdown of the micellar assembly, often through hydration and dissolution of the amphiphile.

Depending on ionizable groups employed to impart pH-responsiveness, amphiphilic polymers of this category may be either anionic or cationic. Polymers bearing pendant acidic functional groups protonate at low pH, yielding neutral molecules that are relatively hydrophobic. At neutral or high pH, the molecules can release protons and become hydrophilic. Such polymers include polyacrylic acid, polymethacrylic acid, polyglutamic acid, and those containing polysulfonamide groups.

For drug targeting, with due regard to the pathological conditions existing within the tumor microenvironment, the more useful pH-sensitive polymers are those bearing cationic groups, since these would tend to become protonated to the charged species at lower pH. Examples of such polymers include poly(*N,N'*-dimethyl aminoethyl methacrylate) (PDMAEMA), poly(4-vinylpyridine), poly(histidine), and poly(β -amino ester) (PAE), of which the latter two are of major interest owing to their biocompatibility. pH-sensitive PEG–PAE-based amphiphiles can be synthesized via a Michael addition reaction between monoacrylated PEG, 1,6-hexane diol diacrylate, and 4,4'-trimethylene dipiperidine (Gao et al., 2010a). Micelles based on these amphiphiles have been investigated for diversified applications, including delivery of doxorubicin to cancerous tissue (Wu et al., 2010), imaging agents like ferric oxide for magnetic resonance imaging (MRI) of acidic ischemic regions in the brain (Gao et al., 2011), tetramethyl rhodamine for imparting discernible fluorescence to the tumor site (Min et al., 2010), and photosensitive agents such as the hydrophobic protoporphyrin (PpIX) for tumor-targeted theranostic applications (Koo et al., 2010).

Similarly, micelles based on amphiphilic polymer composed of pH-sensitive poly(histidine) as hydrophobic block and PEG as hydrophilic block have been probed for targeted delivery. Copolymerization of histidine (His) with another amino acid L-phenylalanine (Phe) is reported to reduce the pK_a of resulting PEG-*b*-PHis/Phe based micelles (Kim et al., 2005). Triblock copolymer composed of poly(L-lactic acid) (PLLA)-*b*-PEG-*b*-PHis is reported to form micelles at physiological pH; however in acidic pH (6.6), the micelles swell to release the loaded

doxorubicin (Liu et al., 2011). The addition of a hydrophobic PLLA segment stabilizes the micellar state (Lee et al., 2007).

pH sensitivity can also be imparted by tethering appropriate pendant groups to the amphiphile backbone. Micelles were designed using di-block copolymer of poly(ethylene glycol) and poly(γ -benzyl L-glutamate), with *N,N*-diisopropyl ethylenediamine (DIP) conjugated to poly(L-glutamic acid) (PGA) blocks as side groups. *N,N*-diisopropyl ethylenediamine enhances the pH-sensitivity of poly(ethylene glycol)-block-poly[*N*-(*N'*,*N'*-diisopropylaminoethyl) glutamide]. This PEG-PGA(DIP) polymer can self-assemble into micelles with an acid-responsive PGA-DIP core and hydrophilic PEG corona. The authors have reported faster acid response with increased grafting percentage of DIP. In acidic condition, the hydrophobic block PGA (DIP) transforms to a hydrophilic block, leading to a gradual expansion and disassembly of micellar structure, resulting in the release of entrapped doxorubicin (Li et al., 2016).

Finally, pH-selective disassembly of micelles can be tailored by preparation of mixed micelles, composed of combinations of amphiphiles. This approach is exemplified by mixed micelles composed of di-block copolymer of cationic poly(5-propyl-1,3-dioxan-2-one)-*b*-dimethylamine modified polycarbonate (PC(MPpC-MMA)) and anionic poly(ethylene glycol)-*b*-carboxylated polycarbonate (PEG-PCCOOH). The oppositely charged copolymers self-assembled into mixed micelles at physiological pH (7.4). The micelle formation is driven by electrostatic and hydrophobic interactions. The hydrophobic core was formed by cationic polycarbonate, whereas PEGylated polycarbonate forms the hydrophilic corona of micelles. The dimethylamine moiety is responsible for imparting pH-responsiveness and disassembly of micellar aggregates occurs at pH below 5 (Yu et al., 2016b). Various types of pH-responsive block copolymers, along with the pH-sensitive linkages employed in drug delivery applications are summarized in Table 8.1.

The complexity of the disease condition prompts the need for more effective control on drug release to enhance therapeutic outcome. Therefore, besides the pH-based systems discussed, literature also abounds in studies where dual, or sometimes multiple, approaches are simultaneously explored to achieve greater drug targeting potential of the developed system(s); pH change is also sometimes an arm of this multi-pronged approach. Tian K et al. developed a multi-responsive (pH-sensitive, reduction-sensitive and folic acid-mediated targeting) micelles for targeted delivery of anticancer drug doxorubicin. Here, a double end functionalized PEG with folic acid as one end group served as the hydrophilic part to which a hydrophobic block of *t*-butyl acrylate was added via atom transfer radical polymerization. A definite proportion of *N,N'*-bis (acryloyl) cystamine was incorporated in the hydrophobic block to enable the core to undergo disulfide-mediated cross-linking and consequent stabilization. The core cross-linked micelles were finally subjected to hydrolysis for removal of *t*-butyl group leaving the acrylic acid groups, which further conferred pH sensitivity in the core. The micelles loaded with doxorubicin exhibited only 13.4% premature drug release in 36 hours, while release in media stimulating a tumor microenvironment: acidic and high Glutathione

Table 8.1 pH-Sensitive Micelles and Their Applications in Drug Delivery

Sr. No.	Amphiphilic Copolymer	pH-Sensitive Linker	Therapeutic Agents	Results	Ref
1.	PLGA-PEG-PLGA	N-Boc-histidine	Doxorubicin	Higher in vitro drug release in acidic pH 6.2 in comparison to pH 7.4, higher uptake of drug into MDA-MB-435 cells.	Chang et al. (2010)
2.	Poly (ethylene glycol) methyl ether- <i>b</i> -(poly lactic acid)-co-poly (β-amino esters)	Poly (β-amino esters)	Doxorubicin	In vitro drug release from the micelles at acidic pH. The micelles exhibited higher cytotoxicity towards HepG2 cells	Zhang et al. (2012)
3.	Mixed micelles composed of poly (HEMA-co-histidine)- <i>b</i> -PLA and di-block copolymer of PEG–PLA containing folate for active cancer targeting	Core composed of HEMA-co-histidine	Doxorubicin	Acidic pH-mediated destabilization of the core resulted in significant drug release. Higher folate-mediated cellular uptake in HeLa cells. In vivo tumor targeting.	Tsai et al. (2010)
4.	Mixed micelles of AP-peptide bearing PEG-poly(D,L-lactic acid) and methyl ether poly(ethylene glycol)-poly(β-amino ester)	poly (β-amino ester)	Doxorubicin	pH-dependent micellization/de-micellization transition at the tumoral acidic pH. pH-dependent drug release profile. Tumor targeting of doxorubicin	Wu et al. (2010)
5.	Poly(poly(lactide-co-methacrylic acid)- <i>b</i> -poly(acrylic acid))	Methacrylic acid/acrylic acid	Nifedipine	Rapid drug release in simulated intestinal fluid (pH 7.36). Enable oral delivery of hydrophobic drug with specifically releasing the drug in intestine.	Yang et al. (2016)
6.	Derivatized chitosan such as N-octyl- <i>N</i> -O-succinyl chitosan	Succinic acid	Meloxicam	Higher release of meloxicam at intestinal pH 6.8 in comparison to acidic pH 0–2.	Woraphatphadung et al. (2016)
7.	Hydrazide-modified poly(oligo ethylglycol methyl ether methacrylate)- <i>b</i> -poly(hydroxyethyl methacrylate)	Hydrazine	Cisplatin	10 times faster degradation of hydrazone bond at pH 5.5 than at pH 7.4. Higher cytotoxicity of drug loaded micelles.	Binauld et al. (2012)

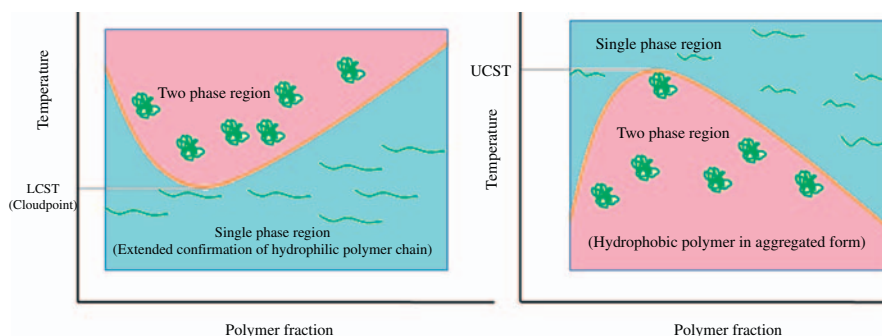
(GSH) level; was observed to be 78.7% (Tian et al., 2016a). In vitro studies in ovarian carcinoma cell line SKOV3 demonstrated the ability of the micelles to accumulate doxorubicin into the cell nuclei.

Mixed micelles, composed of (PEG-poly(D,L-lactic acid) block copolymer) bearing a tumor-targeting peptide as one component and the other comprising of pH-sensitive methyl ether poly(ethylene glycol) (MPEG)-poly(beta-amino ester) (PAE) block copolymer (MPEG-PAE) have been prepared. Both tetramethylrhodamine (TRITC) as fluorescent marker and doxorubicin as chemotherapeutic agent were separately entrapped in the micelles. In vivo studies in tumor-bearing mice demonstrated excellent anticancer efficacy, compared to free doxorubicin and doxorubicin-encapsulated MPEG-PAE micelles (without targeting ligand) which provides further justification for the use of multiple strategies in achieving selective tumor targeting (Wu et al., 2010).

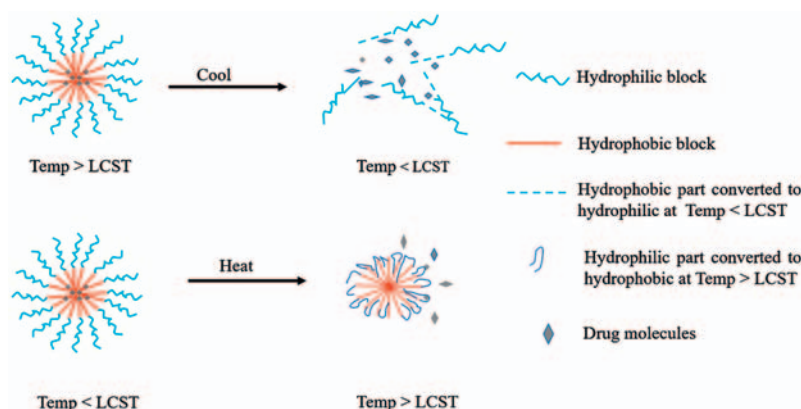
Besides the approaches outlined above, change in pH as a trigger to bring about other changes in the micelle properties have also been explored. e.g., for removal of the PEG segment from the micelles. PEGylation offers advantages of longer systemic circulation (avoid RES uptake) and selective accumulation at tumor site by EPR effect. However, its utility is rendered limited by recent reports that PEGylation inhibits cellular internalization and subsequent endosomal escape (Hatakeyama et al., 2013). Therefore, attempts are in progress to design “shed-dable” micelles by conjugating PEG in the polymer through a pH-sensitive linker. Zhao and coworkers conjugated phosphatidylethanolamine with PEG using benzoic imine. The PEG chains offer the steric hindrance and protect micelles at physiological pH, whereas disassembly of the PEG chain occurs in an acidic microenvironment of lysosomes after intracellular uptake. Although complete removal of PEG was not observed in extracellular pH, in vitro cell uptake was significantly higher when incubated at pH 6.8, compared to pH 7.4 (Zhao et al., 2016).

8.6.2 THERMORESPONSIVE MICELLES

Thermoresponsive micelles rely on localized application of mild hyperthermia as external stimulus for bringing about spatial and temporal release of a drug at target site. The micelles should retain the drug load at physiological temperature and release rapidly to the locally heated area. Polymeric micellar systems which respond to temperature changes are based on the use of thermoresponsive polymers in the fabrication of either block or the amphiphilic block copolymer. These polymers are characterized by their ability to exhibit a difference in physicochemical and conformational features, because of a change in solubility, across their phase transition temperature. They fall into two broad categories, depending upon their temperature dependent miscibility in a solvent. The first class is distinguished by the existence of a lower critical solution temperature (LCST), below which the polymer is completely miscible in the solvent, while polymers belonging to the second class exhibit an upper critical solution temperature (UCST) above which polymer is miscible in the solvent. A typical plot of phase behavior of polymer-solvent binary mixture is presented in Fig. 8.4.

**FIGURE 8.4**

Phase behavior plot of binary mixture of polymer and solvent exhibiting a) lower critical solution temperature (LCST) and b) upper critical solution temperature (UCST). The curved boundary represents the cloud point (adapted from (Phillips and Gibson, 2015)).

**FIGURE 8.5**

Typical disassembly behavior of thermoresponsive micellar systems upon temperature changes across LCST.

Polymers exhibiting LCST in aqueous systems are mainly used in designing thermoresponsive micelles. These polymers are highly hydrated and have an extended random coil conformation in aqueous solutions below LCST. Above LCST, they form hydrophobic globules due to dehydration of polymer; the change being reversible across the LCST.

The amphiphilic polymers used in the fabrication of thermoresponsive micellar systems are largely block copolymers. Accordingly, the LCST-type polymers are incorporated to constitute either the hydrophobic or the hydrophilic block of the copolymer at physiological temperature depending upon the LCST (Fig. 8.5).

8.6.2.1 Thermoresponsive polymer as hydrophobic block of the amphiphile

The LCST of such polymers is below 37°C, so that at physiological conditions (above LCST) it behaves as a hydrophobic block. When these polymers are coupled with another hydrophilic block, a micellar structure forms at a temperature above LCST, due to hydrophobic interactions among the dehydrated polymer chains. On cooling (below LCST), hydrophobic block undergoes transformation to a hydrated entity, resulting in collapse of the micelle structure and consequently release of the entrapped drug. This approach is exemplified by the double hydrophilic block copolymers composed of poly(*N*-isopropylacrylamide-co-polyethylene glycol) (pNIPAAm-co-PEG) (Wei et al., 2009). pNIPAAm exhibits LCST ~ 33°C, below which both components are hydrophilic and no micelles can be formed. However, at temperatures above LCST of the pNIPAAm block, (about 33°C), this block is hydrophobic, enabling the formation of micelles. The resulting micelles can be quickly destabilized due to an induced hypothermia (below 33°C) (Fig. 8.5). However, these micelles require physiological temperature for formation so that their storage during shelf life is at an unacceptably high temperature. For drug release, the temperature is also required to be lowered to destabilize the micelle, an approach not in line with classic thermal therapy, which entails elevation of temperature at the target site.

8.6.2.2 Thermoresponsive polymer as hydrophilic block of the amphiphile:

In contrast, a block copolymer where the hydrophilic block is composed of a polymer possessing an LCST would, above this phase transition temperature, undergo dehydration to a hydrophobic segment, resulting in the collapse of the micellar structure and subsequent drug release (Fig. 8.5). The collapse of the drug-carrying entities is also believed to facilitate increased interaction with the target cells, mediated through hydrophobic interaction (Akimoto et al., 2010).

The selection of polymer to formulate thermoresponsive micelles is of paramount importance, since, to enable utility in drug delivery, the phase transition should be sharp and within the range of clinically achievable hyperthermia (elevation of temp. to 39–43 °C). Ideally, the most useful copolymer would be one with an LCST slightly above physiological temperature which is readily achievable by external heating of the target site. Several polymers with thermoresponsive properties have been identified and representative examples along with their phase transition temperatures are listed in Table 8.2 (Gandhi et al., 2015).

However, as seen from the Table 8.1, the LCST of most of the known polymers are not in required range. Since the LCST of poly(*N*-isopropyl acrylamide) at about 32–33 °C is the closest to the desired value and owing to the fact that the polymer has a history of use in several biological applications such as scaffold for cell culture etc. (Mellati et al., 2016), it is the most widely investigated in designing thermoresponsive micelles. pNIPAAm has been employed as both

Table 8.2 Thermoresponsive Polymers and Their Phase Transition Temperature

Polymer	Phase Transition Temperature
Polymer exhibiting LCST	
Poly(<i>N</i> -isopropylacrylamide)	32 °C
Poly(<i>N,N</i> -diethylacrylamide)	25–32 °C
Poly(<i>N</i> -vinylcaprolactam)	25–35 °C
Poly(methyl vinyl ether)	37 °C
Poly[2-(dimethylamino)ethyl methacrylate]	50 °C
Poly(ethylene glycol) (PEG)	85 °C
Block co polymer of PEO and PPO	20–85 °C
Poly(pentapeptide) of elastin	28–30 °C
Polymer exhibiting UCST	
Polyacrylamide and polyacrylic acid	25 °C

hydrophilic (below LCST) as well as hydrophobic (above LCST) block of amphiphilic copolymers. With pNIPAAm as the hydrophilic block, the polymeric components employed as the hydrophobic block as found in literature, can be classified into three classes:

- (1) conventional hydrophobic, nonbiodegradable polymers such as polystyrene (PSt), poly(butyl methacrylate) (PBMA) and poly(methyl methacrylate) (PMMA);
- (2) biodegradable polymers, like poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL), or their copolymers;
- (3) polymers capable of imparting additional features e.g. those exhibiting pH sensitivity such as poly(10-undecenoic acid (PUA), polyoleic acid, poly[2-(diethylamino)ethyl methacrylate] (PDEA), poly(acrylic acid) (PAA) or methacrylic acid (MAA), etc. to impart dual sensitivity to the micelles (Wei et al., 2009).

Accordingly, pNIPAAm di-block copolymers with polymethacrylate (PMMA), polybutylmethacrylate (PBMA), polylactide (PLA), and polycaprolactone (PCL) as hydrophobic blocks have been synthesized and evaluated for various drug delivery applications (Wu et al., 2015; Wei et al., 2006b; Zhu et al., 2015b; Chung et al., 2000). Furthermore, linear triblock copolymers, graft copolymers, nonlinear star-shaped and Y-shaped copolymers, and dendritic unimolecular micelles have been synthesized based on pNIPAAm as the hydrophilic segment (Li et al., 2006; Wei et al., 2007; Luo et al., 2006).

Efforts directed at facilitating clinical use of pNIPAAm for the preparation of thermoresponsive micelles have revealed that the phase transition temperature, drug entrapment, and drug release can be tailored in several ways; for example,

copolymerization of PNIPAAm with *N,N*-dimethylacrylamide or methyl methacrylate was found to result in an upward shift in the LCST, to about 40 °C (Lakkadwala et al., 2015).

Other approaches, such as the tethering of functional groups such as trimethoxysilyl, which could be converted to crosslinks leading to the formation of shell crosslinked polymeric micelles (Wei et al., 2008) and the use of acidic comonomers or end caps such as undecanoic acid to render the resultant micelles sensitive to both pH and temperature (Wei et al., 2006a), are few of the additional strategies employed.

In the case of dual sensitivity of micelles to both pH and temperature, the sequence of influence of these two triggers on drug release has also been tailored. Accordingly, thermo- and pH-sensitive micelles composed of poly(10-undecenoic acid-*b*-*N*-isopropylacrylamide) (PUA-*b*-pNIPAAm) were prepared, where undecanoic acid (UA) represents the hydrophobic and pH-sensitive block. An in vitro drug release study revealed that, below the LCST of the amphiphile (30.8°C), the highly hydrated pNIPAAm shell surrounded the hydrophobic PUA core tightly, thereby deterring contact of carboxylic acid groups in PUA with water, rendering the micelles insensitive to pH changes. However, above LCST, pNIPAAm shell collapses, rendering the carboxylic acid groups exposed to the water so that responses to a change in pH can be elicited (Wei et al., 2006a). In contrast, Liu and coworkers have reported an interesting copolymer poly(*N*-isopropylacrylamide-*co*-*N,N*-dimethylacrylamide-*co*-2-aminoethyl methacrylate)-*b*-poly(10-undecenoic acid) (poly(NIPAAm-*co*-P(NIPAAm-*co*-DMAAm-*co*-aminoethyl methacrylate)-*b*-PUA), wherein the polymer possess LCST above physiological temperature at pH 7.4, which drops down at acidic pH, enabling micelle collapse and drug release in an acidic environment (Liu et al., 2007).

Tri-block copolymers with one block being pH-responsive, the second one being thermally responsive and a third hydrophobic block are explored for dual stimuli (pH and temperature)-responsive micelles. Active targeting by means of anchoring monoclonal antibodies (biotin, folate, etc.) to the pNIPAAm-based amphiphiles are also reported (Cheng et al., 2008; Liu et al., 2007).

Hydrolysis-activated micellar systems, based on modification of LCST of the thermosensitive amphiphilic copolymers by introducing hydrolysable side chains, have also been proposed. Block copolymer of PEG and poly(NIPAAm-*co*-(*N*-(2-hydroxypropyl) methacrylamide-dilactate) (poly(NIPAAm-*co*-(HPMAm-Lac), exhibits LCST at 15°C in phosphate-buffered saline. Hydrolysis of the labile ester bond in HPMAm-Lac, results in HPMAm which is a more hydrophilic monomer than NIPAAm. Consequently, the LCST of the hydrolyzed poly(NIPAAm-*co*-HPMAm) blocks was increased to about 45°C. As a result, the polymer became soluble at body temperature and underwent a shift from the hydrophobic (collapsed) to the hydrophilic (stretched) state (Neradovic et al., 2001).

Dual responsive micelles composed of double hydrophilic di-block copolymer, aptly named “schizophrenic micelles” because of complete inversion of core and corona according to the surrounding pH and temperature conditions, are reported

by Rao and coworkers. This unique micellization property was exemplified by micelles composed of polypeptide hybrid double hydrophilic block copolymer, based on pNIPAAm-*b*-poly(l-glutamic acid) (pNIPAAm-*b*-PLGA), wherein pNIPAAm-*b*-PLGA was able to self-assemble into micelles with pNIPAAm-cores at alkaline pH and elevated temperatures, whereas at acidic pH and room temperature, the self-assembly consists of PLGA core and pNIPAAm corona (Rao et al., 2007).

8.6.3 PHOTORESPONSIVE MICELLES

Light is an appealing stimulus, as it can be applied and controlled remotely from outside the body and can trigger on-demand drug release by simply switching on/off the light source. Moreover, the intensity and wavelength of light can be readily and precisely controlled. It can penetrate up to a certain extent into the skin and can be applied to a very precise area.

Photoresponsive polymeric micelles are formulated by incorporating functional groups (photochromophore) that undergo structural transformation upon irradiation with light of the appropriate wavelength. Light irradiation results in structural and consequent functional changes in the micelle. A photochromophore can be included in the backbone of an amphiphilic block copolymer or in the side chain. Chemistry responsible for the photosensitivity emerges from the ability of the photochromophore to absorb the photons of a certain wavelength range and initiate photoreactions such as isomerization, dimerization, rearrangement, or bond cleavage. The structural changes resulting from the photoreactions include: change in the hydrophilic/hydrophobic balance of the micelle forming block copolymer, cleavage of bonds in the main chain of polymer resulting in degradation of polymer, and/or reversible crosslinking in the polymer chains (Alatorre-Meda et al., 2013). These structural transformations bring about a release of the entrapped drug by diffusion or by complete disassembly of the micelles (Fig. 8.6). Depending upon the mechanism of photo reaction and resultant physicochemical changes in amphiphilic block polymer, the photo responsive micelles can be classified as those based on:

1. photoisomerization
2. photo-induced cleaving / photolysis
3. photo-induced rearrangement
4. photo-induced crosslinking
5. photo-induced energy conversion

Based on these photo reactions, several designs and variants of photosensitive micelles for drug delivery applications have been reported and representative examples from each class are discussed below. However, an exhaustive account of the literature can be found in a few excellent reviews written by Gohy and Zhao, and Huang et al. (Gohy and Zhao, 2013; Huang et al., 2014).

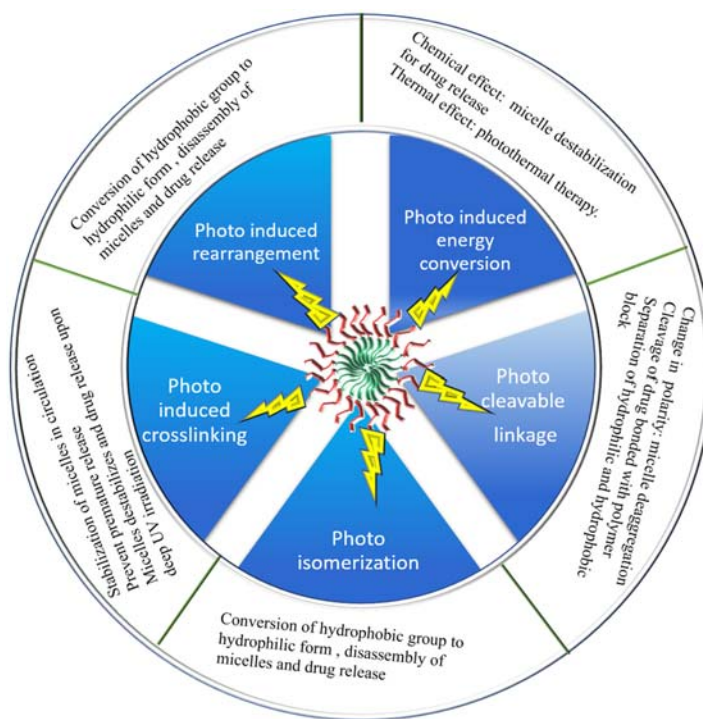
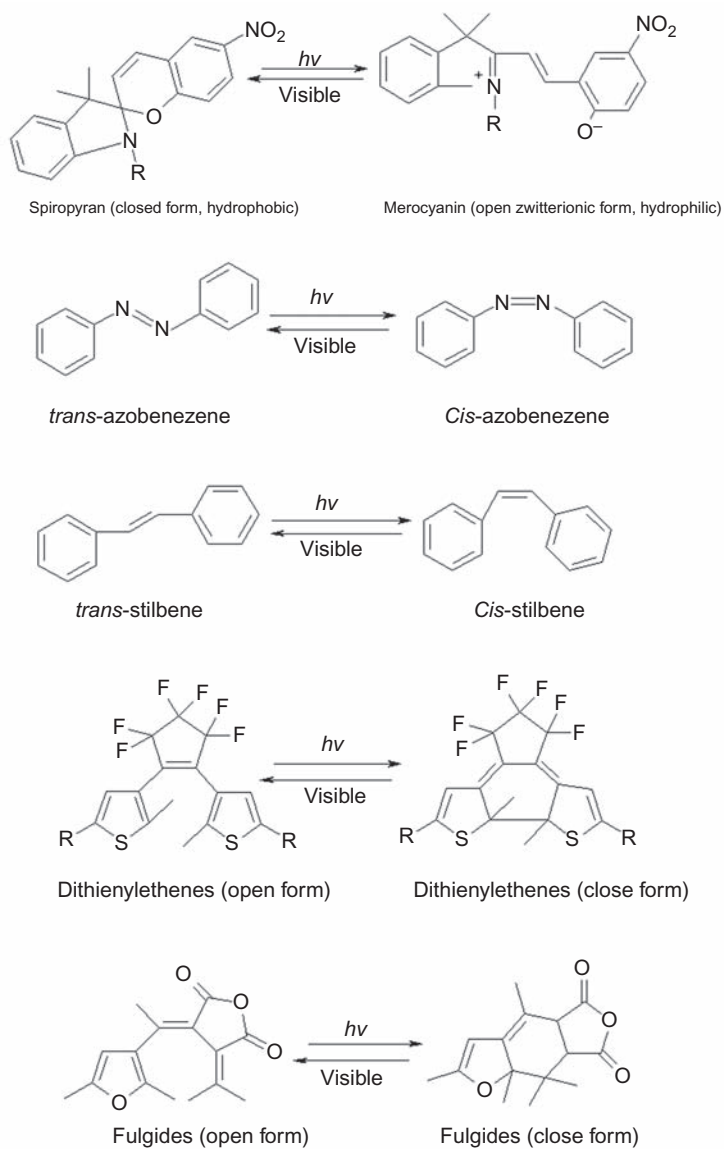
**FIGURE 8.6**

Photo reactions exerted by photoresponsive polymers and consequent drug delivery mechanism of micelles.

8.6.3.1 Photo-induced isomerization

Photoisomerizable groups such as azobenzene, spiropyran or dithienylethene, stilbene and fulgides are tethered to the block copolymer. The isomers are significantly different in their polarity, e.g., stilbene and azobenzene convert from hydrophobic trans form to hydrophilic cis form, upon irradiation by UV light (340–380 nm) (Fig. 8.7). This transformation is reversible upon irradiation by visible light (420–490 nm). Similarly, conversion of spiropyran to more hydrophilic merocyanine form results in disassembly of micelles, followed by the release of entrapped drug molecules.

Amphiphilic di-block copolymers composed of hydrophilic poly(*tert*-butylacrylate-co-acrylate) and hydrophobic poly(methacrylate) containing azobenzene in the side chain have been synthesized. The micelles formed in aqueous medium possess an azobenzene-containing hydrophobic core. Upon irradiation with light, the core-forming polymer is converted to its hydrophilic congener, which triggers disassembly of micelles and drug release (Harnoy et al., 2016).

**FIGURE 8.7**

Photochromophore and their isomer forms upon irradiation with light, commonly employed in preparation of photoresponsive micelles.

8.6.3.2 Photo-induced cleavage

Here, photosensitive links susceptible to lysis are incorporated in the micelle-forming amphiphilic block copolymer. In principle, the photochromophore can be incorporated either in the core forming hydrophobic block, at the hydrophobic hydrophilic interface, or in the hydrophilic corona forming blocks. Many investigations around this central idea have employed PEG as the hydrophilic group and polymethyl acrylate-photolabile group as the hydrophobic component (Jiang et al., 2006). Irradiation with light cleaves the side chain chromophore and results in generation of hydrophilic polymethacrylic acid. This change in the polarity of the hydrophobic core-forming block to a hydrophilic one results in disruption of micellar aggregates. A similar outcome may be brought about by insertion of a photocleavable group/link, such as o-nitrobenzyl esters or truxillic acid between the hydrophilic and hydrophobic fractions of the amphiphile (Cabane et al., 2010; Yang et al., 2010a). The photolabile groups imparting hydrophobicity to the core-forming polymer are typically pyrenyl methyl, o-nitrobenzyl, coumarin, or p-methoxy phenacyl derivative. Insertion of these groups in one of the blocks of copolymer using ester linkage imparts hydrophobicity to the block, which forms micelles when copolymerized with a hydrophilic block. However, upon irradiation with UV light, the photolabile groups cleaves from the hydrophobic block, rendering it hydrophilic and subsequent disruption of micellar structure and drug release. Schematic representation of such a strategy is shown in Fig. 8.8.

8.6.3.3 Photo-induced rearrangement type micelles

These types of photoreactive micelles are based on typical photo-trigger groups, such as the hydrophobic 2-diazo-1,2-naphthoquinone (DNQ) molecule which can be changed into a hydrophilic 3-indenecarboxylic acid (3-IC) molecule through a UV-induced Wolff rearrangement reaction. Accordingly, inclusion of DNQ as hydrophobicity-inducing moiety in the core of the micelle permits the conversion of the hydrophobic segment of the amphiphilic polymers into a hydrophilic one on exposure to specific light, resulting in the disassembly of polymeric micelles. An amphiphilic copolymer was prepared by modification of hydrophilic dextran, a highly water-soluble polysaccharide with excellent biocompatibility, through covalent attachment of the hydrophobic DNQ at the 2-hydroxyl of glucose units in dextran, as depicted in Fig. 8.9 (Huang et al., 2014). Irradiation resulted in conversion of the DNQ to IC, reverting the derivatized dextran back into a hydrophilic entity, thereby sacrificing the micellar structure to release drug.

8.6.3.4 Photo-induced cross-linkable polymeric micelles

While the previous approaches are aimed at destabilization of the micelle under appropriate conditions to facilitate drug release, the rationale behind the use of cross-linkable micelles is to stabilize the otherwise dynamic micellar structure and thereby prevent premature release of drug. Here, light-induced cross-linkable moieties are introduced into the amphiphile, e.g., cinnamic acid forms upon

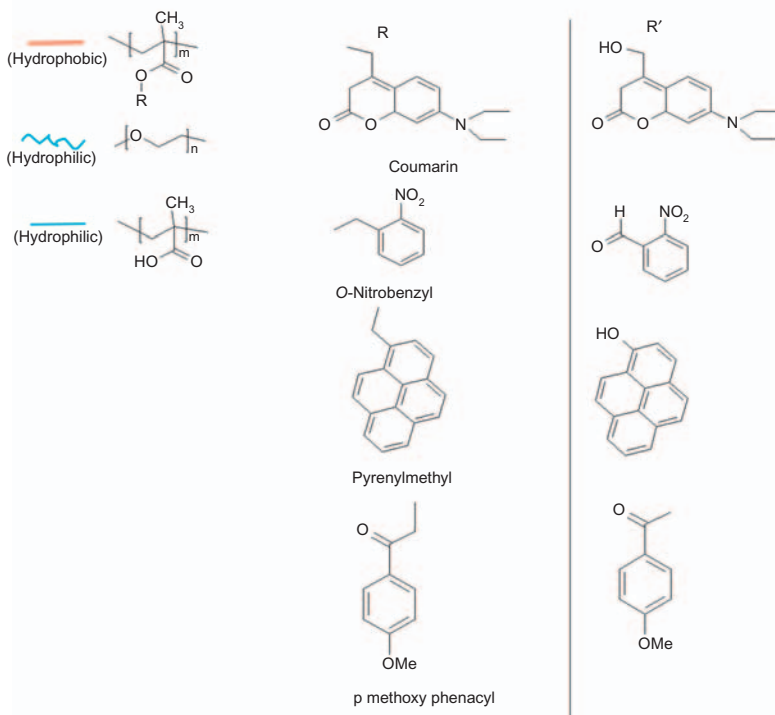


FIGURE 8.8

Schematic representation of irreversible disruption of micelles by light induced conversion of hydrophobic block to hydrophilic-mediated by photocleavable ester linkages.

illumination with UV, a dimer, truxillic acid, which in turn is photolabile under deep-UV light, giving back the original cinnamic acid. Shell cross-linkable micelles have been prepared by the self-assembly of polystyrene-block-poly(2-cinnamoyl ethyl methacrylate) with the polystyrene forming the core and the latter forming the shell in a tetrahydrofuran–acetonitrile medium (Ding and Liu, 1998). Besides this, coumarin-based photocross-linkable micelles can also be envisaged (Zhao et al., 2009). A light-reversible cross-linking adds additional possibilities to

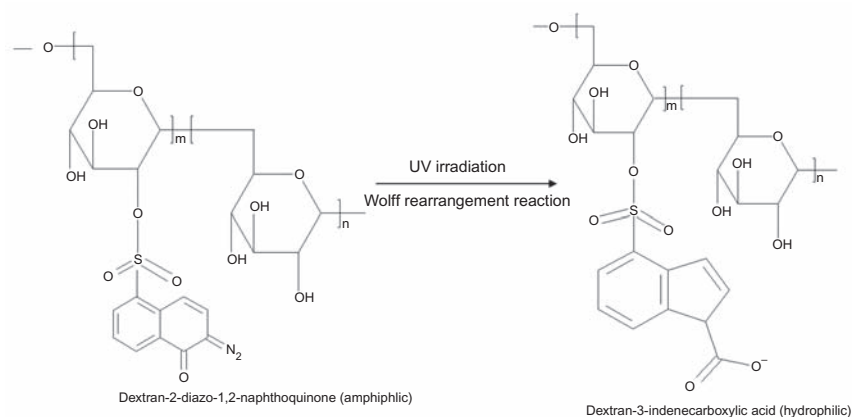
**FIGURE 8.9**

Photo-triggered conversion of dextran conjugated hydrophobic 2-diazo-1,2-naphthoquinone (DNQ) to hydrophilic 3-indenecarboxylic acid (3-IC), adapted from (Huang et al., 2014).

this strategy; while initial irradiation allows stabilization of the polymeric micelles via photo-crosslinking, subsequently, light can also be used to trigger release of encapsulated guest molecules through photo-induced disruption of the micelles.

8.6.3.5 Photo-induced energy conversion

These systems are designed to harness both the chemical effects as well as the heating effects of light on micellar structures. This approach is exemplified by the preparation of micelles composed of cholesteryl succinyl silane, loaded with doxorubicin as chemotherapeutic agent, Fe_3O_4 nanoparticles for magnetic resonance imaging, and further coated with gold nanoshell to enable photothermal therapy. Accordingly, the multifunctional objective of magnetic resonance imaging, magnetic-guided drug delivery, light-triggered drug release, and photothermal therapy could be achieved. While incorporation of Fe_3O_4 allows for MR imaging and magnetic targeting, the outer gold shell operates as light-absorbing agent, leading to an increase in local temperature for photothermal therapy (Ma et al., 2013).

Although several studies have been reported in the area of photosensitive polymeric micelles for drug delivery applications, most of the papers deal with *in vitro* work and the development of a clinically useful system is fraught with difficulties.

In case of the photocleavable systems, the extent of cleavage that occurs is not always sufficient to improve hydrophilicity to an extent resulting in disruption of micelles, although the extent of photoinduced polarity changes in the core-forming block may result in swelling/ shrinkage of the core. Furthermore, the

photo-induced changes are not limited to the intended changes, but may also result in morphological transitions, such as change in micelle size, swelling of core, etc. The overall effect of light depends on other parameters, such as molecular weight of polymer and length ratio of hydrophilic and hydrophobic blocks. Similarly, other features, such as the kinetics of chain reorganization that might be considerably slow in the case of high glass transition “frozen” micellar cores, could also play a crucial role (Gohy and Zhao, 2013). A major hurdle in *in vivo* applications of these systems is also the limited knowledge garnered regarding the biodegradability and biocompatibility of the selected photolabile systems and the toxicity of the products resulting from the photo-induced reactions. Several of the reported studies have measured the photosensitivity of the micelles under *in vitro* conditions and also in nonaqueous environments, which is far removed from physiological conditions, making the transit from bench to bedside a long, drawn-out process.

An additional issue is that of damage to living tissue due to irradiation. While UV light has been the source of energy in most of the studies, it is too harmful for *in vivo* use. Consequently, recent developments are aimed at enabling the use of near-infrared laser radiation to bring about the changes, wherein two photons of the lower energetic IR rays are absorbed in place of a single UV photon (Goodwin et al., 2005).

As a result of these issues hindering the successful development of photosensitive micelles, multiple approaches have been simultaneously undertaken, such as the use of pH or temperature sensing elements, in addition to the photosensitive approach. This was demonstrated by micelles composed of poly(ethylene oxide) block-poly(ethoxy tri(ethyleneglycol) acrylate-co-o-nitrobenzylacrylate with an LCST of 25°C. The hydrophobicity is increased by irradiation as a result of the cleavage of o-nitrobenzyl moiety. This consequently results in an increase in LCST by 10°C, micelles are thus dissociated, releasing the entrapped dye (Jiang et al., 2008).

With the growing options of photochromic moieties and the ever-increasing list of polymeric systems, the pharmaceutical scientist as well as those in other fields of research such as agriculture, material sciences, cosmetics etc., are certain to evolve useful systems based on photoresponsive micelles.

8.6.4 MISCELLANEOUS

Additional promising, albeit relatively less frequently explored, strategies for developing stimuli-responsive micelles, include the use of ultrasound, enzymatic activity, or redox differences to trigger drug release. Ultrasound as a stimulus can penetrate deep in the body in a focused manner and can facilitate absorption and membrane passage of drugs. Ultrasound with a frequency higher than 20 kHz can be employed as an external trigger to generate ultrasonic waves in tissue (Zhao et al., 2013). In general, exposure of tissue to ultrasound results in two effects: thermal effects (hyperthermia, due to absorption of energy), and nonthermal

effects associated with cavitation. Although a number of studies for ultrasound-triggered drug release are based on polyoxyethylene-polyoxypropylene block copolymers (Gao et al., 2004; Marin et al., 2002), micelles composed of polyoxyethylene (ethylene oxide) and poly(2-tetrahydropyranyl methacrylate) (PEO-*b*-PTHPMA) in aqueous solution have also been demonstrated to be disrupted by high-frequency ultrasound (1.1 MHz) (Wang et al., 2009).

Identical to the rationale for the preparation of pH-sensitive micelles for delivery of drug to tumor, which makes use of the fact that the pH within the tumor environment is lower, other characteristic features of tumors, such as the presence of certain enzymes or the difference in redox potential have also served as means of eliciting a response from appropriately designed polymeric micellar vehicles. Enzyme-responsive micelles are attractive due to the higher substrate specificity of their catalytic activity. Furthermore, disease-associated expression of certain enzymes makes it an amenable approach. Enzymes overexpressed in tumors include certain proteases (cathepsins, matrix metalloproteases), glucuronidases, cytochrome P450, glycosidase such as amylase, etc. (Rodriguez-Antona and Ingelman-Sundberg, 2006; Su et al., 2014). Membrane-bound alkaline phosphatase is also overexpressed in tumor cells (Sharma et al., 2014). A clear synthesis strategy for the fabrication of enzyme responsive micelles was demonstrated by Harnoy and coworkers, wherein the amphiphile was composed of hydrophilic PEG and a core-forming stimulus-responsive dendron-containing hydrophobic end group, which could be cleaved by a specific enzyme, thus destabilizing micellar aggregates and enabling release of encapsulated cargo. This was exemplified by attaching phenyl acetamide end groups on dendrons that could be cleaved by penicillin G amidase. In the presence of enzyme, the micellar disassembly was evident by release of entrapped dye, Nile red (Harnoy et al., 2014).

Micelles composed of polymer peptide block copolymer having suitable substrate for enzymes, namely protein kinase A, protein phosphatase, and matrix metalloproteinases were demonstrated to undergo morphological transition upon enzymatic activity (Ku et al., 2011).

Finally, micellar systems have also been designed based on the difference of the redox potential between the oxidative extracellular and the reductive intracellular space, a difference arising due to the difference in glutathione concentration (Bui et al., 2015; Li et al., 2015; Zhu et al., 2015a).

8.7 DIAGNOSTIC APPLICATIONS

A large proportion of the diagnostic tools available for detection of pathophysiological disorders comprises of imaging techniques, which provide noninvasive assessment of anatomical, functional, and molecular information, thus aiding in diagnosis of various disease conditions. The technological advances in these imaging techniques coupled with the development of new molecules,

radiopharmaceuticals, and contrast agents have allowed identification of molecular targets responsible for progression of disease and enabled visualization of disease process and therapy at molecular level. Since these techniques are based on signal emitted from the tissue/organ of interest, the intensity of such signals should be sufficient to differentiate between surrounding area (noise) and tissue structure under investigation (signal). Contrast agents are employed during imaging for their ability to adsorb signal more strongly than neighboring tissues. Clearly, such an application requires sufficient concentration of contrast agent/radiopharmaceutical/diagnostic agent in the target area. Several reports have suggested the ability of polymeric micelles to enhance visualization through noninvasive diagnostic modalities such as MRI, positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography (CT), ultrasound and optical imaging (Xiao et al., 2012). In this context, targeted delivery of diagnostic agents using micelles enables restriction of its distribution to the area of interest thereby improving specificity of emitted diagnostic signals. Owing to the properties of polymeric micelles elaborated at the outset, these nanocarriers remain a suitable vehicle for attaining targeted delivery of diagnostic agents, however, it is noteworthy that while it is acceptable for a drug-carrying vehicle to remain in the body for relatively prolonged periods of time, in the case of products being developed for diagnostic purposes, rapid biodegradation and/or elimination is desirable because these are not only administered to patients but also to the healthy individuals (Kiessling et al., 2014).

In the recent past, the paradigm is shifting towards the concurrent administration of therapeutic and diagnostic agents and so-called theranostic applications are gaining importance in real-time monitoring of therapy. Integration of molecular imaging and therapeutic application requires the design of the multifunctional micelles that can facilitate delivery of both therapeutic as well as diagnostic agents. Such theranostic micelles are not only useful for disease diagnosis but also valuable in noninvasive tracking of delivery system following administration. They can accordingly prove useful in the conduct of pharmacokinetic studies, i.e., in the tracing of distribution, metabolism, and excretion of drug in the carrier system (Ding and Wu, 2012). A few investigations of theranostic application of polymeric micelles have been listed in Table 8.3.

All the stimuli-responsive micellar systems, including pH-responsive, thermo-responsive, photoresponsive systems described earlier, as well as the systems developed based on multiple stimuli can also serve as agents for delivering diagnostic agents. Applications of micelles as carriers in different imaging modalities and those developed for theranostic purposes are discussed below with representative examples.

8.7.1 NUCLEAR IMAGING

This diagnostic modality is based on the visualization of gamma-emitting isotopes. The image may be garnered using either of the two techniques: single photon

Table 8.3 Stimuli-Responsive Micelles Envisaged for Therapeutic and/or Diagnostic Applications

Sr. No.	Composition of Micelles (Amphiphilic Block Copolymer)	Stimulus	Diagnostic Agent	Therapeutic Agent	Results	Ref.
1.	Maleimide-terminated PEG-block-poly(D,L-lactide) and methoxy-terminated PEG -block-poly(D,L-lactide) copolymer.	pH	SPIO nanoparticles	Doxorubicin	In vitro MRI and cytotoxicity studies demonstrated the ultrasensitive MRI imaging and cytotoxic response.	Nasongkla et al. (2006)
2.	Methoxy PEG as a hydrophilic component, and poly(L-amino ester) as a pH- sensitive segment	pH	Fe ₃ O ₄	—	Simultaneous targeting and imaging of the acidic pathologic tissue in both tumor and ischemic tissue was achieved in vivo.	Gao et al. (2010b)
3.	Oligo(ethylene glycol) methyl ether methacrylate- <i>b</i> -poly(2-(diisopropylamino)ethyl methacrylate-co-glycidyl methacrylate)	pH	Gd as MRI contrast agent and tetrakis[4-(2-mercaptoethoxy) phenyl]ethylene as fluorophore	Camptothecin	Acidic endosomal pH triggeres the drug release. The internalization was monitored by fluorescence, enhanced MRI contrast was achieved.	Tian et al. (2016b)
4.	PEG-block-poly(aminolated glycidyl methacrylate)-block-poly (2-(diisopropyl amino) ethyl methacrylate)	pH	—	Cisplatin and siRNA	Drug release in acidic endosomal pH. Tumor growth inhibition and suppression of metastasis achieved by down regulating NF-kappa B.	Yu et al. (2016a)
5.	Boltorn(®) H40-poly(L-glutamate-hydrazone-doxorubicin)- <i>b</i> -PEG	pH	⁶⁴ Cu-labeling and PET imaging	Doxorubicin cRGD for active targeting	pH-sensitive drug release, higher accumulation at tumor site was measured by PET imaging.	Xiao et al. (2012)
6.	Pluronic-lactose modified chitosan-spiropyran/boronic acid-conjugated poly(dimethylamino ethyl methacrylate-co-methacrylic acid)	pH/ redox/ photo	Fluorescence imaging	Paclitaxel	Drug release in mildly acidic and high reductive condition.	Lee et al. (2014)

emission computed tomography (SPECT) or positron emission tomography (PET), where the former uses radioactive isotopes such as technetium-99 (^{99m}Tc), indium-111 (^{111}In) and iodine-125 (^{125}I), whereas the latter employs isotopes such as fluorine-18 (^{18}F), copper-64 (^{64}Cu), and zirconium-89 (^{89}Zr).

Nuclear imaging is highly sensitive as it requires very low concentration of the imaging agent (10^{-10}M) at the tissue/organ site to be imaged (Oerlemans et al., 2010). With the move towards theranostic applications, use of isotopes with relatively longer half-life is desirable for longer period of imaging. Consequently, use of isotopes such as ^{64}Cu , ^{89}Zr , ^{67}Ga is increasing. These metal isotopes can be incorporated into polymeric micelles using chelating agents and this is the chief strategy in the preparation of polymeric micelles for targeted delivery of radioisotopes. Hydrophilic chelating agents such as diethylenetriamine pentaacetic acid (DTPA), (1,4,7-triazacyclononane- N,N',N'' -triacetic acid (NOTA) or 1,4,7,10-tetraazacyclodecane-1,4,7,10-tetracetic acid (DOTA) are conjugated to the micelle forming polymer. DTPA acid is by far the most popular chelating agent, as it can be linked to any amine-containing moiety using the commercially available DTPA anhydride (Trubetskoy, 1999). Additionally, labeling of micelles with ^{99}Tm can be achieved using N -(N -(3-diphenylphosphinopropionyl) glycyl) cysteine linker (Visentin et al., 2004). These hydrophilic chelating agents are tethered to the corona and are found to result in unfavorable changes in the biodistribution and pharmacokinetic properties of the micelles (Patil et al., 2011). The problem is circumvented by incorporating a lipophilic ligand, such as tropolane, that undergoes deprotonation to complex with the metal cation. The tropolane-radionuclide complex is entrapped in the hydrophobic core of the micelles, leaving the corona unaffected (Laan et al., 2016).

An example of a pH-responsive system developed for theranostic application has doxorubicin as the therapeutic agent conjugated via an acid labile hydrazone moiety and ^{64}Cu as the diagnostic aid, integrated using the macrocyclic chelator (1,4,7-triazacyclononane- N,N',N'' -triacetic acid) [NOTA] to enable PET tracking. The micelles are composed of the hyperbranched amphiphilic polymer, Boltorn H40-poly(L-glutamate-hydrazone-doxorubicin)- b -poly(ethylene glycol) and are also tethered with cyclic peptide cRGD (Arg-Gly-Asp-D-Phe-Cys) for active tumor targeting towards integrin $\alpha_v\beta_3$ receptors. Positron emission tomography imaging showed higher accumulation of micelles at the tumor site (Xiao et al., 2012).

8.7.2 MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging is based on magnetization of hydrogen nuclei under the influence of a strong magnetic field. It is a powerful diagnostic tool, owing to the fact that it can report small physiological changes in the tissue (Zhu et al., 2015b). However, the application of MRI is limited, due to intrinsic low sensitivity of the signal detection (Zhu et al., 2015b). Chemical exchange saturation transfer (CEST) is used to enhance the contrast between protein bound water and bulk water by proton exchange between them (Zhang et al., 2013). Paramagnetic

substances, such as superparamagnetic iron oxide nanoparticles (SPIO), manganese, gadolinium, dysprosium, europium (III), and holmium, are used as contrast agents that give stronger MRI signals than surrounding tissues.

The use of polymeric micelles to improve MRI signals is based on either of two approaches: the encapsulation of SPIO in micelles or complexation of paramagnetic metals to the hydrophilic block of micelle-forming block copolymers using suitable chelating agents.

Thermoresponsive mixed micelles composed of pNPAAM block methyl methacrylate copolymer, wherein two types of unimers composed of short chain and long chain pNPAAM thermosensitive hydrophilic block, were employed in the fabrication of mixed micelles. These micelles were intended to signal the presence of a tumor through phase transition across the LCST of the polymer. The premise here is that the tumor microenvironment is at a slightly elevated temperature in comparison to the rest of the body. The short chain of micelle-forming polymer was modified by linking the paramagnetic CEST contrast agent, europium(III) tetra amide complex (Eu-DOTAmCE). The core was formed by hydrophobic poly-methyl methacrylate, while the short and long hydrophilic pNIPAAm forms the corona. The pNIPAAm corona exhibited a thermo-responsive phase transition in aqueous solution. The hydrophilic chains remained stretched at temperature lower than the LCST, so that the contrast agent is embedded within the micellar corona. However, at a temperature above the LCST, the hydrophilic chains aggregate and therefore the short chains modified with Eu-DOTAmCE are exposed to the external aqueous medium, allowing the interaction of PARACEST agent with bulk water. In this way, temperature-induced MRI signal modulation is achieved (Zhu et al., 2015b).

Iron oxide nanoparticles are highly effective as MRI contrast agents and stimuli-responsive micelles to target these to the tissue of interest (e.g., pH-responsive to tumors and ischemic tissues) have been prepared and evaluated in vivo studies with promising results (Gao et al., 2010b).

pH-responsive micelles composed of methoxy poly(ethylene glycol)-*b*-poly(L-histidine) (PEG-p(L-His)) and methoxy poly(ethylene glycol)-*b*-poly(L-lactic acid)-diethylenetriaminopentaacetic acid dianhydride-gadolinium chelate (PEG-p(L-LA)-DTPA-Gd) with a size of ~40 nm have been prepared by Kim et al. The micelles destabilize in the acidic microenvironment of the tumor due to protonation of imidazole group of histidine blocks, leading to disruption of micellar structure and enabling detection of small tumors (Kim et al., 2014).

8.7.3 X-RAY COMPUTED TOMOGRAPHY

X-ray computed tomography is based on differential absorption of X-ray by different tissues to enable distinction between different anatomical structures. X-ray absorbing heavy metals such as iodine, barium and bromine are employed as contrast agents for high spatial and temporal resolution. The major drawback of this imaging modality is a requirement of higher concentration of the contrast agent

(10^{-2} M), however this can be overcome by targeting the contrast agent using micelles as carrier systems (Oerlemans et al., 2010). Iodine-loaded long-circulating micelles composed of poly(L-lysine)-PEG have shown enhanced contrast in imaging of aorta, heart, liver, and spleen in CT scan (Torchilin et al., 1999).

8.7.4 FLUORESCENCE-BASED IMAGING

Fluorescence-based imaging is highly useful for in vitro and in vivo applications, as it allows for tracking of the nanocarriers using a variety of imaging techniques, such as fluorescent microscopy, confocal laser scanning microscopy, and two-photon microscopy. In vivo biodistribution of nanoparticles, cellular internalization, and intracellular trafficking can be visualized using fluorescence microscopy.

Fluorescent nanocarriers are designed by loading micelles with fluorescent dyes or inorganic fluorescent nanoparticles such as quantum dots. Organic dye-based dual (pH and thermo)-responsive polyion complex micelles composed of copolymers, poly(*N*-isopropylacrylamide-co-*N*-hydroxymethylacrylamide)-*b*-poly(L-lysine) and poly(*N*-isopropylacrylamide-co-*N*-hydroxymethylacrylamide)-*b*-poly(glutamic acid), were designed by Liu and coworkers. The core was formed by electrostatic interaction between cationic poly(L-lysine) and anionic poly(glutamic acid). As the electrostatic interaction is pH-dependent, the core was susceptible to pH changes. However, the shell composed of poly(*N*-isopropylacrylamide-co-*N*-hydroxymethylacrylamide) imparted thermo-responsiveness to the micelles. Two fluorescent dyes, green 5-aminofluorescein and red Rhodamine B, were encapsulated in the micelles. The micelles showed intense pH- and thermo-responsive fluorescence when observed via confocal laser scanning microscopy. Accordingly, multicolor fluorescent imaging techniques may find utility in bioimaging of complicated tissues and cellular microenvironments (Liu et al., 2012).

However, the major drawback of using fluorescent agents is the limited photostability of organic fluorescent dyes and their dissociation from the nanocarriers. The high toxicity profile of metallic fluorescent nanoparticles limits their applications (Chen et al., 2015). To overcome these limitations, self-fluorescing micelles composed of a core-forming hyperbranched polymer Boltron®H40, conjugated to a photo luminescent polymer and PEG has been designed with tumor-targeting ligand cRGD peptide (Chen et al., 2015). These self-fluorescent micelles represent an effective tumor-targeting theranostic probe.

8.8 CONCLUSION

Developments in the area of polymeric sciences is driving progress in drug delivery and vice versa. Newer methods of polymer preparation, such as the use of click chemistry, is making it feasible to engineer the structure and thereby the

properties of polymeric materials precisely. These advances have paved the way for preparation of smarter amphiphilic polymers capable of self-association into micellar structures which can respond to appropriate stimulus. The promising results of studies both at in vitro and in vivo levels indicate that these nanovehicles with tunable properties stand a strong potential to serve as the next generation drug delivery systems.

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Design of targeting peptides for nanodrugs for treatment of infectious diseases and cancer

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CHAPTER OUTLINE

9.1 Introduction	344
9.2 Targeting	344
9.2.1 Passive Targeting.....	345
9.2.2 Active Targeting.....	346
9.3 Targeting Ligands	348
9.3.1 Monoclonal Antibodies.....	348
9.3.2 Antibody Fragments.....	349
9.3.3 Nanobodies as Targeting Therapeutics and Targeting Ligands	349
9.3.4 Aptamers.....	350
9.3.5 Proteins.....	350
9.3.6 Peptides	351
9.4 Nanomedicine for Targeted Delivery of Natural Compounds	353
9.4.1 Paclitaxel Nanodelivery Systems.....	353
9.4.2 Peptide-Targeted Paclitaxel-Loaded Nanoparticles.....	354
9.5 Nanomedicine and Infectious Diseases.....	355
9.6 Identification of Peptide-Based Targeting Ligands.....	356
9.6.1 Selection of Targeting Peptides From Biological and Chemical Libraries ...	356
9.7 Computational Design of Peptide Ligands	358
9.7.1 The Concept of Long-Range Intermolecular Interactions in Biological Systems	359
9.8 Nanoinformatics, Databases and Targeting Information.....	369
9.9 Conclusion	370
Acknowledgments	371
List of Abbreviations	371
References	371
Further Reading	379

9.1 INTRODUCTION

Nanomedicine is the application of nanotechnology in medicine for the diagnosis and treatment of disease. Progress in the field made extensive impact on healthcare, resulting in more than 200 nanomedicine products that have been either approved or are under clinical investigation (Etheridge et al., 2013). Nanomedicine applications include drug delivery, in vitro diagnostics, in vivo imaging, therapy techniques, biomaterials, and tissue engineering (Shi et al., 2010). Advancement in nanomedicine has shown that nanoparticles (NPs) have great potential as drug carriers. Until now, drug delivery system engineering has been the priority in nanomedicine research (Sun et al., 2014). Significant application of nanotechnology to drug delivery systems had accounted for more than 75% of overall sales in nanomedicine (Wagner et al., 2006). The global market trend for NPs in biotechnology, drug development, and drug delivery was valued at US\$17.5 billion in 2011 and is estimated at US\$53.3 billion in 2017 (Baptista et al., 2015). Concurrently, the number of patents concerning NP-based drug delivery systems from 2009 to 2012 had 185% growth rate in the field of cancer-targeting nanoparticle technology (Martins et al., 2013). Application of nanotechnology in drug delivery systems could ease reformulation of existing drugs on the market. It can extend the lifetime of products, and improve performance of drugs, reflected by increased effectiveness, safety, and patient adherence (Bamrungsap et al., 2012). Various nanoforms have been applied as drug delivery systems, from biological substances, such as albumin, gelatin and phospholipids for liposomes; to substances of a chemical nature, such as various polymers and solid metal-containing NPs. Drugs could be absorbed or conjugated on the particle surface, encapsulated inside the polymer/lipid or dissolved inside the particle matrix. Many of the pharmacological properties of conventional or free drugs (i.e., drugs that are not encapsulated within or bound to any other compound or delivery system) can be improved through the use of drug delivery systems such as bioavailability, pharmacokinetics, and therapeutic efficacy, while at the same time leading to the reduction of side and toxic effects. Advantages of nanodrugs also include improvement of the stability and solubility of hydrophobic drugs (MaHam et al., 2009; Bamrungsap et al., 2012).

More recently, theranostic multifunctional NPs, with both diagnostic and therapeutic potential emerged, representing a promising technological advancement towards personalized medicine (Thakare et al., 2010). These multifunctional NPs can be externally modified with targeting peptides to simultaneously carry a drug with an imaging probe to monitor therapy.

9.2 TARGETING

The primary goal of nanomedicine is to improve clinical outcomes. The central promise of targeted drug delivery technologies is improved efficacy by increasing

drug concentration at the preferred (or target) site, while simultaneously minimizing toxicity by reducing off-target accumulation (Cheng et al., 2015). Targeted drug delivery may be accomplished through passive or active mechanisms. Active targeting can be defined as targeting mediated by specific receptor–ligand interactions. For active targeting, ligands are usually attached to the NP surface to enhance cell- or tissue-specific binding and NP uptake through receptor-mediated endocytosis (Bazak et al., 2015).

9.2.1 PASSIVE TARGETING

Passive targeting occurs due to the physical properties of an NP, such as surface charge or size, that decrease NP opsonization and phagocytic elimination, thereby improving NP circulation time (Cheng et al., 2015). Accumulation of nanocarriers, preferentially at tumor, inflammatory and infectious sites, is due to the enhanced permeability and retention (EPR) effect, first reported by Matsumura and Maeda (1986). The EPR effect implicates site-specific characteristics, which are not connected to normal tissues or organs, and result in clinical improvements by reducing the toxicities and increasing the drug response. The EPR effect is due to leaky tumor blood vessels as a result of the discontinuity of endothelium and poor lymphatic drainage which allows NPs to extravasate (escape) to the extracellular matrix and accumulate in tumor interstitium (Jang et al., 2003; Martins et al., 2013). In comparison, microvessels in most normal tissues (with the exception of kidney and liver) are less leaky; the tight junctions between endothelial cells are usually less than 2 nm, while the pore size in postcapillary venules is larger, at up to 6 nm. The fenestrated endothelium of the kidney glomerulus and the sinusoidal endothelium of the liver and spleen have larger pore sizes of 40–60 and 150 nm, respectively (Jang et al., 2003). In contrast, tumor tissues have an increased permeability to macromolecules and colloidal carriers of diameter up to 600 nm. This feature allows NPs to easily extravasate into the interstitial matrix at the tumor site, reducing drug distribution and toxicity to normal tissues. In normal tissues, lymphatic vessels are more permeable to macromolecules than the blood capillaries, facilitating the removal of these molecules from interstitial spaces.

While passive targeting facilitates localization of NPs at the target site, within the tumor microenvironment it cannot promote their uptake by cancer cells (Bazak et al., 2015).

Solid tumors depend on rapid angiogenesis to keep sufficient supplies of nutrients and oxygen (Bergers and Benjamin, 2003). The rapid proliferation of endothelial cells during angiogenesis usually results in a reduced density of endothelial cells, loss of tight junctions and formation of large gaps between the cells in the size range of 100–800 nm, depending on the type and stage of a tumor (Haley and Frenkel, 2008). Only NPs with a size smaller than the gap between adjacent endothelial cells can extravasate from the blood vessels into the tumor interstitium. The nanometer size range of therapeutic molecules is

responsible for concentrating the drug in the tumors. Particles smaller than 5 nm, intravenously injected, are removed from the blood by rapid renal clearance through the kidneys, while large microsized particles are filtered mechanically by the sinusoids and cleared by the reticuloendothelial system (RES) of the liver and spleen (Petros and DeSimone, 2010). Nanoparticles with sizes between 30 and 200 nm are believed to be optimal for passive targeting of most types of solid tumors using EPR effect and those larger than 200 nm were cleared from the blood stream more rapidly by the spleen (Jain and Stylianopoulos, 2010). Once extravasated, the penetration of NPs into the tumor tissues is a diffusion-mediated process, which is inversely correlated with the particle size (Sun et al., 2014). For macromolecules or polymer conjugates, the molecular weight >40 kDa is preferred to benefit from the EPR effect (Maeda et al., 2000). Longer circulation times are also necessary for the passive targeting of nanotherapeutics in the tumors by the EPR effect and strategies were developed to improve the circulation times of the NPs using surface hydrophilicity-imparting agents like poly(ethylene glycol) (PEG) (Romberg et al., 2008). The PEGylated adjuvants prolong circulation and reduce side effects, such as allergies and toxicities.

It is reported that numerous vascular mediators that facilitate the EPR effect, including bradykinin, nitric oxide (NO), peroxynitrite (ONOO⁻), prostaglandins, angiotensin-converting enzyme inhibitors, vascular endothelial growth factor (VEGF), and numerous other cytokines, are also mediators of inflammatory processes (Kamaly et al., 2012; Maeda et al., 2013). Consequently, it is not unexpected that the EPR effect is also involved in many inflammatory diseases (Kamaly et al., 2012; Maeda et al., 2013).

9.2.1.1 *Passively targeted nanoplatforms approved for clinical use*

Doxil (Caelyx in Europe), PEGylated liposomal doxorubicin, was the first FDA-approved nanodrug in 1995, with prolonged drug circulation time and avoidance of the RES, due to the use of PEGylated nano-liposomes (Barenholz, 2012). Doxil is passively targeted to tumors due to EPR effect. A very important improvement, when compared to conventional doxorubicin, is the dramatic reduction of cardiotoxicity which allows increasing the accumulated dose and thereby extending the treatment duration (Barenholz, 2012). Other passively targeted nanomedicines approved for clinical use for cancer treatment include: Myocet (non-PEGylated liposomal doxorubicin), DaunoXome (non-PEGylated liposomal daunorubicin), Depocyt (non-PEGylated liposomal cytarabine), Abraxane [albumin-based paclitaxel (PTX)], Genexol-PM (PTX-containing polymeric micelles, approved in Korea), Oncaspar (PEGylated L-asparaginase) and most recently, Onivyde nanoliposomal formulation of irinotecan (Jin et al., 2014; Hare et al., 2017).

9.2.2 ACTIVE TARGETING

Active targeting, also described as ligand-targeting or receptor-mediated targeting includes the attachment of ligands, such as small molecules, peptides, proteins, antibodies, antibody fragments, and nucleic acids (e.g., aptamers) to the surface

of NPs to improve their targeting efficiency. This targeting mode allows the NP to bind to the surface of a specific type of cells efficiently *in vitro* and to a certain extent *in vivo* (Sun et al., 2014). Specific interactions between the targeting ligands on the surface of nanocarriers and receptors expressed on the tumor cells may facilitate NP internalization as a result of receptor-mediated endocytosis, but only after the NPs have extravasated from the vasculature (Nori et al., 2003). In this way, active targeting can help to achieve a higher intracellular drug concentration (Piollo and Chang, 2008). The increase in intracellular drug concentration can increase cellular cytotoxicity and improve the therapeutic efficacy of drugs with intracellular targets. Contrary to expectations, the improvement in tumor accumulation attained through the introduction of an active targeting ligand is disputable (Piollo and Chang, 2008). Mostly, such approaches do not increase the overall concentration in the target tissue, but rather change the distribution within the tissue. An important exception is active vascular targeting for which extravasation is not required and therefore does not represent the rate-limiting step (Crielaard et al., 2012). Active vascular targeting has been shown as a promising option for tumor targeting and numerous active vasculature targeting approaches using NPs have emerged (Siegler et al., 2016). In this case, the targeting ligand is of crucial importance, as tumor accumulation is no longer determined by the EPR effect, but by the binding affinity to blood vessels. By targeting and killing the endothelial cells of the tumor vessels, tumor cells are eradicated by cutting off their supplies of oxygen and nutrients (Neri and Bicknell, 2005). NPs have been developed for various targets on the vascular walls, such as fibronectin extra-domain B, large tenascin-C isoforms, integrins, annexin A1, and VEGFs receptors (Neri and Bicknell, 2005).

In addition, active targeting has allowed overcoming a number of hindrances such as a drug delivery across blood–brain barrier (Leonor Pinzon-Daza et al., 2013) and one of the most common forms of multidrug resistance (MDR) caused by drug efflux transporters such as the P-glycoprotein, frequently overexpressed in cancer cell (Yu et al., 2010; Talekar et al., 2011). P-glycoprotein, extrudes a number of positively charged xenobiotics out of the cell, including some of the commonly used anticancer drugs (Krishna and Mayer, 2000).

9.2.2.1 Actively targeted nanoplatfoms in clinical trials

Many clinically approved NP formulations are used in treating various cancers at various stages. All of these formulations are passively targeted, besides the confirmed advantages of active-targeting in preclinical studies (Byrne et al., 2008; Anselmo and Mitragotri, 2016). To date, only a few targeted NPs have reached the stage of clinical development and none have been clinically approved (Kamaly et al., 2012; Anselmo and Mitragotri, 2016). One particular formulation (Cornell Dots) in clinical trial, using integrin-targeting RGD-based peptides, may point toward a NP future based on personalized medicine (Anselmo and Mitragotri, 2016). A first-in-human clinical trial of inorganic hybrid NPs, Cornell dots (C dots), in patients with metastatic melanoma for the imaging of cancer,

suggested safe use of these particles in human cancer diagnostics. These silica particles were labeled with radioactive iodine for positron emission tomography (PET) imaging and modified with cRGDY peptides for molecular targeting. The particles are inherently fluorescent, containing the dye, Cy5, so they may be used as hybrid PET-optical imaging agents for lesion detection, cancer staging, and treatment management in humans. Other active-targeted NPs in clinical trials introduce antibody targeting (HER2, EGFR, or transferrin receptor targeting) (Anselmo and Mitragotri, 2016).

9.3 TARGETING LIGANDS (FIG. 9.1)

9.3.1 MONOCLONAL ANTIBODIES

Monoclonal antibodies (MAbs) are commonly used as targeting ligands. MAbs have been conjugated to different types of NPs, such as Superparamagnetic iron oxide nanoparticles (SPIONs), quantum dots, liposomes, and Au nanocages, providing them with site-specific targeting ability (Sun et al., 2014). Conjugation of poly(lactic acid) to trastuzumab and rituximab, mAbs currently in the clinic, resulted in conjugates that exhibit a six-fold increase in the rate of particle uptake, compared to related particles lacking Mab-targeting molecules (Nobs et al., 2006; Kamaly et al., 2012). However, MAb-targeted NPs have many limitations, due to immunogenicity, rapid elimination, poor stability, redundant constant region, and large molecule size (approximately 150 kDa); when conjugated to NPs, the increase in overall size is up to 30 nm (Kamaly et al., 2012; Richards et al., 2017).

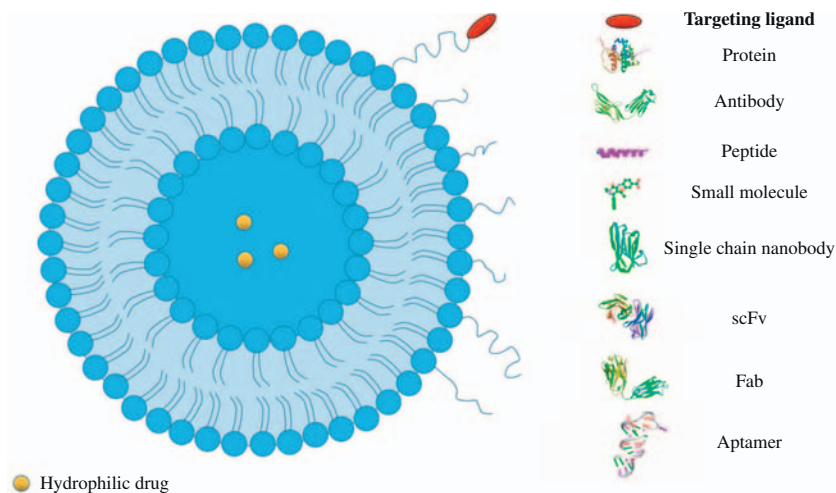


FIGURE 9.1

Schematic presentation of targeting ligands conjugated to a liposomal NP.

9.3.2 ANTIBODY FRAGMENTS

Antibody fragments, comprising antigen-binding fragments (Fab) and single-chain variable fragments (scFv), were therefore developed to diminish the unwanted effects of antibodies (Kamaly et al., 2012). Single-chain variable fragments are fusion proteins of the variable regions of the heavy and light chains of an antibody (VH and VL) connected with a short linker peptide of 10–25 amino acids, with the molecular weight of ~ 27 kDa (Sun et al., 2014). In addition to being less immunogenic, the small size of antibody fragments allows for higher loading capacities and superior orientation of targeting ligands, leading to overall improvements in efficacy (Richards et al., 2017). Affibodies are a class of polypeptide ligands originated from an antibody binding domain of staphylococcal protein A. They were employed as the scaffolds for the construction of combinatorial phage libraries for selection of affibody variants that target a specific cell marker (Kamaly et al., 2012). The small size (6–15 kDa) of affibodies enables penetration into tumor tissue (Sun et al., 2014). The NPs with affibody ligands that bind the extracellular domain of the trans-membrane human epidermal growth factor receptor 2 (HER-2), overexpressed by breast cancer cells, showed increased uptake, compared to nontargeted NPs (Alexis et al., 2008). Various pre-clinical NP–antibody conjugates are reported for the targeted delivery of cytotoxic payloads (Shargh et al., 2016; Richards et al., 2017).

9.3.3 NANOBODIES AS TARGETING THERAPEUTICS AND TARGETING LIGANDS

Nanobodies are single domain antibodies derived from llama heavy-chain only antibodies that have unique properties such as nanoscale size, robust structure, stable and soluble behaviors in aqueous solution, reversible refolding, high affinity and specificity for only one cognate target. They represent important research tool for the development of sophisticated nanobiotechnologies (Wang et al., 2016). Nanobodies' molecular weight is less than 15 kDa polypeptides with similar antigen-binding affinity to traditional mAbs but lower immunogenicity (Kamaly et al., 2012).

Different Nb-derived structures, including the Nb itself, the radionuclide or fluorescent-labeled nanobodies, Nb homo- or heteromultimers, Nb-coated NPs, and Nb-displayed bacteriophages, are reported as powerful nanobiotechnological tools for a basic biomedical research, a targeted drug delivery, and therapy (Wang et al., 2016). Owing to their small size and extended CDR3, nanobodies have advantages over conventional antibodies as therapeutics against infectious diseases with access to hidden and essential epitopes on pathogens (Wang et al., 2016). According to the reported findings, there is potential for application of variable domains of llama heavy-chain antibodies (VHH) as anti-HIV-1 microbicides (Strokappe et al., 2012). Nanobodies are also reported as potential inhibitors of HIV1 entry (Hinz et al., 2010) and therapeutics against influenza (Ibanez et al.,

2011). Moreover, nanobodies as targeting therapeutics have the capacity to distinguish the cognate target from closely related variants thus outperforming small organic antagonists or the conventional antibodies which cannot reach such high specificity (Van Roy et al., 2015). Nanobodies can be genetically engineered to extend their half-life in vivo and serve as shuttles for the delivery of therapeutic agents across the blood-brain barrier (Rissiek et al., 2014).

Successful application of Nb as targeting ligand was reported for the active targeting of pentamidine-loaded functionalized PEGylated-chitosan NPs for the treatment of African trypanosomes. This Nb, known as the NbAn33, specifically recognizes a conserved N-linked high mannose oligosaccharide present in most variant surface glycoproteins of trypanosoma (Unciti-Broceta et al., 2015).

9.3.4 APTAMERS

Aptamers are short, single-stranded, synthetic nucleic acid oligomers, DNA, or RNA that can form complex three-dimensional structures able to bind the target with high affinity and specificity. They have been used in biosensing, drug delivery, disease diagnosis, and therapy (Jo and Ban, 2016). Target-specific aptamers are selected by the systematic evolution of ligands by exponential enrichment (SELEX) technology which generates high-affinity nucleic acid ligands (Stoltenburg et al., 2007). Advantages of aptamers include availability, ease of chemical synthesis, low molecular weight, and lack of immunogenicity (Jo and Ban, 2016). Many publications reported the specific binding ability of aptamers to cancer-related markers and cancer cells ensured their high performance for early diagnosis of cancer (Wu et al., 2015).

9.3.5 PROTEINS

Endogenous proteins with known membrane-bound receptors can be employed for targeting via receptor-mediated endocytosis. There are many examples of proteins as targeting ligands, including the endogenous iron-transporting protein, transferrin, that has been employed to deliver NPs into a variety of cell types (Chou et al., 2011). Transferrin binds to its receptor and induces internalization via clathrin-dependent endocytosis. Transferrin receptor is an attractive molecule for the active targeted therapy of cancer, since it is upregulated on the surface of many cancer types and is efficiently internalized (Daniels et al., 2012). Another highly important receptor found in several cancers is the epidermal growth factor (EGF) receptor, important for tumor aggression and proliferation. Parallel with antibody-mediated EGFR targeting, there are reports on using native ligand, EGF, for EGFR-targeting for delivery of drugs and imaging agents (Master and Sen Gupta, 2012). Endogenous ligands, such as EGF, and transferrin, can bind to their respective receptors with low immunogenicity and high affinity. They are therefore attractive for tumor targeting and several protocols have been reported to conjugate EGF and transferrin to various types of NPs (Sun et al., 2014).

9.3.6 PEPTIDES

Peptides have an important role in nanomedicine, owing to their biological specificity, therapeutic function, as well as structural properties that can be incorporated through rational design. Moreover, intrinsic biocompatibility and biodegradability of peptides are additional advantages for biomaterials. Most NPs provide multiple binding sites for different cargo and targeting peptides which can be used for development of novel approaches for cancer targeting, diagnostics, and therapy (Raha et al., 2011).

Peptides as targeting molecules facilitate NPs entry into cells and have some advantages and drawbacks, compared to antibodies. Advantages include: their low immunogenicity, low cost, stability, long-term storage, easy handling, and ease of conjugation to polymers, lipids, and NP surfaces (Kamaly et al., 2012). The small size of peptides is less likely to change the optimized physicochemical properties of NPs (Ladner et al., 2004). The drawbacks are correlated to a low target affinity and metabolic instability, due to enzymatic degradation. Relatively lower affinity for their target site, can be in-part alleviated through ligand avidity by incorporating multiple peptides on the NP surface (Kamaly et al., 2012). Modification of the peptide sequence, such as the introduction of D-amino acids or multivalent sequences, are strategies to improve the metabolic stability and pharmacokinetics (Accardo et al., 2013).

Many NPs with targeting peptides interact with the target receptors classified in three groups: integrins, growth factor receptors (GFRs), and G-protein coupled receptors (GPCRs). The best studied peptide targeting sequence is RGD, which binds to $\alpha v\beta 3$ integrin receptors highly upregulated on both tumor cells like in ovarian cancer, melanoma, and breast carcinoma, and angiogenic endothelial cells (Desgrosellier, and Cheresh, 2010; Accardo et al., 2014). Nonspecific adhesion is an unsolved challenge concerning RGD-targeted therapies, as $\alpha v\beta 3$ integrin is also expressed on normal tissue and noncancerous inflamed tissues (Kamaly et al., 2012). The most extensively studied cell-targeting peptide is the RGD peptide and is also the first discovered tumor-homing peptide (Pasqualini et al., 1997; Gehlsen et al., 1988). Homing peptides represent peptides that home to the specific sites in the vasculature in vivo (Ruoslahti, 2001). Tumor homing peptides as targeting probes enable the delivery of diagnostic material and drugs to tumor blood vessels and to tumor parenchyma, thus improving tumor detection and increasing the efficacy of the treatment while reducing unwanted side effects (Ruoslahti et al., 2010). The two most widely used homing peptide motifs for drug delivery are RGD and NGR (Ruoslahti et al., 2010; Nazir et al., 2014).

A large number of peptide molecular targets implicated in tumor growth and progression are either solely expressed or overexpressed on both cancer vasculature and cancer cells (Reubi, 2003). 10^5 – 10^6 receptor molecules per cell have been reported for some of the GPCRs, such as somatostatin receptors in neuroendocrine tumors, cholecystokinin receptors in medullary thyroid cancer, and bombesin receptors in prostate and breast carcinoma (Accardo et al., 2014). Peptides

targeting GFRs have been used in peptide-targeted liposomes that represent a great advance in the use of liposomes encapsulating chemo-therapeutics (Accardo et al., 2014). Other important representatives of this class of peptides are: LyP-1 that targets lymphatic vessels in some tumors, and F3, an N-terminal fragment of human high-mobility group protein 2 that can bind to tumor vasculature. All three peptides (iRGD, F3, and LyP-1) include the cell penetration motif, R/KXXR/K or CendR sequence that is assumed to interact with neurophilin-1 facilitating extravasation, tissue penetration, and cell entry of NPs (Chrastina et al., 2011).

Another class of targeting peptides that have been selected from phage display libraries are characterized by the Asn-Gly-Asp (NGR) motif known to bind CD13 isoforms expressed in tumor vessels. They have been used for tumor targeting, recognizing the endothelium and other cells of neoangiogenic vessels, and also to deliver a variety of drugs, cytokines, NPs, viruses, and imaging agents to tumor blood vessels (Corti and Curnis, 2011).

There are some concerns regarding active targeting originating from the level of expression of targets between cancer patients; by the cancer type that limits the benefit to a subset of patients with the adequate level of target expression; and the various spatial distribution of the target within a single tumor (MacEwan and Chilkoti, 2013). A suggested alternative approach for providing targeted delivery of drugs and imaging agents in a variety of cancers and for a large subset of cancer patients is cell-penetrating peptides (CPPs) due to their nonspecific mechanism of cellular uptake that is applicable to a variety of cell types and tumor classes. Their nonspecificity, conversely, presents a challenge for their use in systemically administered applications for targeted delivery (MacEwan and Chilkoti, 2013). More than two decades ago, short peptides from HIV trans-activator of transcription (TAT) protein were first discovered to penetrate cell membranes and efficiently internalize into cells (Frankel and Pabo, 1988). TAT peptides have been successfully used for intracellular delivery of a broad variety of cargos, including various nanoparticulate pharmaceutical carriers (liposomes, micelles, NPs). The weakness of CPPs is still their unclear functional mechanism (Cheng et al., 2015). CPPs are extensively used NP modifications to improve cell uptake but the lack of cell specificity is the major weakness for their clinical development (Vivès et al., 2008). A reported approach to increase CPP specificity in the delivery of drugs to desired tissues and cells is the conjugation of CPPs to homing peptides, combining the cell-penetrating capacity of CPPs with the capacity of homing peptides (most of which are inherently cell impermeable), to recognize specific cell types (Raucher and Ryu, 2015). By conjugating the targeting ligands to CPPs, the specificity of CPP-based drug delivery systems has been increased, which was shown for coupling CPPs with RGD peptides, folic acid, or hyaluronic acid as active targeting ligands (Ruoslahti et al., 2010; Raucher and Ryu, 2015).

9.4 NANOMEDICINE FOR TARGETED DELIVERY OF NATURAL COMPOUNDS

Although natural products have shown exceptional potential against cancer, microbial infection, and inflammation, their success in clinical trials has been less impressive, partly due to the natural compounds' inefficient systemic delivery and poor bioavailability. The incorporation of nanomedicine as a strategy for natural product delivery would be a major advance to increase their therapeutic effects (Bharali et al., 2011). Strategies have been made showing that nanotechnology can significantly increase the bioavailability of natural products, both in vitro and in vivo, and improve systemic delivery (Bharali et al., 2011; Watkins et al., 2015). The natural products widely connected with use in nanotechnology are: Epigallocatechin gallate (EGCG), curcumin, resveratrol, taxol, and camptothecin (Bharali et al., 2011).

9.4.1 PACLITAXEL NANODELIVERY SYSTEMS

Taxol is the most profitable chemotherapy drug in history, and the only drug in clinical use identified by the plant screening program. Taxol was isolated from the bark of the Pacific Yew tree in 1971, and is among the first FDA-approved chemotherapy drugs that originated from natural sources (Walsh and Goodman 1999; Weaver, 2014). Taxol is a potent anticancer agent which has been shown to have activity against a number of leukemias and solid tumors in the breast, ovary, brain, and lung (Bharali et al., 2011). Despite its clinical and commercial success, Taxol suffers from the fact that it is water insoluble. Ethanol or cremophor EL are generally used for solubilization, but they have their own side effects (Bharali et al., 2011). In 1992, Bristol-Myers Squibb trademarked the name "Taxol" and created the new generic name PTX (Walsh and Goodman 1999). Abraxane is an established PTX albumin bound NP system made by Celgene of an average size of 130 nm with the outer layer of albumin and the inner core that contains the water insoluble cytotoxic agent PTX, employed in the treatment of cancer. Abraxane was the first protein-based nanoscale drug formulation approved by the FDA (in 2005) for breast cancer treatment (Desai et al., 2006). It was demonstrated to be less toxic to its free drug equivalent, PTX, and also has higher anti-tumor activity compared to free PTX and cremophor formulations (Desai et al., 2006). It was proposed that Abraxane was transported over the endothelial barrier by binding to Gp60 and consequent caveolae-mediated transcytosis inside the tumor interstitium, where secreted protein acidic and rich in cysteine (SPARC) increases the uptake of Abraxane into tumor cells (Desai et al., 2009). Abraxane is one of the major success stories of a nanomedicine approach to treat cancer. As an injectable suspension, Abraxane evades the hypersensitivity reaction associated with Cremophor EL, the solvent in traditional PTX, and thus has been successful in addressing the solubility problem associated with PTX (Bharali et al., 2011).

9.4.2 PEPTIDE-TARGETED PACLITAXEL-LOADED NANOPARTICLES

9.4.2.1 *Vasoactive intestinal peptide-targeted paclitaxel-loaded nanoparticles*

It was reported that PTX loaded in sterically stabilized, biocompatible, and biodegradable sterically stabilized mixed phospholipid nanomicelles (SSMM) and actively targeted vasoactive intestinal peptide (VIP) grafted SSMM (SSMM-VIP) significantly inhibited drug-sensitive MCF-7 cell growth in a dose-dependent fashion (Önyüksel et al., 2009). VIP is an endogenous 28 amino acid pleiotropic mammalian peptide overexpressed in breast cancer cells but not in endothelial cells (Laburthe et al., 2007; Önyüksel et al., 2009). Actively targeted PTX-loaded SSMM-VIP overcomes MDR of BC19/3 cells significantly more effective than either P-SSMM or PTX (Önyüksel et al., 2009). Later it was shown that administration of clinically relevant doses of PTX (5 mg/kg) as SSMM-VIP and SSMM eradicated carcinogen-induced orthotopic breast cancer in rats, whereas cremophor-based PTX decreased tumor size by only 45%. In addition, a fivefold lower dose (1 mg/kg) of PTX in actively targeted P-SSMM-VIP was associated with about 80% reduction in tumor size, while the response to (PTX) and P-SSMM was significantly less (Dagar et al., 2012). In another study, it was shown that VIP-grafted sterically stabilized phospholipid nanomicellar 17-allylamino-17-demethoxy geldanamycin was successful as actively targeted nanomedicine for breast cancer (Önyüksel et al., 2009). Recently, nanomicellar formulation of VIP-surface grafted curcumin-encapsulated SSM (C-SSM-VIP) showed promising results as actively targeted nanomedicine for potential therapy for cancer stem cells-enriched breast cancer (Gülçür et al., 2013).

9.4.2.2 *Active targeting with transferrin, CREKA, and LyP-1*

The antiproliferative activity of PTX with transferrin (Tf) nanoparticles was shown in human prostate cancer cell line (PC3) and an effect on tumor inhibition in a murine model of prostate cancer (Sahoo et al., 2004). Complete tumor regression and greater survival rate was shown in mice that received a single-dose intratumoral injection of NPs-PTX-Tf, compared to mice that received either NPs-PTX or Paclitaxel-Cremophor EL formulation (Sahoo et al., 2004). In another study, tumor-homing peptides were applied to target abraxane to tumors in mice. The targeting was accomplished with two peptides: cysteine-arginine-glutamic acid-lysine-alanine (CREKA) and LyP-1 (CGNKRTRGC). The CREKA-abraxane activity was not significantly different from nontargeted abraxane. In contrast, LyP-1-abraxane produced a statistically significant inhibition of tumor growth, compared with untargeted abraxane (Karmali et al., 2009).

9.5 NANOMEDICINE AND INFECTIOUS DISEASES

Nanotechnology carrier systems facilitate the body's response to foreign pathogens, including overcoming microbial resistance and improving the innate and adaptive immune response. Antimicrobial agents, with the large surface-to-volume ratio of NPs increase drug penetration by disrupting the microbial cell wall or cytoplasmic membrane. When engineered to carry foreign antigens, NPs may also act as immunostimulatory adjuvants as a result of increased uptake by antigen-presenting cells and a subsequent Th1 or Th2 immune response (Blecher et al., 2011). Nanotherapy employs the chemical and physical characteristics of nanomaterial for the treatment of infectious disease at a molecular level (Look et al., 2010). The nano-engineering of vaccines creates better adjuvants and vaccine delivery systems with new generation NP adjuvants designed to minimize their side effects, prolong the immune response, and concurrently stimulate humoral, cellular, and mucosal immune responses (Feng et al., 2004). NPs can either carry adjuvants or act as adjuvants themselves (Pee et al., 2008). One example is polymethyl methyl methacrylate NPs that have demonstrated adjuvant immunostimulatory effect inducing a 100-fold increased antibody titer response to live HIV2 virus vaccine in murine models (Rajananthanan et al., 1999). Nano-sized carriers delivering antibiotics, including β -lactams such as penicillin, ampicillin and cephalosporins, as well as macrolides (azithromycin), minoglycosides, and fluoroquinolones, were reported to enhance microbial killing (Blecher et al., 2011). It was shown that ampicillin-encapsulated liposomes were more effective than free ampicillin against systemic *Salmonella* infection in mice (Blecher et al., 2011); amphotericin B antifungal encapsulated into NPs also lead to enhanced efficacy against molds and yeasts (Vieira and Carmona-Ribeiro, 2008). NPs targeting intracellular bacteria can release high concentrations of antimicrobial drugs at the site of infection and kill bacteria before they can develop resistance. Likewise, the total dose of administered drug is kept low, decreasing the probability that bacteria outside of the site of action of the NPs will develop drug resistance (Leid et al., 2012).

The NPs conjugated with antibodies against protein A, as a notable example of active targeting, have high selectivity for eliminating *Staphylococcus aureus* (Huh and Kwon, 2011). In another study, the IgG in IgG Fe₃O₄@TiO₂ magnetic NPs targets the NPs to several types of bacteria, including *Streptococcus pyogenes*.

Another promising active targeting approach, with reduced systemic toxicity and without drug resistance, was reported for African trypanosomes. For pentamidine-loaded chitosan NPs coated by the Nb that specifically targets the surface of African trypanosomes, the therapeutic dose was 100-fold lower than pentamidine alone in a murine model of acute African trypanosomiasis. This formulation had the same in vitro and in vivo activity against a trypanosome cell line resistant to pentamidine as a result of mutations in the surface transporter

aquaglyceroporin 2 (Unciti-Broceta et al., 2015). In the next in vitro study, the drug nanocarrier based on pentamidine-loaded PEGylated PLGA NPs coupled to NbAn33 showed a sevenfold decrease in the IC_{50} , in comparison to free drug targeting the surface of trypanosoma. Subsequent in vivo therapy, using a murine model of African trypanosomiasis, showed that the formulation cured all infected mice at a 10-fold lower dose than the curative dose of free pentamidine (Arias et al., 2015).

9.6 IDENTIFICATION OF PEPTIDE-BASED TARGETING LIGANDS

Peptides with affinity and selectivity for specific cell types can be synthesized and screened using diverse libraries.

Peptide-based targeting ligands can be identified via several methods. Most commonly, they are obtained from the binding regions of the protein of interest, or from modeling new sequences on the structure of the target receptor. Peptide libraries are divided into two main categories: biological libraries and chemical libraries are also used to select cell-binding peptides (Gray and Brown, 2013). The surface profile difference between cancer cells and their nonmalignant counterparts can serve as an excellent molecular address for targeted delivery of therapeutic agents, diagnostic agents, or both, to cancer cells.

9.6.1 SELECTION OF TARGETING PEPTIDES FROM BIOLOGICAL AND CHEMICAL LIBRARIES

9.6.1.1 *Biological library methods*

The biological display system is an efficient tool in discovering novel targeting peptides via high-throughput screening. The peptides displayed on the surface of the microbes can be used to screen live cancer cells and/or purified receptor proteins (Liu et al., 2017). The sequence of the peptide hits can be easily determined by DNA sequencing of the microbial plasmid. The biological display system includes phage, yeast, bacteria, ribosome, mRNA, cDNA, retrovirus, baculovirus, and mammalian cell-display. All of these library types are promising, but only phage and bacterial display have been used to isolate mammalian cell-binding peptides (Gray and Brown, 2013).

9.6.1.2 *Phage display*

Phage display methodology can provide different peptides for targeting NPs to desired cells (Molek, et al., 2011). This methodology is also a common combinatorial library technique for the selection of tumor-targeting peptides (Liu et al., 2017). Among many techniques available for investigation of protein-protein interactions, phage display allows with a modest amount of effort, time, and cost

to select peptide ligands to a wide array of targets (Kay et al., 2001). Phage display refers to the ability to genetically modify bacteriophages for introducing specific amino acids on their surface. The technique was reported for the first time by Smith as the expression of a foreign polypeptide on the surface of phage particles (Smith, 1985). This potent strategy for gene/drug delivery studies comes from a physical connection between the phenotype (the displayed ligand) and the genotype (the DNA sequence encoding the displayed ligand) within the same viral particle, producing huge libraries of ligands displayed on the surface of the phage particles. Random peptide libraries are the most broadly used phage display libraries representing a collection of phages carrying on their surface different types of peptides that can be screened against any target of interest for selecting target-specific binders. The procedure of affinity-based library screening is called panning. This screening strategy is similar to natural selection, with dissimilarity of being performed in the test tube instead of nature.

One of the most prominent examples of a phage display-derived targeting peptide is the three-amino acid peptide RGD, which targets tumor vascular endothelial cells by binding to the $\alpha V\beta 3$ integrin exclusively expressed in angiogenic endothelial cells, the first discovered tumor-homing peptide (Pasqualini et al., 1997; Gehlsen et al., 1988; Molek et al., 2011). Phage display technology has been applied successfully to in vivo models of metastatic androgen-independent prostate cancer through the identification of tumor-homing peptides and their target receptors (Mandelin et al., 2015). The same method was applied for identification of peptide ligands with high specificity and affinity to cell-surface hormone receptors, such as luteinizing hormone-releasing hormone gene receptors, and tumor vasculature antigens (Kamaly et al., 2012).

9.6.1.3 Bacterial display libraries

Bacterial surface display is a methodology for the discovery and optimization of peptides with desired functions. Display of heterologous peptides on the surface of bacterial cells was first reported in 1986 (Freudl et al., 1986). Large libraries can be constructed efficiently in *Escherichia coli*, which is ideal for display libraries because it grows quickly and is easy to manipulate (Bessette et al., 2004). In bacteria-display peptide libraries, peptides are typically fused to the C- or N-terminus, or inserted into the middle of one of the four bacterial proteins (FliTrx, CPX, OmpA, or invasion) (Liu et al., 2017). Bacteria that display target-binding peptides can be enriched from large libraries exceeding one billion members using sequential magnetic-activated cell sorting and fluorescence activated cell sorting (FACS) (Kenrick and Daugherty, 2009). De novo design of cyclic peptides with desired properties is achieved through screening of peptide libraries using bacterial display and cell sorting (Shivange and Daugherty, 2015).

9.6.1.4 Chemical libraries

Peptides isolated from biological-display systems are valuable targeting ligands but prone to proteolytic degradation under physiological conditions.

One-bead-one-compound (OBOC) library screening methods are a chemistry-based alternative to peptide ligand discovery and have been used previously to identify novel ligands for molecular imaging, protein inhibition, and directed therapy of diseases (Gray and Brown, 2013). The primary advantage of OBOC technology is the many structural possibilities, e.g., linear, cyclic, branch, and macrocyclic peptide libraries, as well as peptide libraries comprised of both natural and unnatural amino acids (L-/D-, α -/ β -/ γ -amino acids and amino acids with posttranslational modifications such as phosphorylation and glycosylation) (Liu et al., 2017). These mentioned advantages facilitate the identification of peptide ligands that are resistant to proteolytic degradation, making them more suitable for in vivo applications (Gray and Brown, 2013).

Positional scanning synthetic peptide combinatorial libraries (PS-SPCLs) are generated by construction of individual SPCLs with one amino acid held constant while the remaining amino acids are diverse. The peptide sequence is then scanned by creating additional distinctive combinatorial libraries, each holding a different amino acid constant (Gray and Brown, 2013).

9.7 COMPUTATIONAL DESIGN OF PEPTIDE LIGANDS

Considering that the process of identifying targeting peptides by in vitro and in vivo phage display is time consuming and laborious, development of in silico methods for predicting targeting peptides would be very useful for biologists working in the field of peptide-based drug delivery. Many diverse methods currently exist to engineer peptide ligands, ranging from organic chemistry to molecular biology, and from high-throughput screening or directed evolution-based methods to rational approaches (Vanhee et al., 2011; Yin et al., 2007). Rational design of peptides that target proteins includes: structure-free peptide design with sequence modeling using machine learning algorithms that are able to learn complex nonlinear sequence motifs; structure-based peptide design that includes peptide docking; and *de novo* peptide design with structural scaffolds followed by computational site-directed mutagenesis to optimize the specificity of the peptide for the target (Vanhee et al., 2011).

Recently, support vector machine-based models using various features of proteins/peptides such as amino acid composition, dipeptide composition, and binary profile patterns have been developed for prediction and design of tumor homing peptides (THPs). The peptides from the TumorHoPe database (Kapoor et al., 2012) were used as positive dataset. The model was developed in parallel with a user-friendly web server TumorHPD for prediction and design of THPs and their mutants, with better tumor homing and physicochemical properties (Sharma et al., 2013).

The big obstacle in the therapy of cancer and infectious diseases represents mutations of therapeutic targets. This problem mainly concerns the short-range

interactions determining the chemical binding between molecules. Pathogens and cancer cells can prevent by mutations the binding of drugs and antibodies to therapeutic target without affecting the binding of its natural ligand (receptor, growth factor, etc.). In contrast, infectious agents (viruses, bacteria, parasites) and cancer cells avoid mutations that affect the long-range interactions, because these interactions determine recognition and targeting between their proteins and host proteins (Vergara-Alert et al., 2012). For this reason, peptides which modulate the long-range interaction between pathogens, tumor cells, and host represent promising therapeutics and targeting peptides for nanodrugs for infectious diseases and cancer, which are highly selective and resistant to mutations.

9.7.1 THE CONCEPT OF LONG-RANGE INTERMOLECULAR INTERACTIONS IN BIOLOGICAL SYSTEMS

Understanding how biomolecules recognize each other is very important to protein engineering and ligand (drug) design aspects of drug development. According to the current concept, success of this recognition mainly depends on steric complementarity between interacting biomolecules, which is based on two fundamental hypotheses: “lock-and-key” hypothesis and “induced fit” hypothesis. The first hypothesis, which was originally proposed by Emil Fischer in 1894, assumes that a degree of misfit between the enzyme and its substrate is needed to drive the chemical reaction forward. This concept was further extended by the “induced fit” hypothesis suggesting that the enzyme molecule changes shape as the substrate molecules gets close.

In addition to steric complementarity, electrostatic interactions influence ligand binding. Charged receptor atoms often surround the active site, imparting a localized charge in specific regions of the active site. Electrostatic complementarity further restricts the binding of inappropriate molecules, since the ligand must contain correctly placed complementary charged atoms for interaction to occur.

These two complements served as a base for the development of different docking tools for the rational structure-based ligand design (e.g., DOCK, AutoDock, etc.), which serve as a base for the drug discovery. All these approaches are based on the surface complementarity between interacting molecules and an assumption that the first contact between interacting molecules is achieved accidentally by the thermal motions that cause molecular wander. If proteins are considered as spheres of 18 Å radius (typical of a small protein), and if spheres associate with every contact, without regard to orientation, the diffusion-limited association rate constant, calculated according to Smoluchowski's equation is $7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. However, before chemical bond formation takes place, reacting molecular regions must be positioned close enough (at a distance of $\sim 2 \text{ Å}$) and the appropriate atoms must be held in the correct orientation for the reaction that is to follow, because the attractive forces involved in the recognition and binding of molecules include all the weak noncovalent forces. This means

that the protein binding site is only a small fraction of the surface area. Taking into account this limitation, the diffusion-limited association rate constant, predicted from a three-dimensional (3D) “random diffusion” model and calculated according Smoluchowski’s equation is $\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for a protein–ligand and $\sim 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for a protein–protein interaction. Intermolecular interactions in real biological systems generally occur at rates that are 10^3 to 10^4 times faster than would be expected from simple considerations of collision frequencies and strict orientation effects. This assumes that productive binding occurs only when the molecules collide within $2 \text{ \AA}$ of their final binding site (Northrup and Erickson, 1992).

To overpower the inconsistency between theoretically estimated values and real values of the associated rate constant for intermolecular interactions in biological systems, A new concept was proposed (Veljkovic, 1980). According to this concept, the intermolecular interactions in biological systems encompass two basic steps: (1) specific long-distance targeting of interacting molecules and (2) chemical bond formation between interacting molecules. The first step is determined by selective long-range forces which are efficient at a distance longer than one linear dimension of the interacting macromolecules ($102\text{--}103 \text{ \AA}$). These forces are responsible for recognition and targeting between interacting molecules and directly influence the number of their productive collisions. Before chemical bond formation takes place, reacting molecular regions must be positioned close enough (at a distance of $\approx 2 \text{ \AA}$) and the appropriate atoms must be held in the correct orientation for the reaction that is to follow, because the attractive forces involved in the recognition and binding of molecules include all the weak noncovalent forces (van der Waals, hydrogen bonding, ionic interactions, etc.). For this reason, stereochemical complementarity between interacting molecules is essential for the second step. It has been proposed that the number of valence electrons (average quasi valence number, AQVN) and the electron–ion interaction potential (EIIP), representing the main energy term of valence electrons, are essential physical parameters determining the long-range properties of biological molecules (Veljkovic, 1980).

Among 3300 currently used molecular descriptors, AQVN, and EIIP represent the unique physical properties which characterize the long-range interactions between biological molecules (Todeschini and Consonni, 2009). It was showed that small molecules with similar AQVN and EIIP values interact with the common therapeutic target. This finding allowed for establishing of the criteria for virtual screening of molecular libraries for compounds with similar therapeutic properties (Veljkovic et al., 2007a,b, 2011, 2013, 2015a,b; Tintori et al., 2007; Mugnaini et al., 2007; Maga et al., 2013).

Molecular descriptors AQVN and EIIP can be calculated by following simple equations derived from the “general model pseudopotential” (Veljkovic and Slavic, 1972; Veljković, 1973) and are defined by the following equations:

$$\text{EIIP} = 0.25 Z^* \sin(1.04\pi Z^*)/2\pi \quad (9.1)$$

where Z^* is the AQVN determined by

$$Z^* = \frac{\sum m ni Z_i}{N} \quad (9.2)$$

where Z_i is the valence number of the i th atomic component, ni is the number of atoms of the i th component, m is the number of atomic components in the molecule, and N is the total number of atoms. The EIIP values calculated according to Eqs. (9.1) and (9.2) are in Rydbergs (Ry).

9.7.1.1 Informational spectrum method

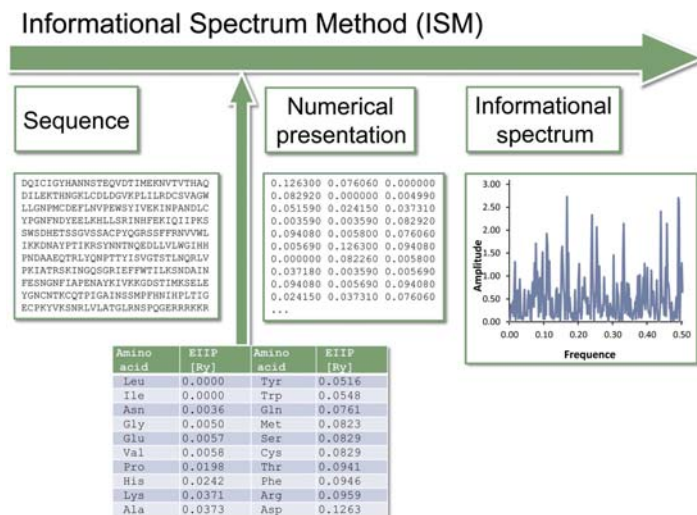
The EIIP molecular descriptor and the concept of long-range intermolecular interactions served as base for development of informational spectrum method (ISM) (Veljkovic et al., 1985; Veljković and Cosić, 1987). The ISM technique is based on a model of the primary structure of a protein, using a sequence of numbers, by assigning to each amino acid the corresponding value of EIIP. The obtained numerical sequence, is subjected to a discrete Fourier transformation which is defined as follows,

$$X(n) = \sum_{m=1}^N x(m) e^{-i2\pi m(m-1)/N}, \quad n = 1, 2, \dots, N/2 \quad (9.3)$$

where $x(m)$ is the m th member of a given numerical series, N is the total number of points in this series, and $X(n)$ are discrete Fourier transformation coefficients. These coefficients describe the amplitude, phase and frequency of sinusoids, which comprised the original signal. The absolute value of complex discrete Fourier transformation defines the amplitude spectrum and the phase spectrum. The complete information about the original sequence is contained in both spectral functions. However, in the case of protein analysis, relevant information is presented in an energy density spectrum (Veljkovic et al., 1985), which is defined as follows:

$$S(n) = X(n)X^*(n) = |X(n)|^2, \quad n = 1, 2, \dots, N/2 \quad (9.4)$$

In this way, sequences are analyzed as discrete signals. It is assumed that their points are equidistant, with the distance $d = 1$. The maximal frequency in a spectrum defined in this way is $F = 1/2d = 0.5$. The frequency range is independent of the total number of points in the sequence. The total number of points in a sequence only influences resolution of the spectrum. The resolution of the N -point sequence is $1/n$. The n th point in the spectral function corresponds to a frequency $f(n) = nf = n/N$. Thus, the initial information defined by the sequence of amino acids can now be presented in the form of the informational spectrum (IS), representing the series of frequencies and their amplitudes. The schematic presentation of the ISM is given in Fig. 9.2.

**FIGURE 9.2**

Schematic presentation of the ISM.

The IS frequencies correspond to the distribution of structural motifs with defined physico-chemical characteristics responsible for biological function of a protein. When comparing proteins which share the same biological or biochemical function, the ISM technique allows detection of code/frequency pairs which are specific for their common biological properties, or which correlate with their specific interaction. This common informational characteristic of sequences is determined by cross-spectrum or consensus informational spectrum (CIS). A CIS of N spectra is obtained by the following equation:

$$C(j) = \prod_{i=1}^N S(i,j) \quad (9.5)$$

where $S(i,j)$ is the j th element of the i th power spectrum and $C(j)$ is the j th element of CIS. Thus, CIS is the Fourier transform of the correlation function for the spectrum. In this way, any spectral component (frequency) not present in all compared ISs is eliminated. Peak frequencies in CIS are common frequency components for the analyzed sequences. A measure of similarity for each peak is a signal-to-noise ratio (S/N), which represents a ratio between signal intensity at one particular frequency and the main value of the whole spectrum. If one calculates a CIS for a two or more proteins, which have different primary structures, and finds strictly defined peak frequencies, it means that the analyzed proteins participate in mutual interactions or have a common biological function.

The ISM was applied for study of the interaction between different pathogens and their hosts (Veljkovic et al., 1992, 2009a,b, 2014; Doliana et al., 2008; Tintori et al., 2010; Glisic et al., 2012; Perovic et al., 2013).

9.7.1.1.1 Design of peptide mimetics of nanobodies by ISM

GPCRs, encompassing more than 800 members, exist in many interconvertible 3D conformations, depending on their activity or ligand-binding state. Agents that specifically bind to a GPCR in a conformationally-specific way can be used to induce a conformational change in this transmembrane receptor. Such agents have therapeutic applications and can be used in studies of the transmembrane receptors. Such agents can also be used to improve drug discovery via compound screening and/or structure-based drug design (Kobilka, 2007; Steyaert and Kobilka, 2011).

Many GPCRs are overexpressed in various cancer types and contribute to tumor-cell growth when activated by circulating or locally produced ligands (Dorsam and Gutkind, 2007). For this reason, agents which modulate the activity of GPCRs might provide unique opportunities for cancer prevention and treatment (Lappano and Maggiolini, 2011). Here we used opioid receptors as a model system for in silico design of peptides modulating activity of GPCRs.

Pain is a composite issue with neurochemical and psychological components adding to the seriousness, the perseverance, and the complexity in satisfactorily treating the condition. Opioid analgesics have been used to treat pain for centuries. Opioids produce their pharmacological effects through activation of GPCRs, which represent an essential therapeutic target for the pain management. There are four distinct genes coding for opioid receptors: the mu-, kappa-, and delta-opioid receptors (MOR, KOR, and DOR, respectively) and the opioid-like receptor-1 (ORL-1 or the nociceptin receptor (NOP). The generation of genetic knockout mice has demonstrated that the majority of clinically used opioids, including morphine, produce their pharmacological effects primarily by activating the MOR. The MOR is widely distributed and expressed in neurons in the brain, spinal cord, and the periphery. The pharmacological and genetic studies of rodents to date suggests that the development of opioid agonists that bias MOR toward G protein signaling cascades and away from β -arrestin interactions may give a novel mechanism by which to create analgesia with fewer serious adverse effects.

The binding of a ligand to the extracellular side of a GPCR results in conformational changes that enable the receptor to activate or to inhibit heterotrimeric G proteins.

Recently, Steyaert et al. proposed nanobodies that specifically bind to an opioid receptor in a conformationally-specific way and can induce a conformational change in the receptor (Patent Application (PCT/EP2015/051991, CA 2936728)). Such agents have therapeutic applications and can be used in X-ray crystallography studies of the receptor, in drug discovery via compound screening, and/or structure-based drug design. Here we used these results to design peptides that represent candidate agents for conformational modulation of opioid receptors.

In [Table 9.1](#) sequences are presented of four human opioid receptors and six nanobodies which bind these receptors. In [Fig. 9.3A](#), the CIS of mu-type opioid receptor (OPRM_HUMAN), kappa-type opioid receptor (OPRK_HUMAN), delta-type opioid receptor (OPRD_HUMAN) and the nociceptin receptor (OPRX_human) is presented. According to the IS concept, the IS spectra of interacting proteins share common frequency component(s). To identify this important informational characteristic determining the interaction between opioid receptors and nanobodies ([Table 9.1](#)), the ISM analysis of these proteins has been performed. [Fig. 9.3A and B](#) presents the CISs of opioid receptors and nanobodies. As can be seen from [Fig. 9.3A](#), the dominant peak in CIS of opioid receptors corresponds to the frequency $F(0.020)$. It means that this frequency component represents dominant information encoded by primary structure of these proteins. The dominant peaks in CIS of nanobodies ([Table 9.1](#)) also correspond to the frequency $F(0.020)$ ([Fig. 9.3B](#)). These results imply that this frequency component determines interaction between opioid receptors and nanobodies, which are given in [Table 9.1](#).

The ISM method was applied in order to identify the domains of nanobodies which are essential for information corresponding to the IS frequency $F(0.020)$. For this purpose, the computer scanning of each Nb from [Table 9.1](#) with peptides of 50 amino acids was performed. Three peptides with the highest amplitudes on the frequency $F(0.020)$ selected in this analysis are given in [Table 9.1](#) and their CIS is presented in [Fig. 9.3C](#).

These Nb peptide mimetics (NPMs), which are significantly shorter than nanobodies, represent candidate binders of opioid receptors that could modulate their biological activity. It is anticipated that the shorter length of NPMs represents significant advantage of NPMs over Nb, due to their better immunological tolerance and cheaper production.

The presented results are significant in at least two major respects: (1) modification of Nb to NPM is the significant contribution to therapeutic modulation of opioid receptors, and (2) as an example of ISM possibility to efficiently generate targeting peptides from proteins, antibodies or Nbs.

9.7.1.1.2 Design of peptides mimetics of vasoactive intestinal peptide

High densities of VIP receptors are present in breast cancer cells ([Gespath et al., 1988](#)). Various evidences indicate that VIP may play an important role in the growth and differentiation of breast cancer. It was shown that VIP elevates cyclic adenosine monophosphate (cAMP) in breast cancer cells, increases expression of c-fos, c-jun, and c-myc oncogenes ([Moody et al., 1998](#)), and functions as an autocrine growth factor in breast cancer tumors ([Moody and Jensen, 2006](#)). VIP induces the expression and secretion of the main angiogenic factor, VEGF, in breast cancer cells, acting as a proangiogenic factor ([Gespath et al., 1988](#)). VIP, acting via VPAC1 receptors, also stimulates a ligand-independent activation of EGFR and HER2 in breast cancer cells throughout tumor progression ([Valdehita](#)

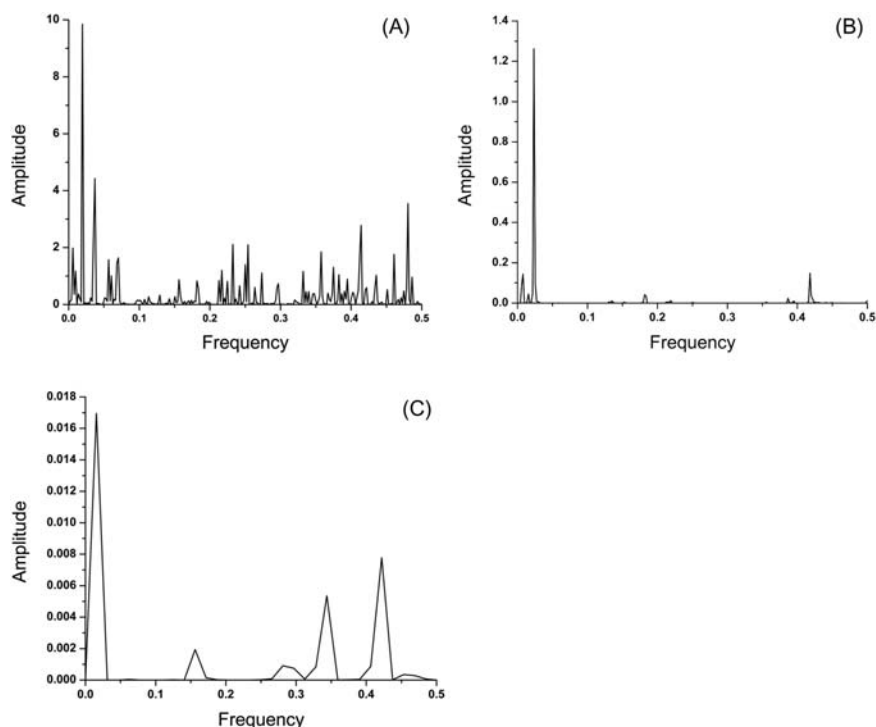
Table 9.1 Opioid Receptors, Nanobodies Which Bind Opioid Receptors (Patent Application (PCT/EP2015/051991, CA 2936728) and Nanobody Peptide Mimetics Designed by ISM

Opioid Receptors	
OPRX_HUMAN	MEPLFPAPFWEVIYGSHLQGNLSLLSPNHSLLPPHLLLNASHGAFLPLGLKVTIVGLYLAVCVGGLLGNC LVMYVILRHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGFWPFGNALCKTVIAIDYYNMFTSTFT LTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVGVPVAIMGSAQVEDEEIECLVEIPTQDY WGPVFAICIFLFSFIVPVLVISVCYSLMIRRLRGVRLLSGSREKDRNLRRITRLVLVVAVFVGCWTPVQ VFVLAQGLGVQPSSETAVAILRFCTALGYVNSCLNPILYAFLDENFKACFRKCCASALRRDVQVSDRVR SIAKDVALACKTSETVPRPA
OPRM_HUMAN	MDSSAAPTNASNCTDALAYSSCSPAPSPGSWVNLSHLDGNLSDPCGNRTDLGGRDLCPTGSPSMITA ITIMALYSIVCVVGLFGNFLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTI LCKIVISIDYYNMFTSIFTLTMSVDRIYAVCHPVKALDFRTPRNAKIINVCNWILSSAIGLPVMFM ATTKYRQGSIDCTLTFSHPTWYWENLLKICVFIFAFIMPVLIITVCYGLMILRLKSVRMLSGSKEKDRNLRR TRMVLVVAVFIVCWTPIHIIYVIKALVTIPETTFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFR EFCIPTSSNIEQQNSTRIQNTRDHPSTANTVDRTNHQLENLEAETAPLP
OPRD_HUMAN	MEPAPSAGAELOPPLFANASDAYPSACPSAGANASGPPGARSASSLALAIATAITYSAVCAVGLLGNVLV MFGIVRYTKMKTATNIYIFNLALADALATSTLPFQSAKYLMETWPFGEILLCKAVLSIDYYNMFTSIFTLT MMSVDRIYAVCHPVKALDFRTPAKAKLINICIWVLASGVGVPIMVMAVTRPRDGAVVCMLOFPSPSWYWD TVTKICVFLFAFVVPILIITVCYGLMLLRSLRSLSGSKEKDRSLRRITRMVLVVVGAFVVCWAPIHIF VIVWTLVDIDRRDPLVVAALHLCIALGYANSSLNPNVLYAFLDENFKRCFRQLCRKPCGRPDSPSSFSRARE ATARERTACTPSDGPGGGAAA
Nanobodies Which Bind Opioid Receptors	
XA8641	QVQLVESGGGLVLRPGGSLRLSCVDSGRTSYPMGWFRRAPGKEREFVASITWSGIDPTYADSVADRFTISRDNANTLYLQ MNSLKHEDTAVYYCAARAPVGQSSSPYDYDWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNGAA
XA8643	QVQLVESGGGLVLRPGGSLRLSCVDSERTSYPMGWFRRAPGKEREFVASITWSGIDPTYADSVADRFTISRDNANTLYLQ MNSLKHEDTAVYYCAARAPVGQSSSPYDYDWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNGAA
XA8635	QVQLVESGGGLVQAGGSLRLSCAASGSISSISTMGWYRQAPGNERELVAAITSGGSTNYADSVKGRFTISRDNANTVY LQMNSLKPEDTAVYYCNFKYYSGSYFYKSEYDWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNGAA
XA8727	QVQLVESGGGLVQAGGSLRLSCAASGFTFDYAIWFRQAPGKEREGVSCISSDGSSTYYADSVKGRFTISRDNAK NTVYLQMNSLKPEDTAVYYCAADLSRSCGRGYRLEVWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNGAA

(Continued)

Table 9.1 Opioid Receptors, Nanobodies Which Bind Opioid Receptors (Patent Application (PCT/EP2015/051991, CA 2936728) and Nanobody Peptide Mimetics Designed by ISM *Continued*

Biv XA8635	QVQLVESGGGLVQAGGSLRLSCAASGSISSISTMGWYRQAPGNERELVAAITSGGSTNYADSVKGRFTISRDNAKNTV YLMNSLKPEDTAVYYCNFKYYSGSYFYKSEYDYWGQGTQVTVSSGGGGSGGGSQVLQESGGGLVQAGGSLRLSCAAS GSISSISTMGWYRQAPGNERELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLMNSLKPEDTAVYYCNFKYYSS GSYFYKSEYDYWGQGTQVTVSSAAAEQKLISEEDLNGAAHHHHHH
XA9644	QVQLVESGGGLVQAGGSLRLSCVASGSIFSISAMGWYRQAPGKQRELVAITSGGTTNYADSVKGRFTISGDTAKNMVYLQMSSLPEDT AVYYCAADMEVWDYTDGDDDDYWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNGAA
Peptide Mimetics of Nanobodies	
Peptide 1	YYSGSYFYKSEYDYWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNGA
Peptide 2	RAPVGQSSSPYDYDYWGQGTQVTVSSAAHHHHHHGAAEQKLISEEDLNG
Peptide 3	FKYYSGSYFYKSEYDYWGQGTQVTVSSAAAEQKLISEEDLNGAAHHHHHH

**FIGURE 9.3**

CIS of human opiod receptors (A), CIS of OPI Nbs (B), and CIS of NPMs (C).

et al., 2009). Several studies demonstrated inhibition of breast cancer growth by VIP antagonists and VIP receptor antagonists (Moody et al., 2001, 2004), suggesting that VIP receptors may be therapeutic targets in breast cancer (Moody and Gozes, 2007).

These results point out VIP as a promising carrier which can be used for delivery of nanodrugs to tumor cells expressing VIP receptors. Possible problems in application of VIP as a targeting peptide for anticancer drug could represent different side effects induced by this pleiotropic peptide. To avoid this obstacle, artificial peptide mimetics of VIP resembling informational properties of this neuropeptide were proposed (Patent Application (US 08/859,699)). The sequences of VIP and two artificial VIP-like peptides are presented in Table 9.2 and IS of VIP and two artificial VIP-like peptides are presented in Fig. 9.4. The presented spectral similarity between designed peptides and VIP suggested similar biological and immunological properties of these peptides. In immunological and binding experiments, designed peptides showed biological properties that are similar to VIP, despite very low structural similarity (alignment score >30) (Table 9.2).

Table 9.2 Sequences of VIP Peptide and VIP Peptide Mimetics and Alignment of Peptides

Peptides	Sequence
VIP	HSDAVFTDNYTRLRKQMAVKKYLSILN
VIP1	RSAHFTDNAKTPESEIP
VIP2	RSAHFTDNAKTHPESEIH
VIP HSDAVFTDNYTRLRKQMAVKKYLSILN	Alignment Score 27.8
VIP1 -RSAHFTDN-AKTPESEIP-----	
. * * * * . : : : : :	
VIP HSDAVFTDNYTRLRKQMAVKKYLSILN	Alignment Score 26.3
VIP2 -RSAHFTDNAKTHPESEIH-----	
. * * * * . : : : : :	

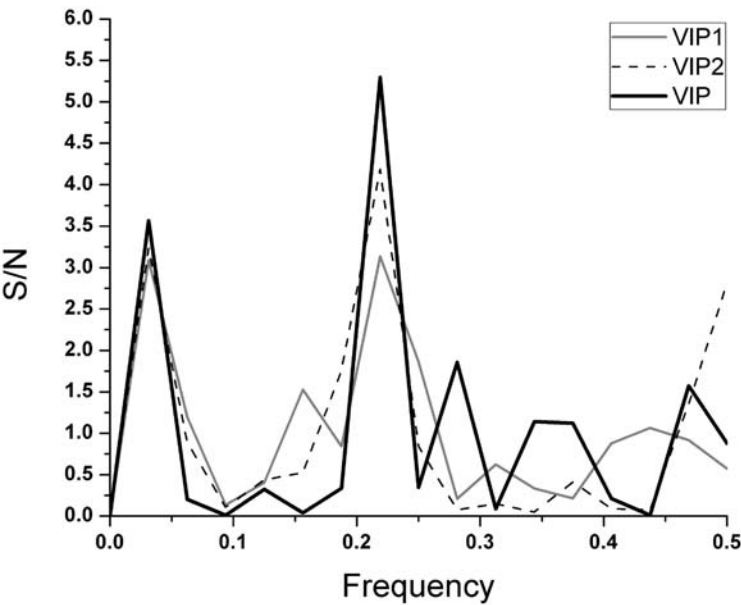


FIGURE 9.4
IS of VIP and two artificial VIP-like peptides.

Presented results demonstrate that informational properties, represented by the informational spectrum, can be used for development of targeting peptides for efficient delivery of nanodrugs to therapeutic targets.

9.7.1.1.3 Identification of targeting peptides for treatment of influenza virus

The influenza is a permanent global health threat since it circulates within and between various host populations, often causing abrupt outbreaks in animals and humans with a unpredictable course of disease and epidemiology. Standard influenza therapy targets the influenza neuraminidase, which facilitates virus release from infected cells, and the viral ion channel M2, which promotes viral uncoating. These drugs, although effective, have a major weakness: their targets have highly variable sequences. As a result, the virus can acquire resistance by mutating the drug targets. Indeed, most seasonal A/H1N1 viruses and the 2009 H1N1 viruses are resistant to M2 inhibitors, and a considerable percentage of the seasonal A/H1N1 viruses are resistant to the neuraminidase inhibitor oseltamivir. Therefore, development of new effective drugs for treatment of disease in the regular influenza seasons and the potential influenza pandemic is a significant goal (Sencanski et al., 2015). Hemagglutinin (HA), the envelope glycoprotein of *Influenza A* viruses (IAV), plays a critical role in viral binding, fusion, and entry. Therefore, HA is an attractive target for developing anti-IAV drugs to block the entry step of IAV infection (Yang et al., 2013).

Although highly variable, HA molecules of influenza viruses encode conserved information, which can be identified by ISM method (Veljkovic et al; 2009a). Identification and characterization of this information contribute to a better understanding of highly pathogenic influenza virus/host interaction. It was demonstrated that the HA subunit 1 (HA1) of H5N1 viruses encodes specific and highly conserved information, diverse from that encoded by other subtypes, which determine the recognition and targeting of these HPAI viruses to their receptor. This characteristic frequency is mainly determined by a well conserved N-terminal domain of HA1. Other subtypes of influenza encode information that corresponds to other domains of HA, including residues 262–295 for H1N1, residues 57–90 for H3N2, residues 28–61 for H7N7 residues 87–120 for Spanish flu, and residues 286–326 for A/H1N1 (Veljkovic et al., 2009a). The identified information corresponding to characteristic virus domains represent candidate targets for diagnostics, vaccines, and therapies (Veljkovic et al., 2009a,b). The specific information characteristic for the influenza subtype is contained in the peptide domains identified by ISM and could be employed for developing targeting peptides for specific subtype of influenza.

9.8 NANOINFORMATICS, DATABASES AND TARGETING INFORMATION

Nanoinformatics is defined as the science and practice of determining relevant information to the nanoscale science and engineering community, in order to develop and implement effective mechanisms for collecting, validating, storing,

sharing, analyzing, modeling, and applying that information (Panneerselvam, and Choi, 2014). Emerging databases and tools available for the nanotechnology community were reviewed recently by Panneerselvam and Choi emphasizing recent databases (Panneerselvam and Choi, 2014).

The Nanomaterial Registry is a recently developed authoritative, web-based tool that includes complex issues regarding coordination, sharing, and analysis of the data generated in nanotechnology (Ostraat et al., 2013).

The Nanotechnology Working Group (Nano WG) of the US National Institutes of Health National Cancer Informatics Program has established ISA-TAB-Nano, a general framework for representing and integrating diverse types of data related to the description and characterization of nanomaterials, assay data (metadata and endpoint measurements), and protocol information using spreadsheet or tab-delimited files. ISA-TAB-Nano supports the usage of ontology terms to promote standardized descriptions and simplify search and integration of the data (Thomas et al., 2013).

To assist in accelerating and validating the use of nanomaterials in biomedicine, the National Cancer Institute (NCI) Center for Biomedical Informatics and Information Technology, in collaboration with the NCI Alliance for Nanotechnology in Cancer (Alliance), has developed a data sharing portal called caNanoLab. caNanoLab provides access to experimental and literature curated data from the NCI Nanotechnology Characterization Laboratory, the Alliance and the greater cancer nanotechnology community. This data repository can be used for the submission and retrieval of information on NPs, including their composition, function (e.g., therapeutic, targeting, diagnostic imaging), physical (e.g., size, molecular weight), and in vitro experimental characterizations (e.g., cytotoxicity, immunotoxicity) (Gaheen et al., 2013). An important thing to point out regarding caNanoLab data portal is availability of information regarding targeting and also sequences of targeting peptides.

TumorHoPe public database is a manually curated database of experimentally validated THPs (peptides that specifically recognize tumor cells and tumor associated microenvironment, i.e., angiogenesis) compiled from published papers, patents and databases. It is freely available at <http://crdd.osdd.net/raghava/tumor-hope> (Kapoor et al., 2012).

9.9 CONCLUSION

One of the greatest issues in nanomedicine is efficient delivery of nanodrugs to therapeutic targets. Therefore, developing effective targeting ligands that can result in specific accumulation of drugs in tumors and antimicrobial drugs at the site of infection, rather than in other tissues, are important challenges. Targeting molecules with application in targeted imaging, diagnosis, and therapy could be selected from combinatorial libraries displaying small molecules, antibodies,

antibody fragments, engineered protein domains and peptides. A lot of work is being presently put into the development of *in silico* methods for predicting targeting peptides. A new concept of the long-range intermolecular interactions for recognition and targeting between drugs and therapeutic targets was employed for development of the bioinformatics platform for design of targeting peptides and for delivering antimicrobial and anticancer nanodrugs to therapeutic targets.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant no. 173001).

LIST OF ABBREVIATIONS

AQVN	average quasivalence number
CIS	consensus informational spectrum
CPP	cell-penetrating peptide
EIIP	electron–ion interaction potential
gp120	glycoprotein 120
HA	hemagglutinin
HIV	human immunodeficiency virus
IS	informational spectrum
ISM	informational spectrum method
NP	nanoparticle
Nb	nanobody
PTX	paclitaxel

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Targeting drugs to cell and organ using nanoparticles

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CHAPTER OUTLINE

10.1 Introduction	384
10.1.1 General Introduction on Nanoparticles.....	384
10.1.2 Cell and Organ Targeting Using Nanoparticles	384
10.1.3 Intracellular Targeting Using Nanoparticles.....	384
10.2 Characteristics of Nanoparticles Affecting Drug Targeting	385
10.2.1 Particle Size	385
10.2.2 Surface Properties of Nanoparticles	385
10.2.3 Drug Release	386
10.3 Modifying Nanoparticles for Cell, Organ Specific Targeting	386
10.4 Nanoparticles Targeting Cells	387
10.4.1 Nanoparticles Targeting Tumor Cells.....	387
10.4.2 Nanoparticles Targeting Lymphocytes.....	391
10.4.3 Nanoparticles Targeting Neutrophils.....	392
10.4.4 Nanoparticles Targeting Cardiac Cells.....	394
10.5 Nanoparticles Targeting Organs	394
10.5.1 Nanoparticles Targeting Lungs.....	394
10.5.2 Nanoparticles Targeting Brain.....	396
10.5.3 Nanoparticles Targeting Vascular Endothelium	398
10.5.4 Nanoparticles Targeting Skin	399
10.5.5 Nanoparticles Targeting Intestinal Epithelium	402
10.5.6 Nanoparticles Targeting Inner Ear	403
10.6 Nanoparticles Targeting Protein.....	403
10.7 Nanoparticles in Diagnosis	404
10.7.1 Detection of Progressive Liver Disease.....	404
10.7.2 Detection of Chronic Kidney Disease	405
10.8 Conclusion	406
References	406
Further Reading	408

10.1 INTRODUCTION

10.1.1 GENERAL INTRODUCTION ON NANOPARTICLES

Nanoparticles are submicron-sized polymeric colloidal particles with a therapeutic agent of interest encapsulated within their polymeric matrix, or adsorbed or conjugated onto the surface. Nanoparticles, nanocapsules, or nanospheres can be obtained by varying the method of preparation. In nanospheres, the drug is physically and uniformly dispersed in a matrix system, whereas in nanocapsules the drug is confined to a cavity that is surrounded by a unique polymer membrane. In recent years, poly(ethylene glycol) (PEG)-coated biodegradable nanoparticles, also known as long-circulating particles, have been widely used as potential drug delivery devices to target a particular organ. The three major goals in designing nanoparticles as a delivery system are: to control surface properties, particle size and release of drugs, in order to achieve site-specific action of a drug at therapeutically optimal rate and dose regimen.

10.1.2 CELL AND ORGAN TARGETING USING NANOPARTICLES

In recent years, significant effort has been devoted to developing nanotechnology for drug delivery, since it offers a suitable means of delivering small molecular weight drugs, and macromolecules such as proteins, peptides or genes by either localized or targeted delivery to the tissue of interest. These systems, in general, can be used to provide targeted (cellular/tissue) delivery of drugs, to improve oral bioavailability, to sustain drug/gene effect in target tissue, to solubilize drugs for intravascular delivery, and to improve the stability of therapeutic agents against enzymatic degradation (nucleases and proteases), especially of protein, peptide, and nucleic acids drugs (Singh and Lillard, 2009). The nanometer size-ranges of these delivery systems offer certain distinct advantages for drug delivery. Due to their sub-cellular and submicron size, nanoparticles can penetrate deep into tissues through fine capillaries, cross the fenestration present in the epithelial lining (e.g., liver), and are generally taken up efficiently by the cells. This allows efficient delivery of therapeutic agents to target sites in the body. By modulating polymer characteristics, one can also control release of a therapeutic agent from nanoparticles to achieve desired therapeutic level in target tissue for the required duration for optimal therapeutic efficacy. Furthermore, nanoparticles can be delivered to distant target sites either by localized delivery using a catheter-based approach with a minimally invasive procedure, or they can be conjugated to a biospecific ligand which could direct them to the target tissue or organ.

10.1.3 INTRACELLULAR TARGETING USING NANOPARTICLES

Intracellular targeting refers to the delivery of therapeutic agents to specific compartments or organelles within the cell. The therapeutic agent could be a

small molecular weight drug or a macromolecule such as protein or DNA. Targeted delivery could result in higher bioavailability of a therapeutic agent at its site of action, simultaneously reducing both total dose and the side effects associated with the drug. When uptake and distribution of nanoparticles in vascular smooth muscle cells and in vascular endothelial cells were studied, the results indicated that nanoparticles were internalized efficiently through an endocytic process and that uptake was concentration- and time-dependent (Panyam and Labhasetwar, 2003). Uptake of particulate systems could occur through various processes, such as by phagocytosis, fluid phase pinocytosis or by receptor-mediated endocytosis. Nanoparticle amounts inside the cell were maintained as long as nanoparticles were present in the outside medium. Once the external concentration gradient was removed, exocytosis of the nanoparticle began, which resulted in a drop in intracellular nanoparticles. By understanding pathophysiology of disease conditions and their cellular mechanisms, customized drug delivery systems can be achieved for optimal therapeutic efficacy (Panyam and Labhasetwar, 2003).

10.2 CHARACTERISTICS OF NANOPARTICLES AFFECTING DRUG TARGETING

10.2.1 PARTICLE SIZE

In vivo biological fate, distribution, toxicity, and targeting ability of nanoparticle systems is determined by particle size and size distribution. To formulate nanoparticles with the smallest size and maximum stability is a major challenge. Particle size can also affect polymer degradation. In in vitro study it was observed that by increasing particle size, the rate of PLGA [poly(D,L-lactide-co-glycolide)] polymer degradation was also increased. It was therefore considered that degradation products of polymer can diffuse out of the particles easily in smaller particles, while, degradation products are more likely to remain within the polymer matrix for a longer period in large particles to cause autocatalytic degradation of the polymer material. Accordingly, it is hypothesized that larger particles contribute to faster drug release by rapid polymer degradation.

10.2.2 SURFACE PROPERTIES OF NANOPARTICLES

The body immune system easily recognizes nanoparticles and clears them from circulation with the help of phagocytes. The in vivo fate of nanoparticles is influenced by the amount of adsorbed blood components, mainly proteins (opsonins), which is dependent upon the surface hydrophobicity of nanoparticles. Binding of these opsonins onto the surface of nanoparticles is known as opsonization, which acts as a bridge between nanoparticles and phagocytes. Consequently, successful

drug targeting by nanoparticles can be achieved by minimizing opsonization and increasing circulation of nanoparticles in vivo. This can be achieved by

1. coating surface of nanoparticles with hydrophilic surfactants/ polymers;
2. nanoparticle formulation with biodegradable copolymers with hydrophilic segments such as poly(ethylene oxide), PEG, polysorbate 80.

10.2.3 DRUG RELEASE

The drug release rate depends on:

1. solubility of a drug;
2. desorption of the adsorbed/surface bound drug;
3. diffusion of drug through the nanoparticle matrix;
4. erosion/degradation of nanoparticle matrix ([Mohanraj and Chen, 2006](#)).

10.3 MODIFYING NANOPARTICLES FOR CELL, ORGAN SPECIFIC TARGETING

One of the emerging goals of nanotechnology is to functionalize inert and biocompatible materials to impart precise biological functions. Union between nanotechnology and small-molecule chemistry can facilitate development of a wide range of nanomaterials for biomedical applications. Several small-molecule modifications (e.g., iodoacetic anhydride, diaminopropane, or diethylenetriamine) show much lower macrophage uptake, which improves in vivo targeting. Small-molecule modification also imparts unique biological functions to nanoparticles. Iodoacetic anhydride modification of the peptide-conjugated nanoparticle greatly decreases macrophage uptake, causing tenfold improvement of target-to-background ratio. Small-molecule modification could be used to optimize linkers, spacers, and conjugation chemistry and to modulate pharmacokinetics and recognition by the reticuloendothelial system (RES) (e.g., for uptake in liver and spleen) ([Weissleder et al., 2005](#)). Polyarginine, which is composed of arginine residues with guanidino groups, helps cellular uptake of nanoparticles. Such targeted drug delivery systems can be useful to deposit anticancer drugs or DNA at desired sites, reducing systemic toxicity and enhancing therapeutic efficacy ([Tian et al., 2012](#)). Active targeting can be achieved by linking targeting ligands, such as antibodies, peptides, nucleic acid aptamers, and carbohydrates, to the surface of long-circulating nanoparticles, to deliver the drug-encapsulated nanoparticles to specially identified sites and to minimize undesired effects. Specific targeting can be induced by conjugation of targeting ligands. Biopolymer-based inorganic probes, such as quantum dots (QDs) and gold nanoparticles (GNPs) (AuNPs), have several advantages, including tunable excitation and emission wavelengths, high quantum yields, specific targeting ability, high quality photos, and chemical

stability compared to organic probes. Biopolymer-conjugated inorganic probes have long circulation time, low immunogenicity, low toxicity, and the ability to penetrate leaky endothelial barriers to overcome the limitation of nude inorganic probes. Tian et al. developed PEG-modified quantum dot-streptavidin (QDstrep) nanoparticles with a biotin-cell penetrating peptide bound to the surface via biotin-streptavidin interactions; it could be specifically dePEGylated in response to the presence of the matrix MMP-2 (matrix metalloproteinase-2) enzyme. The cellular uptake of a modified QD was down to around 20%, compared with that of a nude QD. After the cleavage of the MMP-2-specific substrate in the immobilized PEG chains, the cells took up the QDs by exposing cell-penetrating peptides to the cell membrane. Similarly, GNPs and gold nanorods immobilized by a PLGA-g-MPEG graft copolymer showed excellent stability in aqueous solution at different pH values and elevated temperatures and in serum (Tian et al., 2012).

10.4 NANOPARTICLES TARGETING CELLS

10.4.1 NANOPARTICLES TARGETING TUMOR CELLS

Intense efforts are being made in the use of monoclonal antibodies (mAbs) for targeting cytotoxic drugs and radionuclides to tumors and other diseased cells in the body. The success of this line of therapy has so far been limited, as targeting specificity remains a key problem. One approach to reduce nonspecific tissue toxicity is using light-activated drugs that become toxic to cells only in areas that are exposed to light. Light-absorbing nanoparticles conjugated to antibodies kill cells selectively upon exposure to short laser pulses. High peak temperatures and microscopic mechanical disruption are localized at the cellular level when energy is selectively deposited into the particles. Selective energy deposition is accomplished by using laser pulses at a wavelength that is strongly absorbed by the particles but not by cells, and with a pulse duration that is sufficiently short to minimize heat flow away from the absorbing particles, a condition known as thermal confinement. Short laser pulses ensure that the absorbed energy does not have time to diffuse away from the particles during the laser pulse and is thus confined to the small volume of the absorber. The same principle is being used in human studies, where short laser pulses are employed to target pigmented cells containing endogenous melanin particles. In dermatology, laser removal of pigmented skin lesions is based on selective targeting of melanocytes (Pitsillides et al., 2003).

Silk-based nanoparticles produced by chitosan polymers and silk fibroins were mixed noncovalently to produce nanoparticles for local delivery of curcumin to cancer cells. Similarly collagen, the major constituent of extracellular matrix, has been widely used as biomaterial for years due to its promising biocompatibility, low antigenicity, and biodegradability. Gelatin derived from collagen by alkaline and acid hydrolysis is also used in nanoparticle synthesis. Gelatin nanoparticles

modified with PEG and loaded with a photodynamic agent, hypocrellin b were successfully targeted to cancer cells. Gelatin nanoparticles loaded with cisplatin and surface-functionalized by activated heparin were developed to target breast cancer cells (Nitta and Numata, 2013).

Fig. 10.1 demonstrates the transport of therapeutic drugs to tumor interstitium by using human serum albumin (HSA). HSA is a small protein, abundantly present in blood plasma, and has the ability to bind to albumin-binding proteins such as secreted protein, acidic and rich in cysteine (SPARC), and membrane-associated gp60 (albondon). Overexpressed SPARC results in accumulation of albumin within the tumor interstitium, while an albondon receptor on the endothelial cells of tumor vessels allows transcytosis of albumin across continuous endothelium (Nitta and Numata, 2013).

The integrin $\alpha_v\beta_3$, an important biomarker, is particularly known for its role in cancer progression and overexpressing in sprouting tumor vessels and most tumor cells. The RGD (arginine glycine aspartic acid) short peptides can specifically bind with integrin $\alpha_v\beta_3$ and play a significant role in regulating tumor growth, metastasis, and tumor angiogenesis. The high affinity interaction between RGD peptides and cancer-related integrins has led to the widespread use of RGD peptide sequences as ligands for integrin-targeted drug and gene delivery applications.

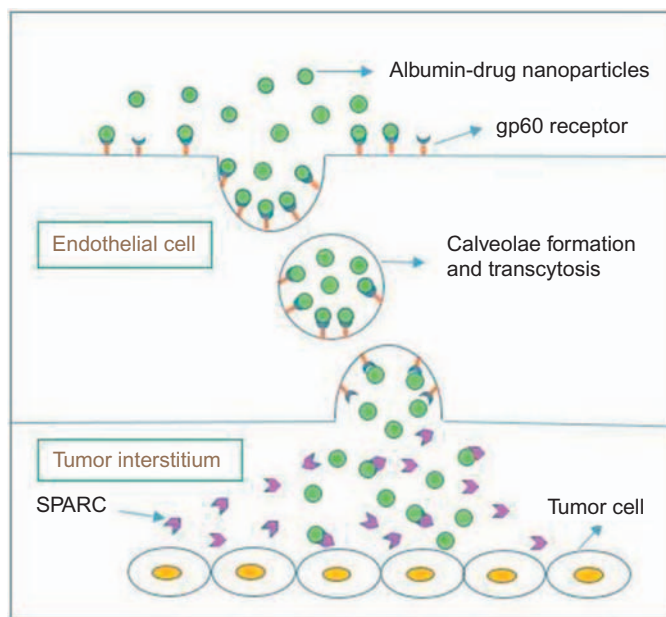


FIGURE 10.1

Uptake of albumin-paclitaxel nanoparticles, mediated by the gp60 transcytosis pathway and subsequent binding to SPARC in the tumor extracellular matrix.

Paclitaxel (PTX), an anticancer drug approved by the FDA, has demonstrated significant antineoplastic activity, including ovarian and breast cancer, nonsmall cell lung carcinoma, melanoma, head and neck cancer, and AIDS-related cancer (Jiang et al., 2011). However, its short circulation half-life, poor aqueous solubility, commonly occurring drug resistance, and serious side effects due to its nonselective distribution in vivo when delivered in conventional formulations, usually compromise its clinical efficacy. An amphiphilic diblock copolymer, α -carboxyl poly(ethylene glycol)-poly (trimethylene carbonate) (HOOC-PEG-bPTMC), was synthesized and the PEG terminus of the copolymer was conjugated to a cyclic RGD peptide. In an aqueous medium, these copolymers self-aggregated to form core-shell type nanoparticles. The hydrophobic core served as a nano-reservoir for loading hydrophobic drugs and the PEG shell endowed the nanoparticles with a lower level of RES uptake and consequently a prolonged circulation half-life. The c(RGDyK)-decorated PEG-PTMC diblock copolymer micellar-like nanoparticles were found to be an efficient vehicle for targeted delivery of hydrophobic chemotherapeutic agents to cancer cells overexpressing integrin proteins (Jiang et al., 2011).

Upon UV radiation, titanium dioxide (TiO_2) particles are able to generate free active radicals ($\text{OH}^\bullet$ and O_2^-) that are responsible for decomposing organics on particle surfaces. Due to this photocatalytic property, TiO_2 has been broadly used for disinfection and killing of viruses, bacteria, fungi, and cancer cells. Photoexcited TiO_2 particles are reported for their biocidal activity and induced cytotoxicity, but the mechanism behind the cell-killing process still remains unknown. Titanium dioxide promotes peroxidation of the polyunsaturated phospholipid component of lipid membrane of *Escherichia coli* and induces a major disorder in the cell membrane, which eventually results in loss of cell viability (Lai and Lee, 2009). Cells are also damaged from inside of the cytoplasm, as TiO_2 particles are taken by cell through phagocytosis. Folate receptors are significantly overexpressed on the surface of human cancer cells, thus folic acid (FA) has been widely used for targeting drugs to cancer cells (Lai and Lee, 2009). In folate receptor-mediated drug delivery, drug is conjugated to FA, and internalized by folate receptor-mediated endocytosis. Additionally, attachment of drug to FA does not interfere with the binding of folate to its receptor. FA has been incorporated into a dendrimer-based therapeutic nanodevice or immobilized on superparamagnetic magnetite, polymer nanoparticles for tumor cell-specific targeting. Lai and Lee et al., in their study for the selective binding to cancer cells, coupled FA on the surface of TiO_2 . The possibility of attaching a wide variety of biomolecules to TiO_2 nanoparticles without loss of photocatalytic activity enables their use in biological and biomedical applications. FA is able to form a stable linkage to photocatalytic TiO_2 . FA modified TiO_2 is partially useful for the cancer therapy since most of the cancer cells are rich in FA receptors on their surface (Lai and Lee, 2009). Photocatalytic TiO_2 nanoparticles damage plasma membranes and cellular components, resulting in cell necrosis, and induce programmed cell death. Titanium dioxide photocatalyst can produce reactive oxygen species such

as hydroxyl radical, superoxide anion, and hydrogen peroxide under UV illumination. These reactive oxygen species led to degradation of multiple cellular components, including lipid membranes which eventually caused the cell death by necrosis, a nonspecific process resulting in cell lysis. In the killing process by photoexcited TiO_2 , membrane blebbing, DNA fragmentation, DNA laddering, and increasing membrane permeability was observed. Some of these are considered to be the characteristics of apoptosis, thus cell death was most likely contributed by both necrosis and apoptosis (Lai and Lee, 2009).

Cancer cells proliferate due to the Warburg effect (altered glucose metabolism characterized by a preference of aerobic glycolysis). In many cancers, hypoxia inducible factors (HIFs) are induced, which frequently promote the expression of protumorigenic pathways. Under hypoxia, mitochondrial function alterations such as stimulation of reactive oxygen species, release of pro-apoptotic proteins from the mitochondrial intermembrane space, decreased production of Adenosine triphosphate (ATP), and loss of membrane potential are observed, while glycolysis becomes enhanced (Chen et al., 2014). Glycolysis is an important pathway under hypoxic conditions, as it allows continued ATP production without O_2 -dependent oxidative phosphorylation. Glycolytic inhibitors are effective against cancer cells with mitochondrial defects; under hypoxic conditions their predominant function is to deplete ATP. Lonidamine, a derivative from indazole-3-carboxylic acid, is a successful compound that has been shown to block aerobic glycolysis in cancer cells, possibly by inhibiting hexokinase. Lonidamine leads to an accumulation of lactate in cells and also disrupts energy metabolism in cells, resulting in ATP depletion. Lactate generation occurs in anaerobic conditions where the oxygen levels are low in normal tissues. In cancer cells, glucose is converted into lactate even under normal oxygen concentrations through glycolysis. The indole nucleus occupies an important position in medicinally relevant heterocyclic systems and is a common substructure of many biologically active compounds. Schiff bases obtained through the condensation of amines and amino acids with 1H-indole-3-carboxaldehyde and with the structure of C N-(azomethine group) have been discovered for their cytotoxic activity against cancer cells. They showed biological applications such as antitumor and antibacterial activity, however some effect was also observed on normal cells. As traditional anticancer compounds cannot differentiate between cancer and healthy cells, the systemic toxicity with undesired side effects is inevitable and the drug dose within cancerous cells is always limited. Ideal drug carriers for tumor-targeted drug delivery are being developed to improve the therapeutic index of drugs in cancer therapy. Drug carriers such as mesoporous magnetic nanoparticles (MNPs) (Fe_3O_4) and hollow mesoporous silica spheres have been used because of their low toxicity. Nano-mimics of catalase, prepared using ionic liquid-modified nano hollow silica nanoparticles as host, decreased the production of lactate, mRNA levels of GLUT1, and expression of HIF-1 α in HepG-2 cells. The mimics of catalase and time-dependent toxicity of the magnetic Fe_3O_4 @indol-IL particle make them candidates for low toxicity inhibitors of HIF-1 and GLUT1. Accordingly, the conjugation of indole

derivative with MNPs provides a new way to develop glycolysis inhibitor for anticancer therapy (Chen et al., 2014).

Significant damage occurs to normal cells due to the nonselective nature of most therapeutic agents. Multidrug resistance is another important limitation related to chemotherapeutic drugs and is mainly the result of two mechanisms *viz.* augmented antiapoptotic mechanism and increased drug efflux pumps on the cell membrane. Increased efflux pumps in cancer cells lead to decreased intracellular concentration of drug, finally resulting in failure of chemotherapy. The one-dimensional mechanism of action of single drug therapy generally leads to activation of alternate pathways, causing development of tumor relapse and chemo-resistance. Accordingly, combination therapy has been recommended for treatment of cancer, as increased efficacy is observed due to additive or synergistic anticancer activity. RNA interference (RNAi), mediated by small interfering RNA (siRNA) (small interfering RNA) and micro-RNA (miRNA) has appeared to be one of the most promising approaches for anticancer therapy (Gandhi et al., 2014). Nucleic acid–derived bioactives such as siRNA can potentially downregulate gene expression and has shown great promise under *in vitro*, *in vivo*, and clinical trials for cancer treatment. Target specificity and inhibition of the mutant carcinogenic protein are major advantages of siRNA strategy. Nucleic acid–based miRNA, also has huge potential to be developed as anticancer therapy. MiRNAs have been revealed to play a vital role in various cellular processes, such as development, differentiation, and apoptosis. Codelivery of siRNA/miRNA with chemotherapeutic agents can be a promising solution to overcome chemo-resistance. The complete nanocarrier system should have the ability to protect the RNAi therapeutic agent and drug and from the circulatory environment and efficiently deliver the therapeutic agents to tumor cells (Gandhi et al., 2014).

10.4.2 NANOPARTICLES TARGETING LYMPHOCYTES

Cell-mediated delivery of nanoparticles (NPs) is promising, in terms of overcoming several barriers based on the tumortropic migratory properties of particular cell types. This guided transport aims to enhance the NP accumulation in the tumor; moreover, it enhances the infiltration of regions that are typically inaccessible for free NPs. Cytotoxic CD8⁺T cells have the ability to migrate to the tumor and intrinsic cytolytic activity against tumor cells and are thus used as carriers in preparing nanoparticles. Many anticancer nanomedicines require tumor cell internalization to mediate cytosolic drug delivery and enhance the anticancer effect. The reversible attachment of liposomes to the surface of cytotoxic T lymphocytes via reduction-sensitive coupling can be used to solve the issue of penetration of nanoparticles. Loading cells with liposomes also does not compromise T cell functionalities such as proliferation and cytolytic function. A siRNA loaded lipid-coated nanogels were anchored to the surface of activated CD8⁺ T cells and successfully released from the T cell surface in presence of glutathione, which mimics the thiolytic tumor microenvironment (Wayteck et al., 2016).

In one of the studies, Pitsillides and coworkers showed that by binding nanoparticles to specific cell surface antigens and by localizing energy deposition into the particles, selective cell damage can be achieved without affecting neighboring cells.

Lymphocytes were initially incubated with anti-CD8 mouse IgG and then with GNPs conjugated to antimouse IgG antibody. Cells were double-labeled with anti-CD8-R-phycoerythrin probe to identify CD8⁺T lymphocytes. Results from the study showed that particle size played an important role in the cellular effect. 30-nm particles with increased particle/cell ratio resulted in greater lethality in targeted cells, without substantial increase in nonspecific killing. The nanoparticles had to be bound to cells to cause damage. Excess, unbound nanoparticles in solution did not cause appreciable damage. Membrane permeabilization was achieved with 20-nm particles without killing cells, whereas with 30-nm particles, most of the cells were either killed or there was no effect. The ability to cause transient increase in the cell membrane permeability opens up the possibility to introduce foreign molecules (such as drugs, genes, or proteins) into selective living cells under light control. Furthermore, selectively inactivating protein molecules bound to the surface of the nanoparticle absorbers offers the prospect of targeting specific cellular components rather than whole cells, a potentially useful tool for basic cell biology research and to achieve a therapeutic effect (Pitsillides et al., 2003).

Detonation nanodiamonds are a widely studied group of carbon nanomaterials. They have the ability to adsorb a variety of biomolecules and drugs onto their surfaces, and additionally their surfaces may be subjected to chemical functionalization by covalent bonds. Analysis of the cytotoxic effect of nanodiamonds was performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay against normal lymphocytes derived from human peripheral blood, the nonsmall cell lung cancer cell line (A549), and the human colorectal adenocarcinoma cell line (HT29) (Adach et al., 2016). The advantages of nanodiamonds are that the drug can be delivered to a specific site, thus maximizing drug bioavailability, and they are one of the most biocompatible materials (Adach et al., 2016).

10.4.3 NANOPARTICLES TARGETING NEUTROPHILS

Chronic obstructive pulmonary disease and cystic fibrosis are chronic obstructive airway diseases with distinct pathophysiological and clinical characteristics. Both these diseases have activated inflammatory responses with elevated levels of neutrophils and/or macrophages. The purpose of mucus lining and activated immune cells is to serve as a protective barrier by rapidly trapping and removing foreign particles and hydrophobic molecules. However, this also limits permeability of drug delivery vehicles and hydrophobic drugs through the airway defense. In order to avoid rapid clearance of nanoparticles and to allow access to underlying epithelia or inflammatory cells, nanoparticle drug carriers must quickly traverse or escape

airway defense. PLGA-PEG nanoparticles are biocompatible and biodegradable, they can be metabolized over time from human body and disposed via excretory system. PEG coating on the nanoparticle provides an hydrophilic and steric surface that minimizes clearance by airway defense mechanisms. Maleimide-terminated PEG copolymer was conjugated with target cell-specific antibody via covalent thiol-maleimide linkage. Antiinflammatory drugs, such as ibuprofen, prednisone, and theophylline, etc. have shown potential in the treatment of chronic obstructive lung diseases at higher doses, which can lead to substantial side effects. Developing a potent nanoformulation allows sustained long-term targeted release of drug to specific cell type, such as lung epithelial or inflammatory cells. Ibuprofen has been reported to be effective in controlling epidural fibrosis by inhibiting prostaglandin (PGE)-mediated mechanisms that can allow lung repair. Vij and coworkers successfully delivered PEGylated immuno-conjugated PLGA-nanoparticle-mediated non-steroidal antiinflammatory drug (ibuprofen) to neutrophils in murine models of obstructive lung diseases, based on its ability to control neutrophilic-inflammation and resulting lung disease (Vij et al., 2016).

Some reports indicate that nanoparticles (NPs) are ingested by cells via different mechanisms, including phagocytosis. The direct role of NPs on the phagocytic process is not well documented. When the effect of titanium dioxide (TiO_2), zinc oxide (ZnO), and cerium dioxide (CeO_2) NPs on neutrophil-induced phagocytosis was studied, it was found that the NPs enhanced phagocytosis, and this occurred by a spleen tyrosine kinase (Syk)-dependent mechanism (Babin et al., 2015). Spleen tyrosine kinase occupies a central role in mediating phagocytic signals in response to NPs and the efficiency of NPs to enhance phagocytosis depends upon their ability to activate this enzyme (Babin et al., 2015).

Nanomedicine provides potential benefits in the fields of drug delivery, medical diagnosis, cancer therapy, and tissue engineering. Within these fields, solid lipid nanoparticles (SLNs) have gained much attention during last decade. Owing to unique features of the particulate structure, SLNs show the advantages of controlled drug release, increased drug bioavailability, improved stability, and industrial-scale production. SLNs are colloidal nanosystems made up of crystalline lipids that are solid at room and body temperature. Despite extensive interests in using cationic colloids for biomedical application, an increasing safety concern is raised due to the possible toxicity of the cationic additives (Hwang et al., 2015). The nanoparticles can be considered as xenobiotics when they are being administered into the body. Polymorphonuclear neutrophil cells (PMNs) are the first line of defense against xenobiotics. The cationic nanocolloids can activate PMNs through the respiratory burst and degranulation pathways. The activation of PMNs can increase the immunity and prevent pathogen invasion; however, overwhelming activation may lead to inflammatory disorders. Hwang and coworkers investigated the interaction between cSLNs cationic SLNs, PMNs, and the subsequent activation of PMNs by cSLNs. Cetyl trimethyl ammonium bromide was employed as the cationic surfactant in preparation of cSLNs and a novel pathogen-killing mechanism of neutrophil extracellular traps (NETs) was

elucidated. This mechanism was involved in the elimination of foreign microorganisms and materials by the highly activated PMNs. NETs were composed of a core DNA element attached with histones and proteases. The experimental results suggested that cationic nanocolloids promoted the phosphorylation of p38 mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinases but not extracellular signal-regulated kinases in the PMN activation via MAPK pathways. Cationic nanoparticles produced NETs within minutes. Development of NETs evoked by cSLNs gradually increased and could be prolonged to 4 h (Hwang et al., 2015).

10.4.4 NANOPARTICLES TARGETING CARDIAC CELLS

One of the leading causes of mortality worldwide is chronic heart failure, occurring due to myocardial infarction. As existing therapies have still limited success, emerging alternatives for the therapy of heart diseases are natural and synthetic nanomaterials. The key challenge in heart targeting is the lack of a specific cardiac marker enabling efficient targeting of nanovectors to the heart. Several factors promote the high cellular uptake of NPs. Internalization of negatively charged NPs has been demonstrated to occur through nonspecific binding and clustering of the particles on cationic sites on the plasma membrane, and their subsequent endocytosis. The spontaneous contraction of cardiomyocytes may also help the uptake of the negatively charged NPs, mainly due to the variations in the cell membrane potential. In one such study, Ferreira and coworkers developed undecylenic acid thermally hydrocarbonized porous silicon nanoparticles using different targeting peptides, to enhance the NP accumulation in different cardiac cells. The study highlighted the future applications of peptide-modified nanosystems in heart diseases. Concentration of atrial natriuretic peptide (ANP)-modified NPs was significantly increased in primary cardiomyocytes and it was further proved that internalization of ANP-modified NPs occurred through guanylate cyclase-A receptor (Ferreira et al., 2016). In different studies, it has been demonstrated that the presence of carboxyl groups may increase the NP uptake. Silica NPs with variable size and surface functionalization showed variable cellular uptake. Particularly, it was observed that $-\text{COOH}$ modified NPs were more extensively captured by the cells than $-\text{NH}_2$ modified NPs (Guarnieri et al., 2014).

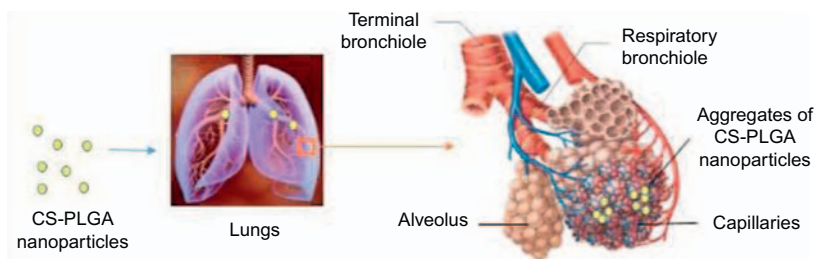
10.5 NANOPARTICLES TARGETING ORGANS

10.5.1 NANOPARTICLES TARGETING LUNGS

Failure in antitubercular chemotherapy occurs mainly because of patient noncompliance. The reason behind this is the requirement of multidrug administration for

at least six months. Development of new drug delivery systems which enhance the bioavailability of drugs to lungs is required, due to the high frequency of pulmonary tuberculosis. A major drawback in using particulate systems, such as liposomes, microparticles (MPs) and nanoparticles, as drug carriers is that they are easily taken up by mononuclear phagocytes from the blood, specially by Kupffer cells (KC) in liver, and thus cannot reach body target sites, such as lungs. In recent years, directly delivery of nanoparticle-based formulations to lungs is being developed via the aerosol route to achieve high drug concentration levels in lungs. Sodium alginate nanoparticles were prepared encapsulating three antitubercular drugs, i.e., rifampicin (RIF), isoniazid (INH), and pyrazinamide (PZA). Fortnightly administration of nebulized alginate nanoparticles for 45 days to *Mycobacterium tuberculosis*-infected guinea pigs resulted in undetectable mycobacterial colony-forming units (CFUs) in lung and spleen homogenates. However, guinea pigs administered with empty alginate nanoparticles and untreated controls exhibited comparable CFU counts (Ahmad et al., 2005). Application of nanoparticle drug delivery systems is a novel strategy. Alginate nanoparticles can thus overcome the daily dosing problem of tuberculosis therapy as a novel drug delivery system, bearing the benefits of sustained release properties of nanoparticles with additional advantages, including, no involvement of toxic molecules, the least use of organic solvents, reduced RES uptake due to stealth nature of alginate, and least use of equipment (Ahmad et al., 2005).

Surface-functionalized NPs, such as chitosan (CS)-coated NPs have been widely investigated for drug delivery. CS is a cationic polyelectrolyte composed of β (1 $\rightarrow$ 4) linked glucosamine and N-acetyl-D-glucosamine. It is a biocompatible and biodegradable polymer, which can be achieved by partial deacetylation of chitin, primarily derived from crustacean and insect shells. In general, cationic NPs tend to be favorable for endocytosis, mainly based on the electrostatic interactions with negatively charged cellular membranes. In addition, CS is one of the mucoadhesive polymers and it can enhance cellular permeability in a reversible and dose-dependent manner. Although micro-sized carriers have been used for the delivery of drug via pulmonary route, micro-sized particles administered intravenously can also be accumulated in the lung; they can thus be used for lung-targeted drug delivery. It is known that MPs can have an enhanced passive pulmonary targeting and improved retention in the lungs. However, MPs can induce embolization and unwanted toxicities after intravenous injection. To overcome these drawbacks, Lee and coworkers spontaneously formed micro-sized aggregates composed of NPs and demonstrated their lung targetability, as observed in Fig. 10.2. In the study, they proved that simple CS coating to NPs can provide an efficient lung targetability after intravenous injection. Accordingly, CS-PLGA NPs can be a promising candidate for injection formulations to treat lung diseases.

**FIGURE 10.2**

Aggregates of CS-PLGA nanoparticles formed after intravenous administration and their entrapment in the capillary bed of lungs.

10.5.2 NANOPARTICLES TARGETING BRAIN

Presently, for neurological disorders such as Parkinson's disease, Alzheimer's disease, brain tumors, or HIV infection of the brain, there is no satisfactory treatment available. Drugs proposed to act in the central nervous system (CNS) need to be introduced directly in CNS by invasive methods or can be delivered systemically, if they have the capability to circumvent the blood-brain barrier (BBB). Colloidal carriers, especially biodegradable polymeric nanoparticles can be a promising way to administer drugs to targets inside the CNS. Surface-modified nanoparticles easily enter the brain via receptor-mediated pathways after intravenous administration. The most important benefit of this technology is that no drug molecule modification is required for the brain delivery.

Most likely, nanoparticles deliver drug to the brain through endocytosis by brain capillary endothelial cells (BCEC). Endocytosis further leads to release of drug in these cells. The surface property of nanoparticles is found to be an important factor for successful delivery of drug to the brain. In one of the studies (Wohlfart et al., 2012), polysorbate 80 coating enabled endocytosis of the negatively charged poly(butylcyanoacrylate) (PBCA) nanoparticles by BCEC and further led to plasma apolipoprotein E (Apo E) adsorption on the surface of these nanoparticles. The Apo E participates in the transfer of lipids to the brain through the low-density lipoprotein (LDL) receptors, which are essential for maintaining cholesterol homeostasis. The Apo E is known to bind a various receptors on the BBB such as LRP-1, very low density lipoprotein receptor (VLDLR), LDLR, megalin/gp330 and apolipoprotein receptor-2 (Apo ER-2), and other receptors in different parts of CNS. In conclusion, it was predicted that the presence of Apo E on the nanoparticle surface can promote nanoparticles internalization in the BCEC through LDL receptors.

The apolipoproteins were covalently attached to HSA nanoparticles using a bifunctional maleimide-PEG-NHS ester (Mal-PEG-NHS) linker reacting with thiol groups, present on the protein and amino groups on the surface of particles. The particles were injected into the jugular vein of rats or mice. Fifteen minutes

after injection, nanoparticles were observed in the BCEC and later, after 30 min, particles could be observed in all analyzed brain regions. Importantly, nanoparticles were also observed in neurons, indicating the presence of transcytosis across the endothelium with successive re-uptake by neurons. After administration of nanoparticles, the integrity of the BBB was confirmed by lanthanum perfusion. Lanthanum diffusion was not observed through the tight junctions, thus confirming the integrity of junctions. The results thus indicated that HSA nanoparticles with covalently attached Apo E were trapped into the cerebral endothelium by an endocytic mechanism, accompanied by transcytosis process into brain parenchyma. Accordingly, the process of intake of nanoparticles into BCEC seems to be receptor-mediated, involving the scavenger receptor class B type I (Apo A-I) or the LDL receptor (Apo E).

As mentioned previously, for receptor-mediated transcytosis of nanoparticles, other specific receptors on the brain capillaries can be utilized. Endothelial cells forming the BBB highly express transferrin receptor and the receptor for insulin. HSA nanoparticles were covalently coupled to the antitransferrin-receptor monoclonal antibodies (TfR-mAB). An opiate receptor agonist, loperamide, that usually cannot cross the BBB, was selected as a model drug. Significant antinociceptive effects were observed after intravenous injection of HSA nanoparticles bonded to TfR-mAB or transferrin, revealing the transport of drug molecules to the brain using the transferrin receptors (Wohlfart et al., 2012).

The PBCA nanoparticles coated with Polysorbate 80 (PS 80) were demonstrated as a successful carrier system for nerve growth factor (NGF) intravenous delivery. After 45 minute of nanoparticle administration, significantly higher concentration of NGF was discovered in the CNS with the PS 80-coated PBCA nanoparticles in the adsorbed NGF. The injection of the NGF solution in PS 80 or in saline solution did not change the NGF concentration in CNS. As demonstrated by the passive avoidance reflex test, intravenous delivery of NGF-adsorbed, PS 80-coated, PBCA nanoparticles successfully inversed scopolamine-induced amnesia and enhanced memory and recognition in an acute amnesic mouse model (Wohlfart et al., 2012). Moreover, this preparation also demonstrated a significant reduction of the primary symptoms of parkinsonism (rigidity, oligokinesia, and tremor).

The quaternary ammonium β -cyclodextrin nanoparticles displayed cationic properties which could be managed by the concentration of choline within the particle core. These nanoparticles enhanced the transfer of doxorubicin through the bovine BCEC monolayers and their permeability improved by changing the surface charge from -11.8 to $+2$ mV. This suggested that uptake into the cells existed mainly through the adsorptive-mediated endocytosis, occurring due to the existence of the positively charged choline quarter ammonium groups on the surface of the particle (Wohlfart et al., 2012).

Localization of PS 80-coated fluorescent PBCA nanoparticles was investigated in rats after carotic and intravenous injections. Nanoparticles were loaded either with fluorescent agents doxorubicin or rhodamine 123 for visualization.

Both agents are P-gp substrates and therefore do not readily cross the BBB in free form. The nanoparticles, when injected into the carotid artery, reached brain quickly. They could be traced in the vicinity of capillary endothelial cells after 30 min and were evenly diffused over the brain tissue at 60 min of administration. In the case of intravenous administration, fluorescent particles could be detected in the endothelial cells within 60 min and after 2 h of administration began to diffuse through the brain parenchyma (Wohlfart et al., 2012).

10.5.3 NANOPARTICLES TARGETING VASCULAR ENDOTHELIUM

Vascular endothelium offers a variety of therapeutic targets associated with cancer, cardiovascular diseases, inflammation, and oxidative stress. Endothelial cells overexpress adhesion molecules, such as intracellular adhesion molecule, vascular cell adhesion molecule, and L-selectin at inflamed sites that may be used for targeting diseases, such as myocardial infarction. Targeting of nanoparticles to endothelium is limited by several factors. Firstly, the target size, the area of diseased endothelium, is often much smaller than that of healthy endothelium. This enhances the contribution of off-target effects and makes the choice of targeting moiety critical. Even if a highly specific targeting moiety is used, the effectiveness of nanoparticles that display these targeting moieties is often limited by their immune clearance. The ability of particles to avoid immune clearance and accumulate at the target depends on several parameters, including size, shape, surface chemistry, and flexibility. The role of shape in vascular dynamics has long been known in terms of its influence on behavior of circulatory cells such as erythrocytes and platelets. The shape of nanoparticles in circulation is of particular interest because it has a significant impact on hydrodynamics, and interactions with vascular targets. Reports state that rod-shaped nanoparticles (nanorods) exhibit higher avidity and selectivity toward the endothelium, compared with their spherical counterparts (Kolhar et al., 2013).

For successful targeting of drugs to the endothelium, the ability of the carrier to localize to the blood vessel wall is the major necessity. It has been confirmed that particle geometry and size notably affects the capability of a particle to localize and bond to the vascular wall in human blood flow. However, it is quite feasible that, with inclusion of size, other particle characteristics may influence the localization tendency of a carrier system. Particle density is one such parameter, which can vary based on the composition of carrier system. Liposomes are usually neutrally buoyant, but can be constructed with diverse densities relying on the density of the medium which is encapsulated into the liposome. It is broadly known that red blood cells (RBC) play an important role in the localization of leukocytes and platelets. By gathering in the center of flow, RBCs force platelets and white cells to converge in the “cell-free layer” (CFL) adjoining the endothelium. This effect helps in the localization and interaction of white cells (8–12 μm diameter) and platelets (2–3 μm diameter) with the vascular wall. Accordingly,

nanoparticles or microspheres with similar size range will have better interaction with the vascular endothelium.

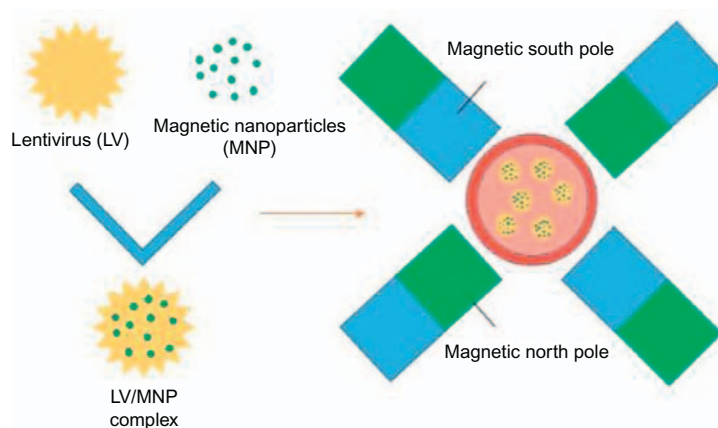
Investigations were carried out to determine the effect of density-dependent forces (gravitational, centrifugal), density-independent forces (hydrodynamic, Brownian), momentum, and adhesion dynamics combined with the presence of blood components, on the targeting of 500 nm diameter nanospheres to inflamed endothelium from human blood flow in a parallel plate flow chamber (Thompson and Eniola-Adefeso, 2015). The targeting ability of polystyrene, silica, and titanium nanoparticles, which have densities of 1.05, 2.0, and 3.9 g/mL, respectively, was determined. Results suggested that particle density, and local hemodynamic conditions including shear rate, flow type, channel height, and the presence of RBCs, all affect targeting of nano-sized carrier system adherence to the target site. Silica particles, with a density approximately twice that of blood, displayed increased adhesion to endothelium relative to the neutrally-buoyant particles of the same size (500 nm diameter). The improved adhesion of silica particles is likely linked to increased localization to the CFL in blood flow, despite having a diameter smaller than that which provides improved CFL localization for neutrally-buoyant spheres. Accordingly, density is a parameter which can be used to improve targeting ability of nanoparticles to the vascular wall, using the wide variety of carrier types that have been proposed as drug delivery systems (Thompson and Eniola-Adefeso, 2015).

At later stages of atherosclerosis, plaques are formed, leading to vascular stenosis, which limits blood flow and thus oxygen supply to organs. Consequently, the exogenous application of angiogenic and vasoprotective growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor is done. This promotes the development of functional collaterals, leading to enhanced tissue perfusion thus preventing end organ damage. To affect the growth of collaterals or endothelial function, an intravascular route of administration is optimal. However, to enhance contact time and transfection efficacy of the vector, vascular occlusion is required, which also increases the risk of ischemic events. As observed in Fig. 10.3, magnetic fields and MNPs can be applied to avoid the interruption of blood flow and retain genetic vectors at the vascular wall. This new approach was established by Vosen and coworkers, wherein lentivirus (LV)/MNP complexes and magnetic fields were used to improve vascular function by radial symmetric gene transfer, specifically to the vascular endothelium.

The endothelial cell biological properties functionally improved due an efficient circumferential transduction of the native endothelium. Application of a radially symmetric magnetic field using an optimized magnet configuration led to overexpression of the therapeutic genes VEGF or eNOS in the native vascular endothelium using LV/MNP complexes (Vosen et al., 2016).

10.5.4 NANOPARTICLES TARGETING SKIN

The protective barrier of skin retards the clinical effects of topical formulations. Atopic dermatitis (AD) is a chronically relapsing and highly pruritic inflammatory

**FIGURE 10.3**

Schematic diagram of the experimental strategy for transduction of the endothelium, using LV/MNP complexes (transversal view).

skin disease that impairs the quality of the life of patients and their families. AD is a frustrating condition for patients because it critically affects daily life of patients physically and physiologically. Topical glucocorticoids (TGs) are still important and widely prescribed treatment choices for acute and chronic exacerbation of AD. However, several studies with TGs have reported local side effects to the skin, such as thinning, bruising, atrophy, telangiectasia, allergy, increased susceptibility to infection, and systemic adverse effects such as glaucoma and Cushing's syndrome. To overcome these disadvantages including reduced toxicity and enhanced drug bioavailability at the target site, it would be expedient to use a newer formulation that can control the drug release at the target site and thereby significantly improve the beneficial drug effects.

Specialized drug therapy, such as nanotechnology can improve efficacy by delivering the pharmaceutical agent to skin, while simultaneously reducing the off-target accumulation to minimize side effects. Moreover, advances in the nanoparticle-mediated delivery and improved understanding of nanoparticle skin interaction have led to clinically relevant improvements in topical drug delivery. Among the polymers frequently used, chitosan (CS) has gained remarkable popularity, due to its excellent biodegradability, biocompatibility, and nonimmunogenicity. The subcellular and micron size of CS particles facilitate their penetration through the tight junctions between epithelial membranes. A mildly potent glucocorticoid, hydrocortisone (HC), was encapsulated into CS particles, which prevented the direct contact of the drug with the skin and created a slow drug release process. When the skin-targeting potential of cationic polymeric chitosan nanoparticles loaded with antiinflammatory (HC) and antimicrobial (hydroxytyrosol, HT) drugs were investigated for the treatment of AD by topical

application, a significant target delivery to the skin was observed with lower systemic drug absorption than the commercial formulation (Siddique et al., 2016).

Polymeric nanoparticles are beneficial for topical antiinflammatory drug delivery or antiinflammatory therapy, due to their ability to retain the drug locally at the site of action by formation of a reservoir. Polymeric materials have a higher potential for local delivery to the skin than lipid carriers. However, aqueous suspensions of polymeric nanoparticles cannot be easily applied on the skin for in vivo studies; the therapeutic drug vectorized by those particles tends to run-off. A new approach for local treatment of inflammatory skin diseases consists of designing innovative antiinflammatory drugs vehicles, such as topical semisolid formulations of poly(lactic acid) (PLA) nanoparticles. PLA nanoparticles are biodegradable and biocompatible nanovectors with a strong safety background for human use (Boisgard et al., 2017).

Skin is an application site for many transdermal and topical drug delivery systems. Development of a variety of topical drug formulations has been done to treat local indications. However, formulations such as UV protection agents, dietary and cosmetic skin supplements, and several skin disorder medicines lack treatment of broad areas of skin because topical application to a broad skin area is associated with drawbacks such as sweating, staining of clothes, skin irritation, and pigmentation. It is not a practical route to cover large areas of skin topically on a daily basis and only a limited number of drugs are responsive to administration by topical application. These limitations are associated with a number of drugs used as medicines for skin disorders as well as cosmetic and dietary skin supplements.

Kadhun and coworkers worked on the improvement of oral bioavailability and skin tissue-targeting of a model compound, p-amino benzoic acid (PABA) with a formulation approach by using liquid crystals (LCs). LCs are semisolids made up of crystalline structure lipids, combining the properties of both liquid and crystal states. Molecules in liquid are free to diffuse in a random way, whereas those in crystal are highly ordered. Accordingly, molecules in LC phases diffuse as liquid molecules but contain some degree of order. A generally used term for LC is mesophase, indicating that such a unique structure is between that of solid crystals and true liquids. PABA a cofactor of the vitamin B complex widely found in foods, was used in the study. Due to its high absorbance in the UVB region, PABA is often used as an ingredient in sunscreen formulations. It is found to be protective against skin cancers and has ability to scavenge reactive oxygen species, which is related to its property to protect against UV and free radical damage. PABA also has antioxidant activity and is thus available as a health supplement (vitamin B10). The potassium salt of PABA is used in the USA as a prescription drug for the treatment of various skin disorders, such as dermatomyositis, scleroderma, and Peyronie's disease. However, in the gastrointestinal (GI) tract, PABA suffers from a narrow absorption window. Increasing attention has been paid to LC formulations, including hexosomes and cubosomes, because of their usefulness in diverse applications and remarkable structural

complexity. Reports stated that LC formulations are a promising approach to improve the oral absorption and skin tissue targeting of PABA. Targeting skin by orally dispersed PABA-LC formulations could thus be a great achievement in dietary, cosmeceutical, and clinical fields (Kadhun et al., 2016).

The *stratum corneum* is the main challenge to administer drugs across the skin because of its architecture, consisting of keratin-rich corneocytes enclosed by the intercellular lipid lamellae. A common approach to increase drug permeation through the skin is to damage and weaken these eminently organized intercellular lipids. Nanocarriers can permit the expansion of improved and novel targeted drug delivery approaches, with an additional value to already existing commercialized therapeutic agents. The improved generation of lipid nanoparticles is nanostructured lipid carriers (NLCs), consisting of solid lipid matrix containing liquid lipids, which leads to a less-organized crystalline structure and thus better loading capacity. The nano-sized NLCs provide a large surface area in strong contact with the *stratum corneum*. The enhanced skin hydration effect of NLCs is because of the lipid nanoparticles occlusive properties, resulting in improved drug penetration into skin. Methotrexate (MTX) is categorized as an antimetabolite drug. It shows immunosuppressant and antiinflammatory activities and is one of the most successful therapeutic agents in healing diseases related with irregularly rapid cell expansion. MTX has been used in hospitals for the management of different tumors (e.g., skin, osteosarcoma, and breast cancer) as well as inflammatory and autoimmune diseases, such as psoriasis and rheumatoid arthritis. Its medical use is restricted because of serious toxic side effects, cellular efflux and short half-time in blood. Ferreira and coworkers, successfully designed topical MTX-containing NLCs for treatment of skin-related diseases, thus avoiding systemic toxicity. MTX was successfully incorporated into NLCs by hot ultrasonication method. Cetyl palmitate and Miglyol 812 lipids combined with polysorbate 80 allowed a theoretical drug loading of 10%, yielding nanocarriers with adequate size for topical administration. Nanostructured lipid carriers loaded with MTX enhanced solubility of the drug and favored permeation of methotrexate through in vitro HaCaT (skin keratinocytes) monolayer permeation model, with enhanced tight junction barrier function (Ferreira et al., 2016).

10.5.5 NANOPARTICLES TARGETING INTESTINAL EPITHELIUM

Diabetes *mellitus* is thought of as one of the five leading causes of death in the world. Insulin is taken as part of treatment in type 1 diabetes. Insulin absorbed by the intestinal epithelium reaches the liver through the portal vein and can directly inhibit hepatic glucose output.

A major shortcoming in insulin therapy includes ineffectiveness of oral administration, as insulin is less absorbed by the GI tract and is strongly degraded by proteolytic enzymes. A short-acting insulin analogue, aspart-insulin, is successfully absorbed from the intestinal mucosa when encapsulated into nanoparticles prepared with a blend of a biodegradable polyester (poly (ε-caprolactone)) and a

polycationic nonbiodegradable acrylic polymer (Eudragit1RS). The insulin-loaded nanoparticles were administered orally in diabetic rats and were effective in reducing glycemia for a longer period of time (Damge et al., 2010). It was demonstrated that insulin was probably absorbed due to the mucoadhesive properties of Eudragit1 RS. This formulation may be applied, in preference, to the treatment of type 2 diabetic patients who do not respond enough to oral antidiabetic agents and who need a long-acting insulin for a glycemic control (Damge et al., 2010).

10.5.6 NANOPARTICLES TARGETING INNER EAR

Inner ear disease and hearing loss is significantly affecting many people worldwide. Strategies to prevent hearing loss are limited, and the cochlea is difficult to access by conventional systemic drug delivery, due to the presence of the cochlea-blood barrier. Roy and coworkers investigated targeting of polymersome NPs to cells of the inner ear by conjugation of neurotrophin-derived peptides. Structurally, polymersomes are similar to liposomes; however, they have several advantages over liposomes, which give them greater potential as drug delivery vehicles. The polymersome surface can be functionalized with groups to promote cellular uptake. Neurotrophins are secreted growth factors and play an important role for the survival, development, maintenance of neurons in both the peripheral and CNSs of vertebrates. The neurotrophin family, including NGF, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin 4/5, signals through two distinct groups of cell surface receptors: tyrosine kinase receptors (Trks) and p75 neurotrophin receptor. Neurotrophins and their receptors are reported to regulate the survival of vestibular and cochlear neurons (Roy et al., 2010). NGF-derived peptide (hNgf_EE)-functionalized nanoparticles (NPs) were targeted to cells of the inner ear. Specific targeting and higher binding affinity to spiral ganglion neurons, Schwann cells, and nerve fibers of the explant cultures was achieved through ligand-mediated multivalent binding to Trks and to p75 neurotrophin receptors. The selective cochlear cell targeting by NPs may be a potential tool for cell-specific gene and drug transport to the inner ear cells (Roy et al., 2010).

10.6 NANOPARTICLES TARGETING PROTEIN

The fibrillation process, in which the self-assembly of proteins leads to the formation of fibrils, is the major cause of neurodegenerative diseases. Suppression of protein aggregation has tremendous implications in biology and medicine. Experimentally, NPs were found to either promote or inhibit aggregation of amyloid proteins, but exact molecular mechanism for their action is still unknown. This can be hypothesized that NPs may bind to the exposed hydrophobic patches of aggregated protein and might lead to their correct folding. It can be suggested that NPs may act as an artificial chaperone and can be a strategy to prevent protein

aggregation related disorders. NPs were found to inhibit the process of fibrillation, due to binding property differences of monomers and oligomers. In neurodegenerative disorders, among A β agglomerates, the oligomers are the most neurotoxic species. NPs may also block these neurotoxic oligomeric species (Parveen et al., 2016).

CD44 is a transmembrane adhesion glycoprotein which participates in cell-cell and cell–extracellular matrix interactions. Hyaluronic acid (HA), fibronectin, and laminin being its natural ligands. CD44 is expressed in many cell lines, including leukocytes and fibroblasts, and it participates in a wide range of physiological processes, such as cell migration, lymphocyte homing, cell activation, and hematopoiesis. Among several surface receptors overexpressed in cancer stem cells (CSCs), including CD133, CD44, CD49, and ITGA6, CD44 is the most frequent molecular marker, being present in a large variety of tumor types. It is prevalent in highly recurrent colon, liver, prostate, or breast cancers. The tumorigenic and metastatic potential of CSCs have been associated to CD44 expression. Alteration of CD44 expression levels promotes tumor cell survival and aggressiveness, and also induces tumorigenesis and metastasis. Not all CD44 + cells constitutively bind HA, as the binding capacity appears to be influenced by structural variation and/or CD44 glycosylation patterns. Accordingly, by applying a nano-architectonic principle based on the combined use of end terminal cationic peptides and polyhistidines five CD44-specific protein ligands as components of multifunctional chimerical proteins, the potential for oligomerization were explored (Pesarrodona et al., 2014). Among them, two peptides from laminin and fibronectin, respectively, efficiently targeted CD44 + cells and also promoted the self-assembling of the carrier protein as functional nanoparticles. Such particulate organization confers added valuable properties to protein constructs, favoring cellular penetrability and opening a plethora of possibilities for the normal designing of protein-based, fully biocompatible nanomedicines, in the form of viral biomimetics, targeting CD44-overexpressing cells. Smart and stable protein-only nanoparticles (A5G27-GFP-H6 and FNI/II/V-GFP-H6) as plastic agents that bind specifically to CD44 + cells were developed and were efficiently internalized by receptor-mediated endocytosis in the absence of cell toxicity (Pesarrodona et al., 2014).

10.7 NANOPARTICLES IN DIAGNOSIS

10.7.1 DETECTION OF PROGRESSIVE LIVER DISEASE

Liver diseases, such as fatty liver, liver fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC) constitute a rapid growing burden to public healthcare. Magnetic resonance imaging (MRI) is generally used for diagnosis of liver fibrosis. Super-paramagnetic iron oxide nanoparticles (SPION), such as Fe₃O₄, have been widely used in MRI as prevailing T2 contrast agents, and show better resolution for focal liver lesions and the liver tumors. However, MRI has several drawbacks, which include: little difference in signal intensity of cirrhotic liver,

compared to that of the normal liver, and the inability to assess the lesion vascular system. Computed tomography (CT), another potent noninvasive imaging modality, has a fast scanning speed and capability to evaluate the hyper vascular.

Both MRI and CT were combined to circumvent the drawbacks of the diagnosis using MRI alone. Liver disease assessment was performed by using Fe_3O_4 -Au NPs. Fe_3O_4 -Au NPs are usually engulfed by the KC of the RES in vivo. The phagocytic activity of KC was greatly weakened in diseased liver, which resulted in an obvious discrepancy of Fe_3O_4 NPs accumulation between normal parenchyma and lesions. Fe_3O_4 -Au hybrid NPs were synthesized with a strawberry-like structure. In vitro studies demonstrated that these NPs rendered superior contrast enhancement for both CT and MR imaging. In vivo findings of CT and MRI confirmed the contrast enhancement potency of Fe_3O_4 -Au NPs. In vivo tests in fatty liver, cirrhotic liver, and HCC-bearing rat models further supported potential application of these Fe_3O_4 -Au hybrid NPs to accurately distinguish the grade of liver disease. Based on both acute pathological study and long term biochemical and hematology studies, the Fe_3O_4 -Au NPs did not cause any acute injuries and chronic inflammation and therefore were highly biocompatible and suitable for future clinical translation (Zhao et al., 2015).

10.7.2 DETECTION OF CHRONIC KIDNEY DISEASE

Progressive loss of kidney function occurs in chronic kidney disease (CKD) through a period of years or months. In recent guidelines, the severity of CKD is classified in five stages in accordance with the decrease in glomerular filtration rate. Stage 1 is a slight disorder identified by few symptoms, while stage 5 a serious disorder specified by a reduced life expectancy if untreated. These restrictions, and the asymptomatic beginning of the disease, currently lead to delayed diagnosis and further treatment of CKD (Marom et al., 2012).

Gold nanoparticle sensors are a novel approach for detecting CKD and disease progression. This novel approach overcomes many constraints of the conventional diagnostic techniques. The method includes detection of volatile organic compounds (VOCs). In the study, sensors depending on organically functionalized GNPs were used. Breath samples were tested using GNP sensors. The selected sensors were crossreactive chemiresistors depending on four types of circular GNPs, having a core diameter of 3–4 nm. The organic ligands were: 2-ethylhexanethiol (S1), tert-dodecanethiol (S2), hexanethiol (S3), and dibutyl disulfide (S4). The ligands were selected based on their ability to absorb certain (classes of) small VOCs that are typically found in exhaled breath as metabolic products. Suitable combinations of GNP sensors could identify, with high accuracy, patients with early-stage CKD and determine disease progression from advanced CKD to ESRD (end-stage renal disease). Breath testing utilizing GNP sensors maintains a future potential as a fast, reliable, and cost-effective, diagnostic test for initial recognition of CKD and observing disease progression (Marom et al., 2012).

10.8 CONCLUSION

Nanodelivery systems are found to have great potential to overcome obstacles and efficiently target drugs to diverse cell types. This represents a powerful possibility to overcome drug resistance and target cells to facilitate the movement of drugs across barriers (e.g., BBB). The challenge, however, is to precisely characterize molecular targets and ensure that these molecules only affect targeted organs. Furthermore, it is also important to know the fate of the drugs after delivery to sensitive cell organelles such as nucleus (Singh and Lillard, 2009). For the development of more potent nanomedicines, an in depth understanding of cellular uptake mechanisms is of paramount importance. Nanoparticles enter the cells via a combination of different internalization routes. Depending on the size, shape, and surface charge of the nanoparticles, a particular cellular internalization route may be preferred. The fact that the cellular internalization routes determine the fate and intracellular localization of nanoparticles emphasizes that the development of reliable strategies to control or at least influence the nanoparticle cellular internalization route can hugely augment the therapeutic outcome (Yameen et al., 2014). Accordingly, nanoparticles have great potential to convert poorly absorbed, labile, and poorly soluble biologically active substance into deliverable drugs. The core of the nanoparticulate system can enclose a variety of enzymes, drugs, and genes. Due to their hydrophilic shell, nanoparticles are not recognized by the reticular endothelial system, thus increasing their circulation time. Greater understanding of particle engineering and the different mechanisms of biological interactions is required to optimize the nanoparticulate delivery system. Further advances are required to turn nanoparticle technology into practical application as a next generation drug delivery system (Mohanraj and Chen, 2006).

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Extracellular vesicles as a recipe for design smart drug delivery systems for cancer therapy

11

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CHAPTER OUTLINE

11.1 Introduction	412
11.2 Extracellular Vesicles: Biogenesis and Composition	412
11.3 Influence of Calcium, pH, and Hypoxia in the Biogenesis of Extracellular Vesicles	416
11.4 Extracellular Vesicle Biochemical and Biological Characterization	417
11.4.1 Exosome/Ectosome Isolation: Differential Centrifugation, Polymer Precipitation, Antibody-Coupled Magnetic Beads, and Microfluidic	417
11.4.2 Extracellular Vesicle Characterization	419
11.5 Methods for Loading Cargo in Extracellular Vesicles	421
11.5.1 Loading of Cells Before Extracellular Vesicle Isolation.....	422
11.5.2 Loading of Extracellular Vesicle After Isolation	422
11.6 Extracellular Vesicle Mimetics.....	423
11.7 Extracellular Vesicles Uptake Into Recipient Cells	424
11.7.1 Participation of Lipid Rafts	424
11.7.2 Clathrin-Mediated Endocytosis	424
11.7.3 Caveolin-Dependent Endocytosis.....	425
11.7.4 Macropinocytosis.....	425
11.7.5 Phagocytosis	425
11.8 Assessing Extracellular Vesicle Uptake Into Recipient Cells	426
11.8.1 Labeling Membrane	427
11.8.2 Labeling Exosome Cargoes	427
11.8.3 Intravesicle Probe.....	428
11.9 Overview of Extracellular Vesicles for Therapeutic Delivery of Small Molecules, Proteins, and RNAs	428
11.9.1 Exosomes as Drug Delivery Platforms for Small Molecules	430

11.9.2 Exosomes as Drug Delivery Platforms for Proteins 431

11.9.3 Exosomes as Drug Delivery Platforms for RNAs 432

11.10 Extracellular Vesicles as Drug Delivery Platforms for Therapeutic Benefit in Clinical Trials..... 435

11.11 Concluding Remarks..... 436

Acknowledgments 438

References 438

11.1 INTRODUCTION

The communication between cell–cell, cell–microenvironment, and cell–distant cells is crucial to the maintenance of the organism homeostasis. This communication occurs by direct contact, for instance, via gap junction or, in the case of long distance, by hormones and extracellular vesicles (EVs) ([Lener et al., 2015](#)).

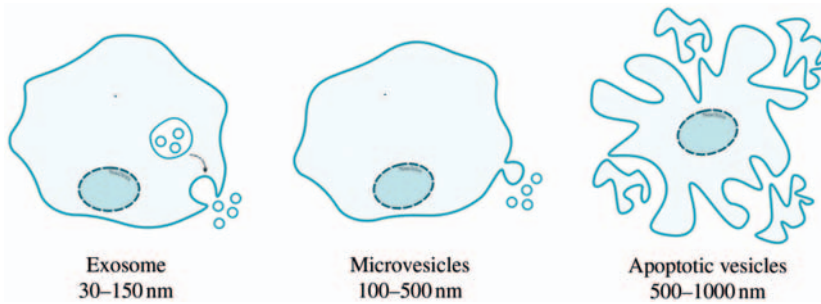
EVs are secreted by most cell types and appear ubiquitously in cell culture, supernatants, and body fluids (blood plasma, serum, saliva, breast milk, urine, amniotic, and cerebral spinal fluids). These vesicles take part in the intercellular communication, since key molecules players (called cargoes) are carried by EVs and transferred to recipient cells. Therefore, EVs exert effects on recipient cells by altering the transcriptome and signaling activity. Based on that, and considering the ability of EVs to cross natural barriers, their inherent cell targeting properties, and stability in the circulation, the use of EVs as drug delivery systems have gained great attention by researchers. EVs have been considered innovative tools for various therapeutic approaches, such as antitumor therapy, vaccination, immune-modulatory and regenerative therapies, and drug delivery ([Wendler et al., 2016](#)).

Despite the potential of EVs as drug delivery devices, there is still a long way to reach the clinical application. This chapter will focus on recent progress and remaining challenges in the engineering of EVs as drug delivery vehicles. However, apoptotic bodies could not be potential drug carriers, since they have a large size compared to other EVs.

11.2 EXTRACELLULAR VESICLES: BIOGENESIS AND COMPOSITION

There are three types of EVs that can be differentiated according to their intracellular origins and sizes: apoptotic bodies (50–5000 nm, containing DNA, RNA and histone proteins), ectosomes (50–1000 nm), and exosomes (30–150 nm) ([Fig. 11.1](#)).

Apoptotic bodies consist of cytoplasm with tightly packed organelles, with or without a nuclear fragment, and are formed during cell death by apoptosis.

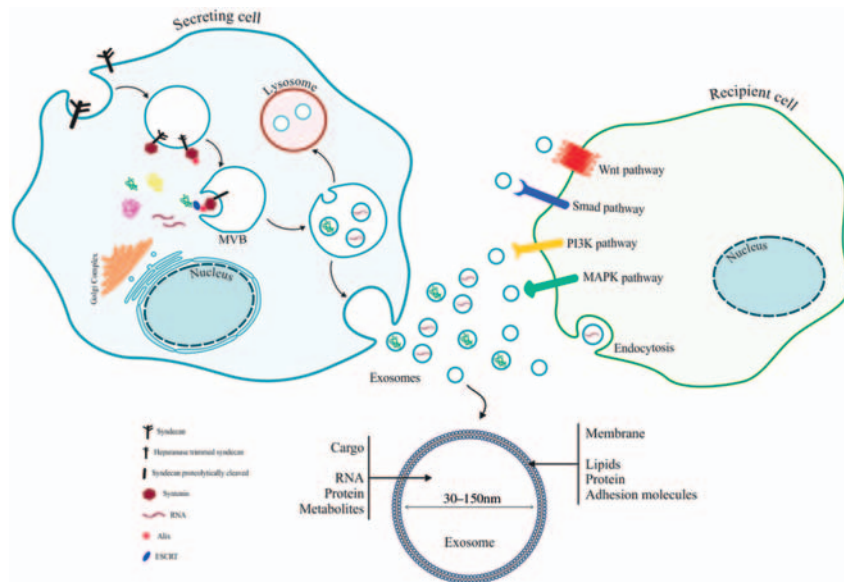
**FIGURE 11.1**

Different types of extracellular vesicles.

Apoptosis occurs in multicellular organisms and depends on biochemical events that lead to characteristic cell changes such as blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. In general, apoptosis is defined as a crucial component of various biological events, including cellular turnover, immune system response, atrophy dependent on hormones, embryonic development, and injury-induced cell death (Akers et al., 2013).

Ectosomes, also referred to with other names (shedding vesicles, microparticles, microvesicles, and others), bud directly from the plasma membrane as small cytoplasmic protrusions covered by specialized membrane rafts, which rapidly grow up and are released by fission of their stalk. It has been shown that specific molecules are enriched in ectosomes (Dolo et al., 2000; Prada and Meldolesi, 2016). These vesicles are the result of dynamic interplay between phospholipid redistribution and cytoskeletal protein. Microvesicle formation is induced by translocation of phosphatidylserine to the outer-membrane through the activity of aminophospholipid translocases. The microvesicle budding depends on ADP-ribosylation factor 6 (ARF6), responsible for initiating a signaling cascade that activates the extracellular signal-regulated kinase (ERK) to the plasma membrane. Subsequently, ERK phosphorylates and activates myosin light-chain kinase (MLCK) promoting the release of microvesicles (Akers et al., 2013). The composition of ectosomes is still unclear, but matrix metalloproteinases, glycoproteins such as GPIb, GPIIb–IIIa, and P-selectin, and integrins seem to be enriched in ectosomes, depending on the cell type. In general, the life span of ectosomes in the producer cell is a few tens of seconds (Cocucci and Meldolesi, 2015).

Exosomes are nano-sized (30–150 nm) vesicles naturally produced by almost every cell type and appear ubiquitously in cell culture supernatants and body fluids. These vesicles represent a physiological mechanism for cell–cell communication, since key molecules players (e.g., proteins, lipids, RNA, and DNA) are cargoes of exosomes and transferred to recipient cells (Akers et al., 2013). Exosome biogenesis is still not well known. It consists of three different

**FIGURE 11.2**

Schematic representation of exosome biogenesis and its effect in recipient cells.

stages: (1) formation of endocytic vesicles from plasma membrane, (2) the inward budding of the endosomal vesicle membrane resulting in multivesicular bodies that consist of intraluminal vesicles, and (3) the fusion of these multivesicular bodies with the plasma membrane, which releases the vesicular contents, known as exosomes (Fig. 11.2) (Milane et al., 2015). In mammalian cells, exosome biogenesis starts with the invagination of the plasma membrane, leading to the formation of primary endocytic vesicles. Subsequently, their maturation creates a late endosomal compartment, also called a multivesicular body (MVB) due to the presence of several intraluminal vesicles. Two molecular mediators play crucial roles in exosome biogenesis:

(1) Endosomal sorting complex required for the sorting/transport (ESCRT) machinery, which is formed by the following steps: exosome formation is triggered by the accumulation of tetraspannins (CD9 and CD63) in the endosomal membrane. Next, extracellular heparan sulfate proteoglycan syndecan interacts with syntenin, which subsequently interacts with ALG-2-interacting protein X (ALIX), and in turn, transmits the signal directly to several ESCRT proteins. The ESCRT system consists of four soluble multi-protein complexes, namely ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III and associated proteins. This machinery complex is recruited to sort selected proteins into intraluminal vesicles and finally triggers the budding and abscission processes in MVBs.

Table 11.1 Main Players in Exosome Biogenesis and Releasing

Molecule	Function
ESCRT-0	Functions in a ubiquitin-dependent way to cluster cargo
ESCRT-I, ESCRT-II, and ALIX	Taking part in budding
ESCRT-III and VPS4	Involved in vesicle scission
Rab, GTPases, and SNAREs	Exosome secretion
Ceramide	Orchestrates an ESCRT independent process of exosome formation, loading and release

(2) Ceramide stimulates the budding process of intraluminal vesicles from the MVB membrane. MVBs can either fuse with lysosomes and their cargo are further degraded; or fused with the plasma membrane of the cell, releasing intraluminal vesicles (called exosomes) into the extracellular compartment. Therefore, ceramide plays an important role to define the fate of MVBs. Furthermore, ceramide, Rab GTPases, synaptotagmin-like 4, exophilin 5 and soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNAREs) also take part in exosome secretion through exocytosis. The first are responsible for trafficking of late endosomes (MVBs) to the plasma membrane, and SNAREs aid the fusion process of MVBs with the membrane (Milane et al., 2015). Table 11.1 summarizes the main players in exosome biogenesis.

In general, exosomes carry different classes of biomolecules (cargoes) such as lipids, proteins, DNA, coding RNAs, noncoding RNAs, and metabolites, protected from degradation by a lipid bilayer (Table 11.2). Development of ExoCarta (<http://www.exocarta.org>), a manually curated database that lists the proteins, RNA, and lipids identified in exosomes (Mathivanan and Simpson 2009; Simpson et al., 2012; Mathivanan et al., 2012), and Vesiclepedia (<http://microvesicles.org>), a community annotation compendium for all EVs (Kalra et al., 2012), have provided a general overview of EVs molecular composition (Simpson et al., 2012). Although EV cargo is defined by the intracellular compositions of its derived cell, the selection of cargoes does not occur in a random way (Schneider et al., 2016). For instance, Nickel and Rabouille (2009) reported that suppressor of cytokine signaling proteins, which do not have N-terminal signal sequences, are preferentially secreted within vesicles. In addition, the ubiquity of a specific protein determines its traffic to endosomal sorting complexes required for transport into vesicles (Hanson and Cashikar, 2012).

The intracellular life span of exosomes is considered long in comparison to ectosomes, tens of minutes or more, necessary for their accumulation within MVBs and for their release (Cocucci and Meldolesi, 2015).

Based on that and considering the ability of EVs to cross natural barriers (such as membranes), their inherent cell targeting properties, and stability in the circulation, the utilization of EVs as drug delivery systems has gained great

Table 11.2 Some Examples of Exosomes Cargoes

Exosome Composition	Examples of Cargoes Found in Exosomes
Lipids	Sphingomyelin Phosphatidylcholine Phosphatidylethanolamine Phosphatidylserine GM3 Phosphatidylinositol Cholesterol
Proteins	HSP70 HSP90 GTPase Annexins Flotilin Tetraspanins: CD9, CD63, CD81, CD82 Thrombospondin ALIX TSG101 Actin Myosin
Nucleic acid	mRNAs7,8miRNA Noncoding RNA
Metabolite	Prostaglandins

scientific interest. For instance, exosomes have been engineered to incorporate therapeutic miRNAs, short interfering RNAs (siRNAs), or chemotherapeutic molecules. Furthermore, examples of protein delivery by exosomes for therapeutic benefit in preclinical models include delivery of catalase, prodrug activating enzymes, and caspase-1.

11.3 INFLUENCE OF CALCIUM, PH, AND HYPOXIA IN THE BIOGENESIS OF EXTRACELLULAR VESICLES

Exogenous and endogenous factors can modulate the type, content, and number of released EVs. There have been several studies demonstrating that an increase in intracellular calcium levels leads to EV biogenesis. For instance, it was reported that the treatment of leukemia cells (K562) with monensin, a Na^+/H^+ exchanger that induces changes in intracellular calcium (Ca^{2+}), generated large MVBs and exosome release was markedly enhanced (Savina et al., 2003). It has

been reported that hypoxic condition is one of the driving forces for vesicle formation. For instance, under hypoxic environment, breast cancer cells released an increased amount of exosomes which was dependent on HIF-1 α (King et al., 2012). In a similar way, hypoxia-resistant multiple myeloma cells secreted higher amount of exosomes, compared to parental cells under normoxia or acute hypoxic conditions (Umezu et al., 2014). Microvesicle biogenesis can also be modulated by hypoxia (Wang et al., 2014). Besides controlling the amount of vesicles formation, hypoxic conditions affect the vesicle cargo composition. Deep and Schlaepfer (2016) have shown that exosomes from prostate cancer produced under hypoxic condition were loaded with a higher number of unique proteins and signaling molecules [including matrix metalloproteinases, heat-shock proteins, transforming growth factor-beta 2, protein kinase B (PKB or AKT), interleukin 6, and tumor necrosis factor alpha (TNF1 α)] and higher amounts of triglycerides in comparison to normoxic prostate cancer exosomes. pH changes in the tumor microenvironment can also contribute to changes of the lipid composition of EVs (Parolini et al., 2009). In addition, it was reported that p53 enhances exosome production (Yu et al., 2006).

11.4 EXTRACELLULAR VESICLE BIOCHEMICAL AND BIOLOGICAL CHARACTERIZATION

The study of exosomes is growing and this field earned lot attention of the scientific community in recent years. Regardless, biochemical or biological characterization is still a challenge. A deep understanding about structure, biochemical function, and cargo uptake/release mechanism may advance research in this field. This section is dedicated to showing the main techniques used so far. EVs characterization can be based in biochemical and biophysical properties that include cargo, lipid and protein content, size, and density (Revenfeld et al., 2014).

11.4.1 EXOSOME/ECTOSOME ISOLATION: DIFFERENTIAL CENTRIFUGATION, POLYMER PRECIPITATION, ANTIBODY-COUPLED MAGNETIC BEADS, AND MICROFLUIDIC

11.4.1.1 Ultracentrifugation

EVs can be isolated using their size and density properties through numerous sequential centrifugation steps with increasing centrifugal force from 10,000 to 20,000 $\times g$ for larger EVs and from 100,000 to 120,000 $\times g$ for smaller EVs. Ultracentrifugation times also vary widely, with 16 h being traditional. Moreover, contaminants like extravesicular protein complexes, aggregates, and lipoproteins may also be pelleted by a high-speed spin. Nonetheless, these have been the most used technique (Witwer et al., 2013; Revenfeld et al., 2014; Caradec et al., 2014).

11.4.1.2 Density gradient centrifugation

To further reduce centrifugation speeds and contaminants, density gradient centrifugation (i.e., sucrose or iodixanol) has gained popularity. However, it should be noted that centrifugation times are longer than ultracentrifugation and vary from 62 to 90 h.

11.4.1.3 Polymer-based precipitation

Exosomes can also be isolated using polymer precipitation method, and the ExoQuick exosome precipitation kit (SBI System Biosciences, Inc.). This kit allows the use of a small sample volume and recovers purified exosome, comparable to ultracentrifugation in electron microscopy (EM) and functional assays (Sohel et al., 2013). Another commercial kit (Total Exosome Isolation Reagent—Invitrogen, Carlsbad, CA, United States) uses hydrophobicity to separate, forcing less-soluble components such as exosomes out of the solution, allowing the collection by a short and low-speed centrifugation (Lane et al., 2015).

11.4.1.4 Size exclusion

EVs are separated based on their size differences by passage through or around physical barriers, such as a porous filters or chromatography resins. Commercially available kits are manufactured by qEV (Izon Sciences, Cambridge, MA, United States) and ExoMir (Bioo Scientific, Austin, TX, United States). EVs from adenocarcinoma cell lines were successfully isolated through sequential centrifugation with an extra filtration step to decrease the amount of contaminants (Koga et al., 2005).

11.4.1.5 Affinity

Isolation of EVs through affinity methods depends on the specific EV surface proteins (antibody–antigen) or physiochemical properties. EVs from adenocarcinoma cell lines were purified using herceptin-coated magnetic beads and the magnetic bead-exosome complexes were used for further assays. In this particular case, the authors intention was to selectively isolate HER2-containing exosome, but the approach can be used to other proteins (Koga et al., 2005).

In addition to previously mentioned procedures, in the last few years, microfluidic devices have emerged for EV isolation (Ingato et al., 2016).

11.4.1.6 General extracellular vesicle isolation problems

Despite many authors reporting EV isolation procedures, we still face some problems in this area. Firstly, it is common to have inconsistencies in the isolates, mainly due to nonstandardized centrifugation protocols. This fact may, at least partially, explain the differences in biological effects of EVs reported by various research groups. Secondly, an inherent problem with isolating exosomes and ectosomes from body fluids is the fact that these potentially contain high amounts of non-EV particles, such as lipoproteins, viruses, and biomolecule aggregates.

Exosomes generally overlap in size with viruses and lipoproteins, whereas ectosomes overlap with the size range of bacteria. Such contaminants need to be removed in order to obtain sufficiently pure isolates. With regard to viruses, sucrose gradients are inefficient at separating them from exosomes, but [Cantin et al. \(2008\)](#) recently showed that the use of iodixanol gradients do allow their separation and purification. Contamination with proteins and protein complexes, such as insoluble immune complexes, also disrupt the isolation of both exosomes and ectosomes ([Rood et al., 2010](#); [Gyorgy et al., 2011](#)). Furthermore, filtration of EV isolates under pressure to remove particular contaminants or fractions carries the inherent risk that fragmentation of vesicles occurs and thus sample might be lost. Finally, specific purification protocols after ultracentrifugation not only affect EV purity and yield, but often lead to protein loss in the preparation ([Webber and Clayton, 2013](#); [Nordin et al., 2015](#)). For further information on currently available isolation methods, the reader is referred to comprehensive reviews ([Choi et al., 2013](#); [Momen-Heravi et al., 2013](#); [Sàenz-Cuesta et al., 2015](#)).

11.4.2 EXTRACELLULAR VESICLE CHARACTERIZATION

Over recent decades, the research field of EVs has expanded exponentially, together with the need for the development of a number of methods allowing for the detection of particles with characteristic sizes below 200 nm. Here, we summarized the methods available for characterizing EVs. For more details we invite the readers to have a look at the review published by [Rupert and collaborators \(2017\)](#).

11.4.2.1 *Electron microscopy*

A classic method, EM has been used since the 1980s for study EVs, providing information about size and shape mainly for validation of purification protocol ([Pan et al., 1985](#)). EM has some limitations, such as influence of chemical fixation, dehydration, resin embedding, and limited substructure details, but is a nano-scale technique capable of detecting those small vesicles (50–90 nm) ([Théry et al., 2001](#)). Electron microscopy uses a high voltage of electron and shows the morphology of EVs. Nonetheless, it gives no information about number of particles ([Dragovic et al., 2011](#)). There are two types of EM that can be used for EVs analysis. The first one is transmission electron microscopy (TEM). TEM visualizes two-dimensional data of a sample, however it can gain three-dimensional data through electron tomography method application or through the reconstruction of the successive sections ([Harding et al., 1984](#); [Taylor and Gercel-Taylor, 2008](#); [Chandler and Roberson, 2009](#)). Scanning electron microscopy (SEM) is the second, and uses a thin, focused electron beam to obtain a high-resolution three-dimensional image built by a raster pattern of the topographical details of a specimen's surface. SEM can be used for thicker samples, instead of TEM ([Kuo, 2007](#); [Park and Choi, 2014](#); [Rupert et al., 2017](#)).

11.4.2.2 Atomic force microscopy

Also known as scanning force microscopy (SFM), atomic force microscopy (AFM) has the advantage of having resolution comparable to that of EM (nano-scale) without chemical fixation or extreme temperatures. AFM provides information about structure, mechanical properties, and bio-molecular composition, by scanning the surface with a sharp tip (Sharma and Gimzewski, 2012; Park and Choi, 2014). AFM was used for the characterization of salivary exosome sub-structural organization, unresolvable in EM (Sharma et al., 2010).

11.4.2.3 Fluorescence microscopy

Fluorescence microscopy uses a microscope specialized in fluorescence that is emitted when the initial light of the microscope interacts with the fluorophore. With this technique, is possible to monitor the trafficked exosomes into living cells and to establish the process of exosome uptake. Using a fluorophore, a specific biomarker for the exosome, an immunofluorescence to image exosomes in vitro and in mouse models can be performed. Therefore, a real time in vivo observation of exosome transportation from donor cell to recipient cell can be done, once the fluorophore, bound to the membrane of exosome, fluoresces. One limitation is that totally isolated exosomes are required (Park and Choi, 2014). This method was used to show exosomes secreted from breast cancer cells by transferring the tumor microenvironment, and their incorporation into other cancer cells and normal tissue cells, suggesting the contribution of exosomes in promoting tumor growth and metastasis (Suetsugu et al., 2013).

11.4.2.4 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is the most commonly used method for characterization of exosome size and concentration. It allows a direct and real-time visualization by light scattering using a light microscope. The size and the total concentration of exosome are calculated according to the rate of Brownian motion of individual exosome (Park and Choi, 2014). Two groups can only be distinguished if their particle diameter differs at least 1.5-fold (Sitar et al., 2015).

11.4.2.5 Western blotting

Western blotting is an analytical technique used to identify whether a specific protein is present or absent (Revenfeld et al., 2014). The first step is to separate the protein bands through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins are transferred to a membrane, where specific antibodies to the target protein are used as probe. The drawback of this technique is the large amount of samples required (Park and Choi, 2014). One main application of Western blotting is to prove that the exosome isolation technique was successful through the detection of protein levels. It is possible to check exosome membrane protein content (e.g., CD63) and if the sample is free

of any protein of cellular origin (e.g., CYCS, a mitochondrial protein) (Sohel et al., 2013).

11.4.2.6 Flow cytometry

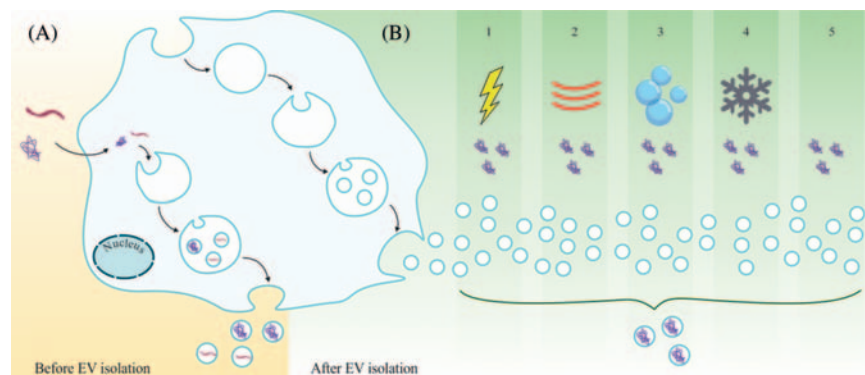
Flow cytometry is a laser-based technology mainly used to measure fluorescence intensity or the scattered light, to obtain information of the cells by analyzing their physical properties. In general, flow cytometry can detect vesicles above 300 nm in diameter. Weak signal makes analyzing EVs via flow cytometry challenging. Therefore, for exosome detection, scientists improved flow cytometry method by using aldehyde sulfate latex beads to adsorb exosomes. The bead-exosome complex is then labeled with a primary antibody. Fluorescence-activated cell sorting (FACS, Becton Dickinson, USA) can detect and analyze fluorescent cell-derived vesicles of 100 nm (Valadi et al., 2007; van der Vlist et al., 2012). Fluorescent compounds can be used to confirm exosome cargo loading by FACS such as the detection of trapping of doxorubicin in exosomes derived from diffuse large B-cell lymphomas cell lines (Koch et al., 2016).

11.4.2.7 Dynamic light scattering

Small particles move in a Brownian motion influenced by solvent, temperature, and particle size. When the laser beam is generated in the particle, the light scatters in different angles according to their size. Dynamic light scattering (DLS) uses this relation to analyze the size of the particles. The light from all particles is detected at a single detection, which means that in an heterogeneous mixture of vesicles, to get the size distribution of particles is not trivial. If the size of vesicles differs at least twofold, DLS can distinguish the population. Therefore, the exosomes should be sorted into an homogeneous size (Park and Choi, 2014). DLS combined with another approach was used successfully for airway epithelial-derived exosomes analysis (Kesimer and Gupta, 2015).

11.5 METHODS FOR LOADING CARGO IN EXTRACELLULAR VESICLES

Specific cargo can be incorporated into EVs aiming to improve the distribution, and these vesicles also can be engineered to achieve enhanced uptake. In this aspect, exosomes have been evaluated as a drug delivery vehicle for a diverse class of therapeutic molecules, including both small molecules (doxorubicin, curcumin, and paclitaxel) and macromolecules (RNA, DNA, and proteins). Generally, two strategies have been employed to incorporate these cargoes: pre-exosome formation (incorporation of the desired molecule into the cells before exosomes formation) and postformation (incorporation of the desired molecule into exosomes after their biogenesis and releasing) (Fig. 11.3) (Kooijmans et al., 2016; Vader et al., 2016).

**FIGURE 11.3**

Overview of extracellular vesicle loading strategies. (A) Loading of cells before extracellular vesicle isolation: specific cells are transfected with nucleic acids (siRNAs, MicroRNAs and mRNAs) or treated with small molecules (e.g. chemotherapeutic), followed by packaging into EVs during their biogenesis; (B) Loading of extracellular vesicle after isolation: different techniques can be used such as electroporation (1), sonication (2), saponin-treatment (3), freeze-thaw cycles and (5) incubation at room temperature.

11.5.1 LOADING OF CELLS BEFORE EXTRACELLULAR VESICLE ISOLATION

Therapeutic cargo is first loaded into the producer cell, followed by packaging into exosomes during their biogenesis. This strategy has been used for packaging paclitaxel (PTX), nucleic acids (siRNAs, miRNAs, and mRNAs), and proteins. However, in the case of nucleic acids, loading into exosomes can be accomplished by transfection of the producer cell with the respective cargo (miRNA) by lipofection or via a plasmid vector encoding for the desired cargo. Proteins can also be sorted into exosomes by creating a fusion construct containing the protein of interest, linked to a protein that is inherently associated to exosomes (Vader et al., 2016).

11.5.2 LOADING OF EXTRACELLULAR VESICLE AFTER ISOLATION

Postformation loading approaches attempt to load drugs in isolated and purified exosomes, and can also be classified as passive and active loading.

Passive loading is usually used for hydrophobic membrane-permeable molecules, simple coincubation at ambient or elevated temperature is often sufficient to load exosomes. Sun et al. (2010) reported the passive loading of exosomes incubated with curcumin. Incubation of exosomes with curcumin for 5 min produced a loading rate of 2.9 g curcumin per 1 g exosomes and allowed for delayed release in vivo. Passive loading of prostate cancer cell-derived exosomes with

PTX was also described. In this study, the authors observed that 9.2% of the drug was loaded after 1 h incubation at room temperature (Saari et al., 2015).

Active loading refers to strategies that enable more efficient penetration of therapeutics through the lipid bilayer than exclusive incubation. In this regard, electroporation is the most frequently reported method, especially for hydrophilic membrane-impermeable components. Hood et al. (2014) applied single-pulse electroporation to exosomes in order to load RNA, and reported a significant loading enhancement, compared to passive loading. Wahlgren et al. (2012) optimized electroporation for loading RNA into exosomes. They reported the optimized conditions as 150 V/100 μ F.

Nonetheless, in the case of hydrophilic cargo, the strategy transiently destabilizes the exosome membrane, by repeating freeze-thaw cycles, sonication, extrusion, or saponin treatment. Haney et al. (2015) compared several methods of active loading: permeabilization with saponin, freeze-thaw cycles, sonication, and extrusion. Sonication of exosomes in the presence of the therapeutic, catalase, proved to be the best formulation with the highest loading efficiency of ~ 200 μ g catalase per mg exosomes and the slowest release. To preserve the exosome structure, sonication was completed at 20% power with brief pulses of 4 s each.

Although postformation loading requires additional purification processes, in comparison to loading EV via their parent cells, they often produce more efficient loading outcomes.

It is important to mention that, as long as the fundamentals of EV biogenesis and cargo sorting are not clear, preformation methods will suffer from limited efficiency. Indeed, when comparing both loading strategies for PTX, the postformation method yields ~ 21 mg/g EV and ~ 7.3 mg/g EV, compared to ~ 2 μ g/g EV for the preformation method (Pascucci et al., 2014; Saari et al., 2015; Yang et al., 2015).

11.6 EXTRACELLULAR VESICLE MIMETICS

The low production yield of naturally secreted EVs, which is difficult for scalable production, remains one of the biggest challenges for their clinical use. Therefore, it has been proposed to develop EV-mimetic nanovesicles derived from cells (Kim et al., 2016a,b). This novel type of EV mimetic has been made by breaking down the cells through serial extrusion using nano-sized filters with diminishing pore sizes, followed by density gradient ultracentrifugation. The loading of small molecules to these EV-mimetics can occur either during the extrusion or to the nanovesicles after ultracentrifugation step. In addition, for the exogenous loading of siRNAs, siRNAs were loaded by electroporation (Kim et al., 2016a,b). Jang et al. (2013) developed bioinspired exosome-mimetic nanovesicles that delivered doxorubicin to the tumor tissue after systemic administration. The doxorubicin-loaded nanovesicles were produced by the breakdown of Raw 264.7 macrophages using a

serial extrusion through filters with diminishing pore sizes. These cell-derived nanovesicles have similar characteristics with the exosomes but have 100-fold higher production yield. These authors reported that, *in vivo*, chemotherapeutic doxorubicin-loaded nanovesicles were taken up by tumor tissue (colorectal cancer) and reduced tumor growth. Importantly, when compared with free doxorubicin, adverse effects were not observed when nanosized formulation was used. Furthermore, compared with doxorubicin-loaded exosomes, doxorubicin-loaded nanovesicles showed similar *in vivo* antitumor activity. Importantly, doxorubicin-loaded liposomes that did not carry targeting proteins were inefficient in reducing tumor growth (Jang et al., 2013). Recently, Lunavat et al. (2016) reported that EV-mimetic carrying c-Myc shRNA was taken up by recipient cells (mouse lymphoma cells), which resulted in downregulation of target gene expression.

11.7 EXTRACELLULAR VESICLES UPTAKE INTO RECIPIENT CELLS

Once generated and released, the outcomes of the two types of EVs are similar. Part of both types of EVs undergoes dissolution of their membrane (acting locally and establishing interactions with target cells) and most EVs navigate in the extracellular fluids (Prada and Meldolesi, 2016). EV uptake has been shown to occur via multiple routes, including a direct fusion between EV-plasma membrane or via endocytosis. The latter is a process that depends on energy and demands a functioning cytoskeleton, and in general happens quickly (around minutes). EV endocytosis occurs by different mechanisms: lipid raft-, clathrin-, and caveolae-dependent endocytosis, macropinocytosis, and phagocytosis.

11.7.1 PARTICIPATION OF LIPID RAFTS

Lipid rafts are specific regions (defined as subdomains or microdomains) of the plasma membrane containing proteins and lipids, such as cholesterol and sphingomyelin. These specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecule mediators, influencing membrane fluidity, and membrane protein trafficking. Specifically, in the case of EV uptake, sphingolipids present in the EV membrane are crucial for vesicle binding and endocytosis. For instance, it was reported that pretreatment of recipient cells with methyl- β -cyclodextrin, filipin, and simvastatin (cholesterol-reducing agents) decreased EV uptake by those cells (Svensson et al., 2013).

11.7.2 CLATHRIN-MEDIATED ENDOCYTOSIS

Cellular EV internalization via clathrin-mediated endocytosis occurs via a sequential assembly of clathrin-coated vesicles, which contain a set of proteins located

into their membranes. Clathrin-coated vesicle formation proceeds through five stages that correspond to ultrastructural and cell biological observations: initiation, cargo selection, coat assembly, scission, and uncoating. The subsequent intracellular vesicle fuses with the endosome where it deposits its contents (Kirchhausen, 2000). Escrevente et al. (2011) reported that the ovarian cancer cells treated with chlorpromazine (a well-known clathrin-mediated endocytosis inhibitor) were less efficient in taking up EVs.

11.7.3 CAVEOLIN-DEPENDENT ENDOCYTOSIS

Caveolae are subdomains in the plasma membrane, containing cholesterol, sphingolipids, and caveolins, which can be internalized by the cell. It was shown that the internalization of exosomes isolated from culture medium of cells infected with Epstein-Barr virus was significantly suppressed by treatment with an inhibitor of dynamin or by the knockdown of caveolin-1. In the same study, it was also reported that labeled exosomes were colocalized with caveolae and subsequently trafficked through endocytic pathways (Nanbo et al., 2013).

11.7.4 MACROPINOCYTOSIS

The macropinocytosis process involves actin-mediated membrane ruffling of the plasma membrane. Most of the lamellipodia formed retract back into the cell, however, some of them may fold back onto themselves and fuse with the basal membrane, creating large, irregular shaped vesicles (Lim and Gleeson, 2011). These vesicles contains extracellular fluid and other molecules that are close to the region around the membrane ruffles. Macropinocytosis is a process dependent on rac1, actin, and cholesterol and it demands Na^+/H^+ exchanger activity. Inhibition of rac1 or Na^+/H^+ exchanger abrogates the macropinocytosis process and consequently, decreased uptake of oligodendrocyte-derived EV by microglia (Fitzner et al., 2011).

11.7.5 PHAGOCYTOSIS

Phagocytosis is a process mediated by receptors that culminates with a formation of invaginations encompassing the material destined for internalization. Rudt and collaborators (1993) reported that particles sized as small as 85 nm have been internalized by phagocytosis. This finding is indicative that EVs can be taken up via this process.

The route of EV uptake is likely dependent on the following factors: (1) lipid and protein composition of the EVs and plasma membranes of donor and recipient cell types; (2) EVs subtypes; (3) state of cells releasing and taking up EVs; and (4) conditions in the extracellular space, such as acidic versus basic, hypoxia, and extracellular matrix components (Schneider et al., 2016).

11.8 ASSESSING EXTRACELLULAR VESICLE UPTAKE INTO RECIPIENT CELLS

Taking into account that most EVs are nano-sized (as opposed to micrometer-sized) cells, an ideal EVs labeling method for imaging at subcellular resolution and consequently tracking them, should attend the following criteria: (1) specific labeling of EVs; (2) a half-life compatible with that of EVs; (3) stable and sufficient signal to detect nanoscaled EVs from background noise; and (4) not be metabolized. The exploration of the role of EVs *in vivo* is a challenge by difficulties in labeling endogenous EVs, tracing their trafficking, and identifying target cells without interfering with their function. Recent progress has been made in imaging single EVs *in vivo* (Huang-Doran et al., 2017). Some of this progress is described further.

The methods for detecting EV uptake involve the use of fluorescent dyes, membrane-permeable chemical compounds, reporters conjugated to fluorescent proteins, bioluminescence reporters, radionuclide labeling, and superparamagnetic iron oxide nanoparticles for magnetic resonance imaging (MRI) (Fig. 11.4).

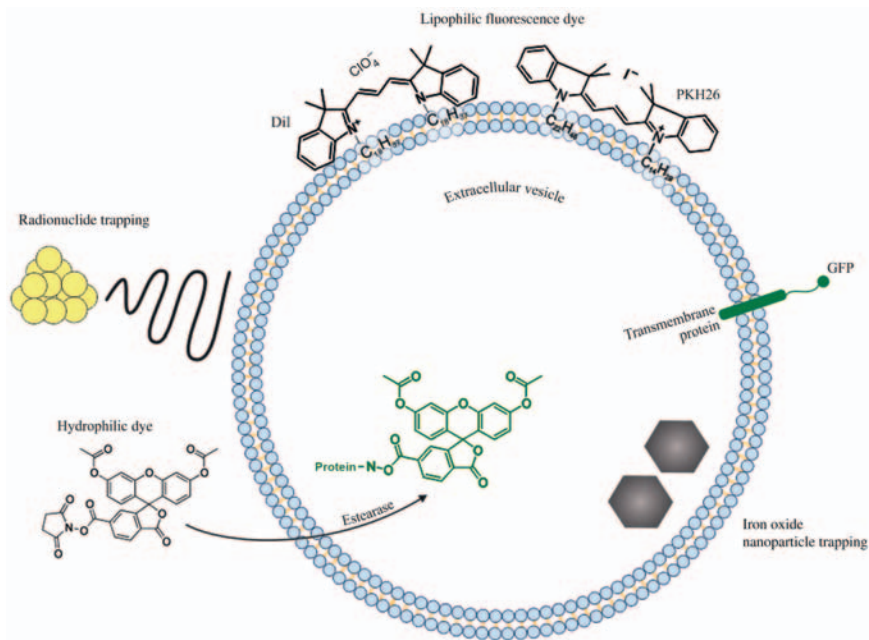


FIGURE 11.4

Different labeling procedures for tracking EVs. Some labeling methods have been standardizing. Lipophilic fluorescence dye and reporter imaging using fluorescence or bioluminescence combined with transmembrane proteins. For *in vivo* imaging, radionuclide imaging or MRI (Magnetic Resonance Imaging) can be employed.

11.8.1 LABELING MEMBRANE

Lipophilic dyes, such as PKH and 1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) are the most commonly used dyes to label EVs. PKH26 is a red fluorescent dye while PKH2 and PKH67 are green fluorescent probes. Therefore, the last two can be used with red fluorescent probes such as propidium iodide (PI), 7-aminoactinomycin D, and R-phycoerythrin, without interfering in the analysis. A disadvantage of using PKH dyes is their unspecificity in labeling EVs. They label other lipid-containing entities in the extracellular space, and presumably in recipient cells, potentially leading to false-positive signals (Li et al., 2013). Furthermore, PKH dyes were reported to display 5 to >100 days of half-life, in vivo. Consequently, the dye may outlast labeled EVs following their processing/degradation by cells, leading to misinterpretation of dye residues and/or recycled dye-labeled membrane as originally labeled EVs (Mulcahy et al., 2014).

S-Palmitoylation through a thioester linkage between the sulfhydryl group on cysteines and the fatty acid palmitic acid enables association of proteins with cellular membranes. By fusing fluorescent proteins with a consensus palmitoylation sequence, these reporters can be transferred to the cellular membrane, enabling whole-cell labeling (Lai et al., 2015).

Genetically engineered bioluminescent proteins, such as *Gaussia luciferase*, combined with lactadherin were employed to track the distribution of EVs, in vivo, without background autofluorescence (Lai et al., 2014; Takahashi et al., 2013). Another approach used a streptavidin reporter expressed in EVs conjugated with ¹²⁵I-labeled norbiotinamide (Morishita et al., 2015; Choi and Lee, 2016).

11.8.2 LABELING EXOSOME CARGOES

RNA and protein cargoes are labeled by using fluorescent probe. The exo-red stain is based on acridine orange, a fluorescent cationic dye. It is cell-permeable and interacts with DNA by intercalation and RNA by electrostatic attractions. ExoRNAs (labeled RNAs inside of exosomes) can then be monitored for delivery into target cells via the exosomes using fluorescent microscopy. Exo-green stain is based on carboxyfluorescein succinimidyl diacetate ester (CFSE) chemistry, which is also membrane -permeable. When CFSE enters exosomes, it is hydrolyzed by exosome esterases that remove the diacetate residues (Escrevete et al., 2011), then coupled to the amino ends of exosome proteins. This approach allows tracking of internal exosome protein transfer into target cells using fluorescent microscopy.

More recently, Zomer et al. (2015, 2016) reported a technique based on using the Cre-loxP system to fluorescently mark Cre-reporter cells that take up EVs released by Cre recombinase-expressing cells in vitro and in vivo.

11.8.3 INTRAVESICLE PROBE

Detection and analysis of EVs by flow cytometry have been a challenge, as general staining with membrane dyes or conjugated antibodies against surface markers fails to discriminate between intact vesicles and debris. Therefore, some strategies have been described for flow cytometry-based analysis of EVs: to use a probe (such as calcein AM) that is membrane-permeant and nonfluorescent until it is cleaved by intravesicular esterases. [Gray et al. \(2015\)](#) reported a calcein AM-based method to detect and quantify EVs derived from a large number of samples (human and mouse, different cell types, cultured cells in various conditions and in the circulation of humans in different states of health). The authors provided evidence that this method was able to distinguish between intact vesicles and disrupted vesicles.

Another strategy is to use superparamagnetic iron oxide nanoparticles for MRI. For instance, it was produced nanoparticle-laden exosomes by electroporation with superparamagnetic iron oxide nanoparticles ([De La Peña et al., 2009](#); [Hu et al., 2014](#)).

11.9 OVERVIEW OF EXTRACELLULAR VESICLES FOR THERAPEUTIC DELIVERY OF SMALL MOLECULES, PROTEINS, AND RNAs

Some diseases, like cancer, are urgently in need of new systemic therapeutics which can be delivered safely and effectively. In the drug delivery systems field, liposomes and polymeric nanoparticles are preferred for carrying different types of drugs. However, the ability of an ideal liposome to evade the host immune system with stability, a long circulating capability, and without toxicity still remains elusive. Conversely, polymeric nanoparticles may have better stability than liposomal systems, but their biocompatibility and long-term potential safety remains a concern. In this context, EVs have been considered desirable vehicles for targeted drug transport, as they encompass many interesting features for drug delivery: (1) a proteo-lipid membrane that protects the encapsulated cargoes; (2) their nano size and specific composition minimizes recognition by the mononuclear phagocyte system; (3) their self-derived nature mitigates activation of the adaptive immune system; (4) they contain specific lipids that help stabilize the vesicles in the blood circulation and stimulate membrane fusion; (5) they seem to possess intrinsic cell- and tissue-targeting properties; (6) they cross the blood brain barrier ([Lener et al., 2015](#); [Strotman and Linder, 2016](#)).

In recent years, several studies have been done to evaluate the use of EV-based therapeutics. Pharmaceutical EV preparations is categorized as a biological medicine, also known as biological drugs, biologicals, or biopharmaceuticals, which are defined as a medicine that contains one or more active substances

made by or derived from a biological cell. Based on the active substances, the International Society for Extracellular Vesicles (ISEV) suggests categorizing EV-based therapeutics as Biological Medicine in the following scenarios: EV-based therapeutics derived from unmodified cells containing native EVs; EV-based therapeutics derived from genetically manipulated cells, but the released EVs do not contain trans-gene products; and native EVs used as drug-delivery systems for chemical drugs or for other molecular components, such as miRNAs or siRNAs (Lener et al., 2015).

Table 11.3 summarizes some studies in which EVs produced by different cells were loaded with small molecules, RNAs, and proteins. The following topics will provide examples of molecules carried by EVs, as well as their biological effects focusing in therapeutic application.

Table 11.3 Studies Designed Using EVs as Platforms to Delivery of Interest Therapeutic Molecules

Cargo	Aim	Reference
Small Molecules		
Doxorubicin	Delivery of targeted exosomes and EMs to cancer cells and tumor tissue	Tian et al. (2014), Jang et al. (2013), Hadla et al. (2016)
PTX	Improve cytotoxicity of PTX encapsulation	Pascucci et al. (2014), Kim et al. (2016a,b)
Curcumin	Exosomes as platforms to improve delivery of curcumin	Sun et al. (2010)
Proteins		
Catalase	Delivery of catalase across blood–brain barrier to treat Parkinson’s disease	Haney et al. (2015)
Caspase-1	Confirm activity of encapsulated caspase-1 into exosomes	Sarkar et al. (2009)
Prodrug converting enzyme CD	Delivery of CD to Schwannoma tumor in a mice model	Mizrak et al. (2012)
Nucleic Acids		
miRNA	Exosomes to deliver miRNA-7b to breast cancer in a mice model	Ohno et al. (2013)
siRNA	Delivery of GAPDH siRNA and BACE-1 siRNA to Alzheimer’s disease both in vitro and in vivo	Alvarez-Erviti et al. (2011)
	Human exosomes to deliver siRNA against MAPK-1	Wahlgren et al. (2012)
	Human exosome to deliver siRNA against PLK-1	Greco et al. (2016)
	EMs to deliver siRNA against CDK4	Yang et al. (2016)

11.9.1 EXOSOMES AS DRUG DELIVERY PLATFORMS FOR SMALL MOLECULES

Recently studies provide evidences of the possibility of loading chemotherapeutics, drugs such as doxorubicin and PTX, into exosomes (Jang et al., 2013; Pascucci et al., 2014; Tian et al., 2014; Saari et al., 2015; Hadla et al., 2016; Kim et al., 2016a,b). In 2014, Tian and colleagues showed for the first time the use of targeted exosomes to delivery doxorubicin to solid tumors in vitro and in vivo (Tian et al., 2014). Mouse immature dendritic cells (imDCs) were the source of exosomes which were targeted to the tumor by engineered ImDCs to express an exosomal membrane protein (Lamp2b) fused to α v-integrin-specific iRGD peptide. ImDCs-exosomes loaded with doxorubicin via electroporation showed an efficient targeting to α v-integrin-positive breast cancer cells in vitro. Systemic administration of iRGD-doxorubicin exosomes inhibited tumor growth in BALB/c nude mice while reducing the heart toxicity. Doxorubicin loaded in exosomes and exosome-mimetic nanovesicles also inhibited the growth of ovarian cancer and colon adenocarcinoma tumors in vivo (Jang et al., 2013; Hadla et al., 2016). In vivo experiments showed that doxorubicin-exosome mimetic nanovesicles were taken up by tumor tissue and decreased tumor growth without adverse effects. The authors showed in the same paper that doxorubicin-loaded liposomes that did not carry targeting proteins were inefficient in reducing tumor growth (Jang et al., 2013). PTX is a common antimitotic cancer therapeutic; however, due to its side effects, the development of nanoencapsulated formulations has gained interest. PTX was encapsulated in different populations of exosomes and was able to enhance the cytotoxicity in vitro and in vivo (Pascucci et al., 2014; Saari et al., 2015; Kim et al., 2016a,b).

A new approach for PTX delivery was evaluated by Pascucci et al. (2014). In this paper, mesenchymal stem cells (MSCs) were proposed for delivering anticancer drugs, despite their ability to take up and release drugs into vesicles, including exosomes. Based on this fact, the authors proposed a simple procedure of exposing cells in vitro to a high concentration of PTX. SR4987 cells were treated with high doses of PTX and acquired resistance to this drug, due to the ability of these cells to package PTX into microvesicles and exosomes. The authors have demonstrated by High-performance liquid chromatography (HPLC) the presence of PTX released by SR4987 cells and significant antiproliferative activity of SR4987PTX microvesicles/exosomes against CFPAC-1 cell line (human pancreatic adenocarcinoma) (Pascucci et al., 2014).

Different sources of exosomes are being used as vehicles to delivery PTX. One of them is autologous prostate cancer cells (Saari et al., 2015). EVs of LNCaP and PC-3 cells were isolated and incubated with PTX. The main finding of this study is that the loading of PTX to autologous prostate cancer cells-derived EVs increases its cytotoxicity (Saari et al., 2015).

Exosome-based drug delivery platforms could be designed for the therapeutic of multidrug resistant (MDR) cancer. The incorporation of PTX increased the

cytotoxicity more than 50 times in MDR cancer cells (Kim et al., 2016a,b). A refined paper published in 2016 by Kim and colleagues showed the efficacy of exosome-PTX in vitro and in vivo experiments. PTX was loaded by different methods in exosomes released in the culture medium by macrophages RAW264.7. The ability to transport exosome-PTX was studied with fluorescent-labeled exosomes in 3LL-M27 cells. Confocal images showed a high accumulation of fluorescent-labeled exosomes in 3LL-M27 lung carcinoma cells, compared to standard fluorescent-liposomes or fluorescent-polystyrene nanoparticles, suggesting that PTX can be efficiently delivered by exosomes. To corroborate these findings, the authors observed an increased cytotoxicity in vitro of exosome-PTX and inhibition of metastases and tumor growth in a mouse model of pulmonary metastases, compared to PTX alone (Kim et al., 2016a,b).

Curcumin is a natural polyphenol found in the rhizome of *Curcuma longa* and exhibits antiinflammatory, antioxidative, and antineoplastic activity (Anand et al., 2008; Aggarwal and Harikumar, 2010). Curcumin was found to regulate and inhibit different oncogenes (Nagaraju et al., 2015) and was also able to modulate the expression of several miRNAs (Teiten et al., 2013). Despite its benefits, curcumin shows poor solubility and low bioavailability, due to its hydrophobic characteristics and interaction with lipid membranes (Anand et al., 2007). To improve solubility and bioavailability, researchers have encapsulated curcumin in exosomes (Sun et al., 2010; Taverna et al., 2016).

Curcumin was incorporated into exosomes by simple incubation with exosomes derived of murine tumor cell line (E1-4). Exosomes were then characterized by Western blotting and the purity was increased by subjecting curcumin-exosomes to a sucrose-gradient ultracentrifugation. A set of experiments were carried out to provide information about the incorporation of exosomes, while increasing solubility and bioavailability. Antiinflammatory activity of curcumin-exosomes was assessed by in vitro and in vivo approaches. In vitro curcumin-exosomes provided slightly pro-inflammatory cytokines in macrophages, when compared to curcumin alone. For in vivo tests, a mouse model of septic shock was produced by treatment with lipopolysaccharides (LPS) and the survival was improved in animals treated with curcumin-exosome, compared to treatment only with curcumin (Sun et al., 2010).

In another study, Taverna et al. (2016) treated K562 cells (CML line) with curcumin and observed that these cells released curcumin-exosomes with a high amount of miRNA-21. Interestingly, HUVEC cells treated with curcumin-exosomes were able to take up the miRNA-21 and had decreased angiogenic phenotype.

11.9.2 EXOSOMES AS DRUG DELIVERY PLATFORMS FOR PROTEINS

In addition to small molecules, exosomes are also able to deliver therapeutic proteins.

Catalase was loaded into exosomes and proposed to be relevant to Parkinson's disease, to reduce oxidative stress and promote neuron survival (Haney et al., 2015). Exosomes were obtained from conditioned media of macrophages Raq 264.7, isolated by ultracentrifugation and characterized by NTA and DLS. The incorporation of catalase into exosomes by sonication was successfully evaluated by hyperspectral microscopy and the antioxidative effects of catalase-exosome were evaluated by *in vitro* and *in vivo* experiments. The uptake of catalase-exosome was confirmed by images of confocal microscopy of stained catalase-exosome with Dil, in different neuronal cells and in mouse brain. The authors have demonstrated that catalase-exosome exposed macrophages displayed lower levels of reactive oxygen species, when compared to nonexposed macrophages, in response to LPS or tumor necrosis- α (TNF- α). In addition, an *in vivo* mice model of Parkinson's disease was developed by administration of 6-hydroxydopamine and catalase-exosomes were able to cross the blood–brain barrier and deliver catalase to the brain, reducing inflammation (Haney et al., 2015).

The delivery of apoptosis-inducing enzyme, caspase-1, has been explored for a number of diseases such as, schwannomas and gliomas (Sarkar et al., 2009; Hall et al., 2016). Encapsulated caspase-1 seems to play a key role in programmed cell death. In a set of experiments, Sarkar et al. (2009) showed LPS-induced monocytes to release caspase-1 into exosomes. It also showed that caspase-1 is active when encapsulated into exosomes and induces programmed cell death in recipient cells (vascular smooth muscle cells). Importantly, the activity of caspase-1 in LPS-induced monocytes was not a direct effect of the endotoxin LPS (Sarkar et al., 2009).

Another important study that showed exosomes as a delivery system for proteins was developed to load a prodrug activating enzyme, cytosine deaminase (CD) (Mizrak et al., 2012). This enzyme converts 5-fluorocytosine (5-FC, a prodrug) to 5-fluorouracil, which is toxic to cells expressing uracil phosphoribosyl-transferase (UPRT). This is due to conversion to 5-fluoro-deoxyuridine monophosphate, an irreversible inhibitor of thymidine synthetase thus reducing DNA synthesis and causing apoptosis. The authors generated engineered stable HEK-293T cells which expressed the suicide therapeutic-mRNA/protein for CD, fused to UPRT in exosomes. Exosomes loading CD-UPRT were able to inhibit schwannoma tumor growth in mice models when injected into tumor, in combination with systemic administration of 5-FC (Mizrak et al., 2012).

11.9.3 EXOSOMES AS DRUG DELIVERY PLATFORMS FOR RNAS

Over the past decade, it is known that exosomes transfer miRNAs and other RNAs from donor to recipient cells (Valadi et al., 2007). The majority of studies use the ability of exosomes as nanovehicles to deliver genetic materials (Johnsen et al., 2014; Lässer, 2015; Ha, Yang and Nadithe, 2016), which can affect gene expression in certain diseases and improve the development of genetic therapy.

Exocarta, a web-based compendium of exosomal cargo, includes more than 7540 different types of RNA in 250 articles (Keerthikumar et al., 2016). The mechanism of loading RNA into exosomes is still unknown, but an interesting review of Ferguson and coworkers collected data from studies with RNA next generation sequencing, and showed differences between the RNA content of donor cells and the exosome produced to focus. Some RNAs could be enriched in exosomes which can or cannot represent the donor cell environment (Ferguson and Nguyen, 2016).

The existence of double-stranded small molecules of RNA was discovered; they are able to interact in a sequence-specific manner, thus silencing the mRNA translation process to a protein. This small molecules of RNA was called RNA interference (Hannon, 2002). It was demonstrated in recent years that the double-stranded small RNA was cleaved by RNases, such as DICER, to originate a small noncoding simple-strand RNA with 22 nucleotides (miRNA). The majority of the studies have been developed to exploit the relation between exosomes and miRNA or siRNA (exogenous synthetically miRNA-like) in therapeutic benefits or as biomarkers of diseases.

11.9.3.1 Short interfering RNA

The use of siRNA is a very plausible strategy to develop genetic therapy, however there are many challenges to overcome. For example, the low stability in the systemic circulation to confer specificity of the therapeutic siRNA to the site, and the internalization of siRNA. As exosomes have been found in vivo, they appear to be a solution, due to their ability to circulate in the blood circulation, low immunogenicity, and specific delivery (El-Andaloussi et al., 2012).

The first study that used exosomes loading siRNA was developed by Alvarez-Erviti et al. (2011), which successfully delivered GAPDH siRNA and BACE-1 siRNA to Alzheimer's disease models both in vitro and in vivo. C57BL/6 dendritic-cell derived immunogenically inert exosomes were engineered to express an exosomal membrane protein Lamp2b fused with a neuron-specific peptide RVG. The preclinical benefit of exosome-delivered siRNA was demonstrated in mice by increased mRNA and BACE-1 protein knockdown, a possible therapeutic target to Alzheimer's disease (Alvarez-Erviti et al., 2011).

By electroporation, a siRNA tagged with a fluorescent probe was loaded and the uptake was measured by confocal microscopy, showing efficient internalization by human mononuclear blood cells, with subsequently silencing of mitogen-activated protein kinase-1 MAPK-1 (Wahlgren et al., 2012).

A similar approach was applied to encapsulate siRNA against RAD51 and RAD52 (Shtam et al., 2013). HeLa cells, HT1080 human fibrosarcoma cells, and ascitic fluid were used for exosome isolation by ultracentrifugation, prior to characterization by NTA, AFM, and Western blotting. The authors showed the efficacy of two methods of loading cargo into exosomes: electroporation and chemical transfection of a fluorescent probe siRNA against RAD51 and RAD52. These formulations decrease the fibrosarcoma cell viability and proliferation.

Polo-like kinase-1 (PLK-1) has a key role in the regulation of mammalian cell proliferation. It is also related to cancer, especially bladder cancer, of which PLK-1 overexpression correlates with poor prognostics (Nogawa et al., 2005). In 2016, Greco and coworkers suggested that exosomes could act as a vector for siRNA against PLK-1 and knockdown this gene (Greco et al., 2016). Human embryonic kidney 293 cells (HEK293) and MSC were the exosome donors. A coculture of invasive bladder cancer cell line (UMUC3) and siRNA-exosomes was obtained and the uptake was observed. The knockdown of PLK-1 was successfully achieved by reduced levels of mRNA and protein, when compared to cells treated with negative siRNA control exosomes. The authors highlighted the attractive and potential use of exosomes as vector to selective gene silencing.

Now, the use of nanoscale exosome-mimics (EMs) as a novel strategy to siRNA delivery has been proposed. Both EMs and exosomes were loaded with siRNA against CDK4, a cyclin-dependent kinase related to regulation of cell cycle progression, by the electroporation technique. EMs and exosomes harvested from the same cell donor have similar physical characteristics, similar uptake, and both are able to deliver the CDK4 siRNA safely and efficiently to the tumor cells reducing tumor growth due to decrease tumor CDK4 mRNA in a mice model.

11.9.3.2 *miRNA*

MiRNAs are small noncoding RNAs that, in general, contain around 20–22 nucleotides and are produced by the canonical or noncanonical biogenesis pathways. Both pathways occur through a multistep process in nuclear and cytoplasmic compartments. The canonical biogenesis pathway synthesizes precursor (pri-miRNA) from intergenic, intronic, or polycistronic genomic loci by RNA polymerase II, which is processed by Drosha and DGCR8 RNase III complex. Noncanonical miRNA biogenesis directly produces miRNAs as endogenous short hairpin RNAs or refolds introns into hairpins (mirtrons). Subsequently, both precursors (pre-miRNA and mirtrons containing around 60–100 nucleotides) are transported by exportin 5 from the nucleus to the cytosol, a process dependent on Ran-GTP. In this cellular compartment, pre-miRNA and mirtrons are processed by DICER and transactivation-response RNA-binding protein RNase III complex, generating the mature double-stranded miRNA. The mature miRNA biological action depends on its incorporation into the argonaute-containing RNA-induced silencing complex (RISC). RISC-miRNA assembly is, in turn, conducted to specific target sequences in mRNAs. Initially, the recognition of mRNAs by the RISC-miRNA complex occurs by base-pairing nucleotides 2 to 8 in the miRNA with specific mRNA target sequences located in the 3' untranslated region. However, additional base-pairing is crucial for increasing the affinity and targeting efficiency. The RISC-miRNA complex acts either in translational repression or decreasing mRNA stability (Abrantes et al., 2014). It has been predicted that miRNAs modulate, at translational level, up to 60% of protein-coding genes in the human genome, and a single miRNA is capable of targeting approximately 200 transcripts simultaneously. Therefore, miRNAs have been implicated in

almost every biological process, including development, cell cycle regulation, cell growth and differentiation, stress response, and apoptosis. In addition, miRNAs play a role in a variety of diseases, particularly in cancer.

Ohno et al. (2013) conducted a study to evaluate the ability of exosomes to encapsulate and deliver miRNA-7b to epidermal growth factor receptor (EGFR)-expressing breast cancer cells. For this purpose, the authors have created a pDisplay vector encoding epidermal growth factor (EGF) or GE11 (EGFR-specific peptide). The vector was transfected to HEK293 cells and it was observed that isolated exosomes carried miRNA let-7, and EGF or GE11 were incorporated on the exosome surface. The expression of GE11 or EGF was assessed by Western blotting. The uptake of PKH67-labeled miRNA let-7 exosomes was observed in various breast cancer cell lines. In addition, the authors provided information about the effects of GE11 or EGF-positive exosomes to inhibit cell proliferation in vitro. GE11-exosomes loading with miRNA let-7a inhibit tumor development in vivo in a mice model of xenograft breast cancer (Ohno et al., 2013).

11.10 EXTRACELLULAR VESICLES AS DRUG DELIVERY PLATFORMS FOR THERAPEUTIC BENEFIT IN CLINICAL TRIALS

Some clinical trials studies on exosome-based therapies are currently either ongoing or completed. Autologous ascites-derived exosomes (Aex) were tested in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) adjuvant therapy in a Phase I clinical trial in the treatment of colorectal cancer (Dai et al., 2008). Ascites-derived exosomes were isolated by ultracentrifugation and subsequently subjected to sucrose gradient. Forty patients with advanced colorectal cancer were enrolled in the study and randomly assigned into two groups: one group were subjected to Aex alone and the second group subjected to Aex combined with GM-CSF. Both groups received a total of four subcutaneous immunizations at weekly intervals. Both therapies are safe and well tolerated but only the combination of Aex with GM-CSF can induce antitumor cytotoxic T lymphocyte response. Because of the fact that Aex contained major histocompatibility complex molecules, it is suggested they can stimulate the activation of T lymphocytes.

Zitvogel et al. (1998) developed a novel cell-free vaccine, which means that they were able to support the implementation of dendritic cell-derived exosomes (Dex) to cancer immunotherapy. Two clinical trials in recent years applied Dex immunotherapy to patients with advanced lung cancer (Morse et al., 2005; Besse et al., 2016) and one study applied Dex to melanoma metastatic patients (Escudier et al., 2005). Dendritic cell-derived exosomes were obtained from patient peripheral blood mononuclear cells by ultracentrifugation and sucrose

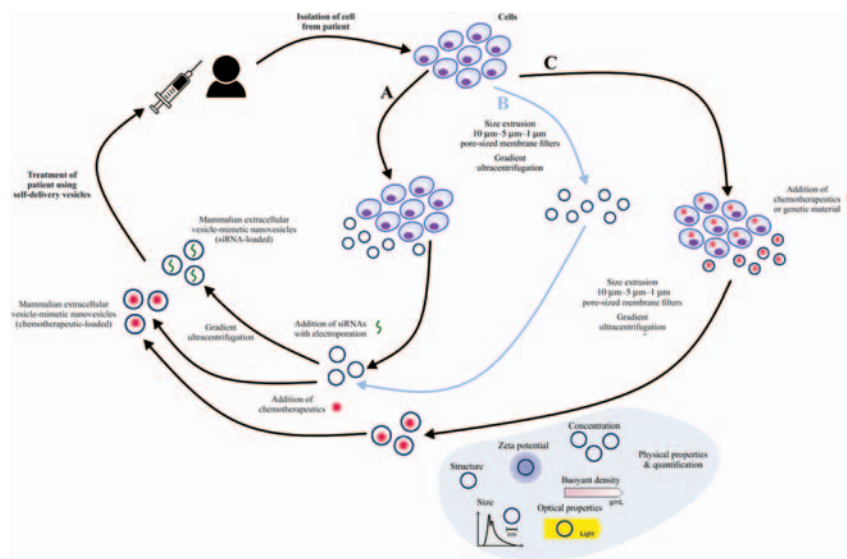
cushion (Morse et al., 2005). Subsequently, melanoma-associated antigen A3 (MAGE-A3) was loaded into Dex and subjected to thirteen patients with nonsmall cell lung cancer (NSCLC) Stages IIb/IV with tumor expression of MAGE-A3 at four doses of Dex or Dex-MAGE-A3 at weekly intervals. The immunotherapy was well tolerated and showed an improvement of survival of patients after the first dose. It is a really interesting study, due to the feasible production of Dex vaccine loading MAGE-A3 and increased cytotoxic T lymphocyte response in 3/9 patients and also increased NK lytic activity in 2/4 patients (Morse et al., 2005).

A Phase II study based on Dex immunotherapy was carried out to: (1) investigate if Dex-MAGE-A3 loading interferon γ (IFN- γ) could improve the survival rate at 4 months after chemotherapy in unresectable NSCLC patients; (2) if Dex-MAGE-A3-IFN- γ could boost the NK cell response. The primary objective was not reached, but it was confirmed that Dex-MAGE-A3-IFN- γ improved NK cytotoxicity in patients with advanced NSCLC. In a study conducted by Escudier et al. (2005), a technique to isolate exosomes from autologous monocyte-derived dendritic cells by ultracentrifugation was reported. High levels of MAGE-A3 have been reported with shorter survival of lung adenocarcinoma (Decoster et al., 2012). Dendritic cell-derived exosomes were then pulsed with MAGE-A3 peptide for immunization of fifteen eligible patients at Stage III/IV of melanoma. Four doses of MAGE-A3-pulsed Dex were administered at weekly intervals. The first exosome Phase I trial highlighted the safety of Dex administration and that the large scale Dex production was easily done.

Another clinical study hypothesized that changes in the levels of total exosomal protein and miRNA in exosomes isolated from patient plasma samples before and after vaccination could serve as biomarkers of response to immunotherapy (Muller et al., 2015). Exosomes were isolated by size exclusion chromatography from pre/postvaccine plasma samples in 20/22 enrolled patients in a Phase I/II trial (Okada et al., 2011). The authors reported changes in exosomal mRNA levels assessed by qRT-PCR in pre-to-post vaccination state and it is correlated with immunological and clinical responses. According to these authors, mRNA expression levels for immune-related genes in plasma exosomes were useful in the evaluation of glioma patient response to vaccination therapy.

11.11 CONCLUDING REMARKS

It is undoubtedly that EVs hold immense promise for cancer therapy as targets and as drug delivery systems. However, it is still necessary to provide more insights about the molecular mechanisms of EV biogenesis, intracellular trafficking, and release in cancer cells, in order to design specific inhibitors and to improve EV production efficiency (in general cancer cells release higher amount of exosomes in comparison with normal parent cell line). For instance, in a paper published by Riches et al. (2014), it was shown that human mammary epithelial

**FIGURE 11.5**

Pipeline for drug delivery systems based on EVs or nanosized vesicles. Specific cells are collected and cultured *in vitro*, subsequently different strategies can be used: (A) the culture medium is withdrawn and EVs are isolated, characterized and used for drug loading; (B) cells are suspended in fresh buffer and are serially extruded with 10, 5, and 1 μm pore-sized membrane filters to form nanovesicles. Then, the nanovesicles are isolated by density gradient ultracentrifugation. For chemotherapeutic-loaded mammalian extracellular vesicle-mimetic nanovesicles preparation, chemotherapeutics are added during extrusion or are added to isolated nanovesicles. siRNA-loaded mammalian extracellular vesicle-mimetic nanovesicles are generated by electroporation after mixing nanovesicles with siRNA. siRNA-loaded nanovesicles are isolated by two-step density gradient ultracentrifugation; (C) cells are treated with the desired drug followed by serial extrusion as mentioned above.

cell line releases 4.5×10^8 exosomes per 10^6 cells, while the same amount of breast cancer cells (clone derived from parental cell line) released 53.2×10^8 . Conversely, the use of EVs as delivery systems requires the establishment of new strategies for scalable EV isolation, approaches for efficient drug loading, *in vivo* trafficking, biological fate, and their impact on targeted organs need to be understood more thoroughly. However, a light in the end of the tunnel is coming by designing artificial EVs or EV-mimetics. Native EVs can serve as an immeasurable source of inspiration for the improvement of synthetic drug delivery systems, as they are believed to comprise a multitude of constituents which facilitate delivery of their cargo to recipient cells. The incorporation of such moieties in synthetic drug delivery systems could be a smart strategy to increase the

biocompatibility and in vivo transfection efficiency of such systems. Another characteristic to consider is that the use of predefined components to generate EV-mimetics could facilitate approval of such systems by regulatory agencies, such as US Food and Drug Administration.

Based on the content of this chapter, we have the opinion that the pipeline summarized in [Fig. 11.5](#) is one of the ways to optimize the application of EVs in the clinic. EVs can be isolated and characterized, or mimetic vesicles can be obtained, from patient-specific primary cells. Subsequently, the drug (e.g., small molecules or RNAs) will be load in the vesicles and characterized. Finally, the EVs are employed as delivery systems for personalized therapy.

ACKNOWLEDGMENTS

Our research on EVs field has been supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)—processo 2015/20412-7, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Advancements in exogeneous techniques for stimuli-sensitive delivery systems

12

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CHAPTER OUTLINE

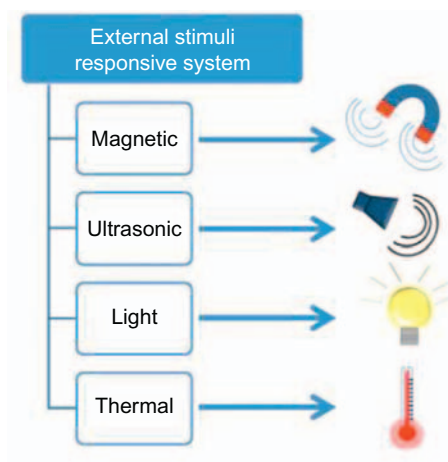
12.1 Introduction	447
12.2 External-Stimuli-Responsive Systems	450
12.2.1 Magnetically Responsive Systems	450
12.2.2 Ultrasound-Responsive Systems	460
12.2.3 Electro-Responsive Drug Delivery Systems.....	465
12.3 Other Classes of Exogenous Stimuli-Responsive Systems.....	467
12.3.1 Light-Responsive Systems (Photo-Sensitive)	467
12.3.2 Thermo-Responsive Systems	468
12.4 External Multistimuli-Responsive Drug Delivery Systems.....	471
12.4.1 External Dual Stimuli-Responsive Systems	471
12.5 General Phenomena in Delivering Anticancer Therapeutic Agents Via Stimuli-Responsive Drug Delivery Systems.....	472
12.6 Future Directions for Drug Delivery Systems that Response to External Stimuli	475
12.7 Conclusion and Perspectives	476
References	476

12.1 INTRODUCTION

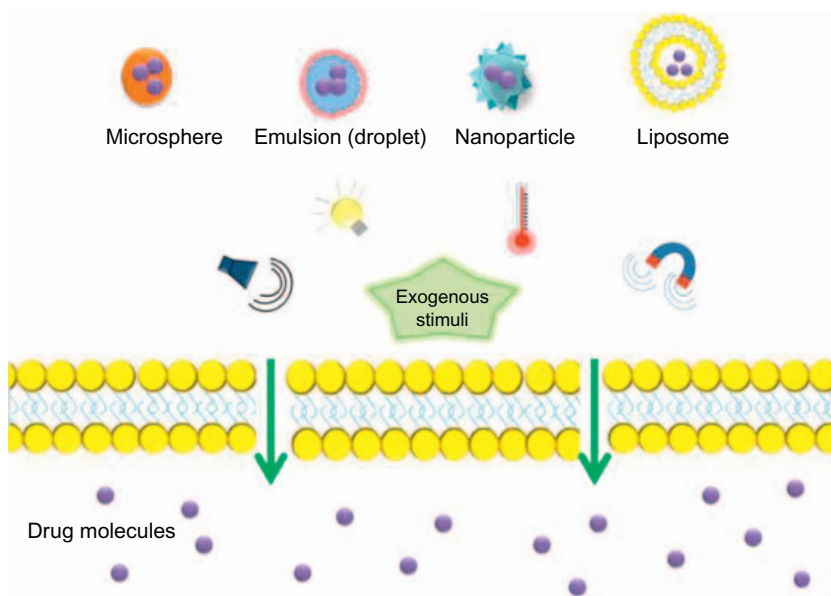
Numerous types of intelligent drug delivery formulations have been designed to deliver the drug for effective treatment. Serious efforts have been made in recent years aiming for formulating and utilizing intelligent drug delivery systems (DDSs) by using nanotechnology that shows indubitable benefits for drug administration. Conventional DDSs have notable side effect limiting their clinical application, due to their nonspecific bio-distribution and uncontrollable drug release

features. In contrast, advanced DDSs are characterized by reduced dosage frequency, and maintained drug concentration in targeted site of action for a longer period of time, in order to take full advantage of their therapeutic potential and diminish the undesirable side effects upon administration. Furthermore, reducing drug toxicities and improving the therapeutic effectiveness of drugs can be other significant properties (Liu et al., 2016). The science of the versatile multiparticulate systems for drug delivery can include a lot of nanoparticle examples, such as: liposomes, polymer nanoparticles, micelles, dendrimers, and inorganic nanoparticles made of iron oxide, quantum dots, and gold. These nanopreparations can be further engineered and adjusted to enhance their performance in various areas (Zhu and Torchilin, 2013). Biomedical applications of nanotechnology in external stimuli-responsive systems as well as internal stimuli-responsive systems offers promise in disease prevention, diagnosis, and therapy. This can be applicable in numerous fields, such as light, electrical, ultrasound and magnetic, or thermo-stimulation as exogenous responsive systems, whereas change in pH, redox-sensitive systems and enzyme-mediated DDSs are endogenous stimuli-responsive DDSs (Yin et al., 2016). They may be also used for repeat injection of short-acting drugs or those with rapid clearance (Fong et al., 2009). Smart nanocarriers can be prepared by several techniques using different organic and inorganic materials, including nondegradable and biodegradable nanoconstructs and lipids (liposomes, nanoemulsions, and solid-lipid nanoparticles) which can be used to synthesis the liposomal nanopreparations for the purpose of thermo-responsive, self-assembling amphiphilic molecules, and inorganic semiconductor nanocrystals. Stimuli-responsive nanomaterials, which can be termed as “smart nanoparticle,” have attracted researchers looking for new methods to tailor, due to their versatility in emerging applications in targeted drug delivery, imaging and sensing applications. In comparison with the internal stimuli-responsive systems that are used within tumor microenvironments, such as lower pH number, or higher temperature, external stimuli-responsive systems could introduce contrast agents to visualize the accumulation of nanoparticles in the target tissues (Dobson, 2006), cells, or organelles, and then activate the nanocarriers from out of the body by magnetic field or other triggers (Yao et al., 2016). Multiple stimuli-responsive materials are also used in current times. These substances are sensitive to various triggers (three or more stimuli), including: redox and thermo-responsive systems, pH and magnetic-responsive nanoformulations, and pH and redox-sensitive nanopreparations (Cheng et al., 2013).

In this chapter, we discuss external stimuli-responsive nanocarriers that control the bio-distribution and effect of drugs in response to specific stimuli, and their broad classification including: (1) magnetic-targeting multiparticulates, which are further subdivided to magnetic nanoparticles (MNPs), microspheres, liposomes, as well as magnetic emulsions; (2) ultrasound-responsive particles, which are classified to microbubbles, nanodroplets, micelles, and microemulsions; and (3) materials that respond to electrical fields, and other classes, such as: external local temperature-induced DDSs and photo-sensitive systems. Moreover, this chapter explores different valuable applications of external responsive nanoparticles,

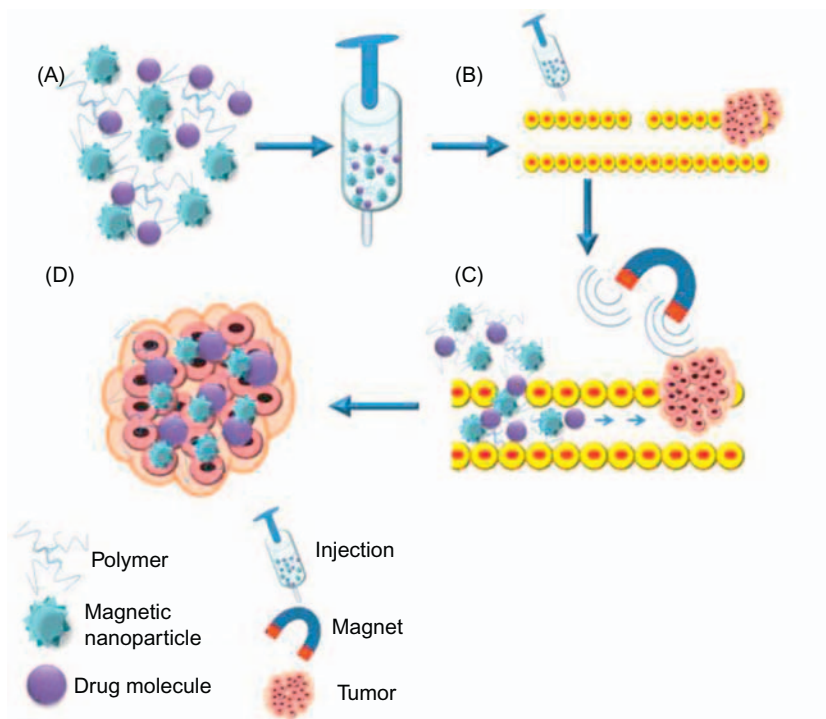
**FIGURE 12.1**

Types of external stimuli-responsive system.

**FIGURE 12.2**

Types of magnetic materials.

including cancer hyperthermia by enhanced tumor targeting, stimulating tissue repair, sensing, delivering of gene by magnetic targeting, and stimulation of neurotransmitter release with the help of electric-responsive moieties, etc. (Figs. 12.1–12.3).

**FIGURE 12.3**

Mechanism of action. (A) Drug incorporation with magnetic nanoparticles in polymer, (B) injecting via intravenous or intra-arterial routes, (C) application of magnetic field, and (D) delivering the drug molecules to the affected tissue (tumor).

12.2 EXTERNAL-STIMULI-RESPONSIVE SYSTEMS

12.2.1 MAGNETICALLY RESPONSIVE SYSTEMS

During the last couple of years, stimuli-responsive DDSs have been extensively developed as new types of smart soft devices for biomedical applications, such as MNPs (Sung et al., 2016). Magnetically triggered drug delivery nanodevices have established high efficacies through either in vitro proof of concept studies or in vivo preclinical applications. However, their use in biomedical applications is still limited, due to their insufficient biocompatibility or biodegradability (Yao et al., 2016). For in vivo applications of MNPs, three basic necessary qualifications must be met: firstly, biocompatibility of the surface is an essential requirement; secondly, nontoxicity, and the third important property is that the particles must be sufficiently dispersed with no aggregation formation (Medeiros et al., 2011). Development of the tools in magnetic-responsive systems has become

significant for molecular detection and in drug delivery pathways. “Theranostic” agents combine these two functions for diagnostic purpose and to deliver targeted therapy to a specific location within a single system (Medeiros et al., 2011; Mura et al., 2013).

12.2.1.1 Mechanism of action of magneto-sensitive agents

Interestingly, nanobiomagnetism has been used in clinics since 1945, mostly in systems which contain different magnetically responsive materials integrated from numerous substances, for example: magnetite, iron, cobalt, nickel, iron-boron, or samarium-cobalt. is one of the productive methods to deliver the drug at diseased site. The drug and the magneto-responsive nanocarrier is injected to the blood circulation system, with the support of a permanent magnetic field at the target site to block it and trigger drug delivery. MNPs are used for the following advantages: ability of utilizing a lesser amount of drug concentration at specific site, which gives correct treatment with fewer unwanted side effects (due to the high concentrations of freely circulating drug in conventional treatments); a decrease in the rate of normal cell tissue damage; and increased inefficiency of drug delivery to the targeted tissues (up to 60%) (Ahmad et al., 2013).

Conversely, effective heating can be reached at the surface of silica nanoparticles when exposed to alternating magnetic field, due to embedded magnetite nanoparticles. This can be another mechanism for the development of efficient DDSs. This discovery demonstrates the possibility for designing new stimuli-responsive devices that are based on a “hot spot” effect. Magnetically triggered drug release, without the need for supplying high magnetic energies commonly required for heating the environment, therefore overcoming the blood-mediated thermal dissipation, is possible. For this goal, the surface of mesoporous silica was with thermosensitive copolymer, which expresses linear-to-globular transition at temperature of 40–43°C (Guisasola et al., 2015).

Magnetic materials can be further categorized as follows:

12.2.1.1.1 Magnetic nanoparticles

a. Preparation of magnetic nanoparticles

Extensive research with magnetic nanotechnology has shown great promise in drug and gene delivery pathway, where the delivery system becomes an active participant, instead of passive medium, in the monitoring or optimization of therapy. This has expanded its scope to oncological, cardiovascular, and neurological diseases. Different nanoparticles, such as iron, nickel, cobalt ferrous oxides, ferrites and metal alloys nanoparticles are prepared in three average sizes less than 100 nm: 10, 22, and 30 nm, and are employed under the influence of magnetic field. Magnetic nanomaterials are prepared by using numerous methods: thermal decomposition, microemulsions, and coprecipitation methods; coprecipitation is the most convenient, and preferred over other methods because of its lower temperature and lower time. For instance, iron oxide MNPs either magnetite (Fe_3O_4)

or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) can exist commonly as a nonstoichiometric combination of the two. Iron oxide particles are synthesized from aqueous salt solutions of ferrous and ferrous, by the introduction of concentrated alkaline solution with or without heating. In this process, MNPs and metal hydroxides are oxidized by the use of oxidizing agents, while in the second one, metal hydroxides are mixed together in the presence of aqueous environment. Finally, there is aggregation within the metal hydroxide to form spherical particles (Medeiros et al., 2011; Mura et al., 2013; Ahmad et al., 2013). Magnetic iron oxide nanoparticles coated with polymer can serve as a versatile platform for combined therapy (Joglekar and Trewyn, 2013). Moreover, to achieve the most effective body targeting, especially close to the surface of the body, scientists designed a new model that incorporates the effects of shear-induced diffusion (Ganta et al., 2008).

- *Preparation of magnetic nanoparticles by microemulsions method:*

Water-in-oil (w/o) microemulsions (i.e., reverse micelle solutions) have the following ideal properties: transparent, can form spontaneously, have a size range of 10–120 nm, are thermodynamically stable, and can accommodate 20%–40% without increase in viscosity. As with any emulsion, these systems are stabilized by emulsifying agents that reduce the surface tension (Acharya and Pise, 2016). Water/oil microemulsions, versatile, effective and simple process to synthesize magnetic nanosized particles (Tartaj et al., 2003). Controlling of the particles size can be by modification of size of the water pool which surrounds the nanodroplets (Ahmad et al., 2013).

- *Preparation of magnetic nanoparticles by thermal decomposition method:*

Synthesis of MNPs by this method is done at elevated temperature, which results in decomposition of the substance by breaking the chemical bonds between the molecules of organometallic compounds such as acetylacetonates and metal cup ferromates. These are placed in organic solvent carbonyls, for instance, using oleic acid, polyvinyl pyrrolidone and hexadecyl amine as surfactants. Ratios of organometallic precursor components facilitate the determination of final size and magnetic nanostructures morphology. Precursors with zerovalent metals form if they undergo oxidation reaction its lead to quality monodispersed metal oxide (Ahmad et al., 2013; Guisasola et al., 2015; Joglekar and Trewyn, 2013; Ganta et al., 2008; Acharya and Pise, 2016; Tartaj et al., 2003; Khan et al., 2015; Faraji et al., 2010).

b. Mechanism of action of magnetic nanomaterials

Magnetic nanosystems could be introduced to the systemic circulation and then accumulated in a specific site in the body with the presence of an alternating magnetic field. The uses of magnetic nanoparticles in different shapes, such as nanodots and nanorings, were investigated for the delivery of drugs and has received attention in research fields. The transportation of particles is facilitated by using the method of magnetically guided drug targeting which involve three steps: first, drug immobilization in MNPs, followed by injection of the complex into the vascular system by two ways, either via intravenous (IV) or intra-arterial routes. Lastly, generation of

external magnetic fields by rare-earth permanent magnets to guide the drug/carrier complex and concentrate it at the pathological site, especially tumors. The therapeutic agent is then freed from the magnetic carrier when the complex is accumulated in the desired area (in vitro). This occurs by activation of the enzyme or via physiological environment alteration, for instance: temperature or osmolality. As a result, there will be an increased drug uptake by the affected cells and lower drug concentration in the systemic circulation (Estelrich et al., 2015; Blachowicz and Ehrmann, 2016). However, the problem of intrinsic instability still exists, which occurs when after exposure to air for longer period, as they can easily oxidize, resulting in deficiency of the magnetic property (Ahmad et al., 2013). Incorporation of nanosized magnetic particles within natural/synthetic polymers, known as encapsulation, aids in regulating the release kinetics. The most widespread synthetic polymers are: polylactides, starch, and liposomes with oxide particles, named magnetosomes, which utilize natural polymers such as chitosan, gelatin, and agarose (Singh et al., 2016).

c. Advantages of nanosized material (Ahmad et al., 2013):

- Less frequent administration, thus patient compliance will be high;
- Utilizing less dosage, resulting in minimum undesirable side effects;
- Pathological site receives prolonged delivery of drug;
- Sustained drug release to the targeted organ.

d. Applications of magnetic nanoparticles (Tartaj et al., 2003):

Among the broad spectrum of nanomaterials being investigated for clinical application, magnetic DDSs shown significant application in medical and bio-physical fields, according to their usage either in the body (in vivo) or outside the body (in vitro):

• *In vivo applications:*

1. therapeutic applications: treatment of tumors. The use of a carrier formed of albumin microspheres (of several μm in diameter) with captured Fe_3O_4 and doxorubicin (DOX) that could be concentrated at the desired site in vivo with the aid of a magnetic field;
2. delivery of nucleic acids, including siRNA and genes;
3. targeting of radioactive compounds;
4. magnetic hyperthermia: magnetic-induced hyperthermia and photodynamic therapy to destroy the diseased tissues with the help of elevated temperature of a region of the body as they are more temperature labile each single treatment inhibit tumor growth. One advantage of this method that is the heating is restricted to the tumor area. Another advantage is increased absorption at site of action;
5. real-time determination and monitoring contamination of pathogenic bacteria, viruses or other microorganisms;
6. diagnostic purpose: nuclear magnetic resonance (NMR) imaging technique for establishment of magneto pharmaceuticals.

- *In vitro applications:*
 1. Detection mainly for diagnostic purpose (magnetorelaxometry);
 2. separation or selection of proteins (magnetic cell separation) with micron-sized polymers used as a material for separation.
 3. magnetic solid phase extraction method it is a method that facilitate isolation and have a role in impurities determination in test samples. This method proved faster and less time consuming compared to the conventional treatment.

12.2.1.1.2 Magnetic microspheres

Different materials can form various magnetic microparticles with strong magnetic moments that are able to efficiently transport the nonmagnetic components, such as antibodies, drugs, nucleic acids, and enzymes. These are small systems, with size range of 4 μm , that can show an efficient rate of flow to pass within the capillaries, with no formation of embolus. Microparticles made up of proteins or synthetic polymer which bond with the drug. They are manufactured in such a way that gives a localized effect, avoiding undesired drug distribution to nontargeted tissue or organ, thus the toxicity will be bypassed (Ahmad et al., 2013).

a. Method of preparation of magnetic microparticles

Preparation of magnetic microparticles can be done by using a single nozzle electrospray (ES) method. Poly caprolactone (PCL) porous particles are accurately weighted, then at ambient temperature dissolved in 30 mL of dichloromethane (DCM). One hour of constant magnetic stirring is essential to achieve uniform and total dissolution of the particles by using VELP ARE heating magnetic stirrer. In a plastic syringe, the solution will be extracted and fed into the ES nozzle through a metallic sterilized needle. Product is collected in a nonsolvent bath made from ethyl silicate. Synthesis of drug-loaded particles is obtained by the same conditions and parameters. The chosen drug, for example, indomethacin, is incorporated into the PCL-DCM solution. The drying process is done by using a desiccator after collecting the resulting product (Faraji et al., 2010).

Magnetic microspheres are mainly synthesized by two techniques: phase separation emulsion polymerization method and continuous solvent evaporation method (Kakar and Singh, 2014). Both methods can be stabilized by albumin addition and are biocompatible with the blood. For a fast moving arterial systems, a magnetic field with high strength is applied (Poovi et al., 2015) (Table 12.1).

- *Phase separation emulsion polymerization method (PSEP):*

There are four major steps involved in magnetic microsphere particle preparation (Liu et al., 2005):

 1. aqueous phase dispersion, that contains magnetite sol and homopolymer poly(2-hydroxyethyl acrylate) (PHEA) in a mixture of toluene and chlorobenzene as the organic phase;

Table 12.1 Drugs and Materials Used in Preparation of Magnetic Microspheres (Chandna et al., 2013; Patil et al., 2016; Kakar et al., 2013; Liu et al., 2005; Gao et al., 2016; Ramteke et al., 2012; Bhange et al., 2015; Batra et al., 2012; Mukherjee and Bandyopadhyay, 2012)

Material Used	Types	Examples of Polymer	Example of Drug	Application
Synthetic Polymers	Biodegradable Nonbiodegradable	Poly lactic acid Polymethylmethacrylate	Nimesulide Indomethacin	Antirheumatics Magnetic resonance imaging
Natural Polymers	Proteins Carbohydrates Chemically modified carbohydrates	Albumin Chitosan Polystarch Polydextran	Doxorubicin Oxantrazole NA	Cancer therapy Cancer therapy NA

2. distillation of water from the droplets, using suitable emulsifying agents to reduce the surface tension and to form spheres in emulsion system. Examples of emulsifying agents: PtBA-PHEA and PtBA-PSA-PHEA;
 3. succinyl chloride is used for crosslinking of the microspheres in the presence of pyridine;
 4. hydrolysis of PtBA by organic-based media and, in the case of triblock microspheres, the hydrolysis process will be done for PSA: poly(solketal acrylate) (PSA). During this step, the solubility of Fe^{2+} or Fe^{3+} at minimum level which help in the reduction of magnetite dissolution.
- *Continuous solvent evaporation method (CSE):*
 1. both the drug and polymer are dissolved in volatile organic solvent
 2. magnetite is added with continuing stirring in order to form a homogeneous suspension.
 3. the suspension is added to an immiscible additional solution along with vigorous stirring at temperature of 22–30°C for slowly evaporation of the volatile organic solvent to form microspheres.
 4. microspheres are separated by centrifugation then freeze-dried and stored at 4°C (Chandna et al., 2013).

b. Mechanism of action magnetic microspheres

Once the magnetic carrier is administered, either by intravenous or oral route, the drug is accumulated at specific site of action and augmented by application of magnetic field and magnetic agglomeration. At suitable magnetic field, the magnetic microspheres incorporate with the tissue endothelial cells. The accumulation at the target site facilitates the local delivery of drug-carrier complex. Various physiological factors can affect the efficacy of the targeting, including microsphere particle size and the strength of the applied field (Patil et al., 2016).

- c. **Advantages of magnetic microspheres** (Poovi et al., 2015; Liu et al., 2005; Chandna et al., 2013; Patil et al., 2016):
 1. reduce circulating concentration of free drug;
 2. different option for the bimolecular malformation;
 3. incorporate smaller doses of drug, thus predictable rate of drug release can be achieved;
 4. Improve patient compliance.
- d. **Disadvantages of magnetic microspheres** (Poovi et al., 2015):
 1. drug cannot be targeted to deep organs so this approach is restricted only for targeting at surface of the body organs;
 2. undesired localization of the drug in different organs especially liver;
 3. costly and requires specialized technical approach.
- e. **Biomedical applications of magnetic microspheres:**
 1. Mesalamine (5-aminosalicylic acid) as colon-targeted DDSs in treatment of diseases such as Crohn's disease, ulcerative colitis, and inflammatory bowel syndrome (Kakar et al., 2013);
 2. Diclofenac sodium minimizes joint swelling (Chandna et al., 2013);
 3. Work as carrier system for different of anticancer agents, such as radionuclides (Poovi et al., 2015);
 4. DNA analysis and bacteria detection (Poovi et al., 2015);
 5. The microspheres prepared should be beneficial in immunoassays (Liu et al., 2005);
 6. magnetic porous polymeric particles used for pulmonary drug delivery (Gao et al., 2016);
 7. Protein purification and drugs targeting to tumor sites (Ramteke et al., 2012);
 8. Treatment of liver tumor by delivering chemotherapeutic agent (Bhange et al., 2015).

12.2.1.1.3 Magnetic liposomes

Targeted DDSs for cancer treatment can be accomplished by magnetic liposomes (MLs). Liposomes are tiny particles consisting of bilayered vesicular-shaped structures made up from a biogenic hydrophobic lipid layer and an aqueous layer, arranged in different patterns, and biocompatible and suitable for biomedical applications (Nawroth, et al.). Furthermore, magnetic liposomes are used to encapsulate water soluble and oil soluble pharmaceutical drugs and transport them with the help of blood circulation through the body to specific targeted tissue, thereby minimizing adverse effects, unwanted side effects, and toxicity of potent anticancer therapeutic agents (Ahmad et al., 2013; Guisasola et al., 2015; Joglekar and Trewyn, 2013; Ganta et al., 2008; Acharya and Pise, 2016; Tartaj et al., 2003; Khan et al., 2015; Faraji et al., 2010; Estelrich et al., 2015; Blachowicz and Ehrmann, 2016; Singh et al., 2016; Kakar and Singh, 2014; Poovi et al., 2015; Liu et al., 2005; Chandna et al., 2013; Patil et al., 2016; Kakar et al., 2013; Gao et al., 2016; Ramteke et al., 2012; Bhange et al., 2015; Batra et al., 2012; Mukherjee and Bandyopadhyay, 2012; Nawroth, et al.; Shah et al., 2016a,b).

a. Method of preparation of MLs:

Magnetic liposomes can be synthesized by two techniques, namely: thin film hydration (TFH) and double emulsion (DE) method. THF was found to be more preferred and with greater efficiency than DE for the encapsulation of lauric acid-based magnetic fluid. In the first method, the magnetic particles are encapsulated with phospholipid linkage, which function as a nanoreactor. Whereas in the other preparation method, phosphatidylcholine/phosphatidylethanolamine are used to cover the magnetic particles, which results in production of magnetic particles agglomeration. Pullulanhydrazide is used to enhance the stability of these linkages (Medeiros et al., 2011). Moreover, MLs can be prepared with three average sizes: 40, 70, and 110 nm by MNPs which were prepared previously by alkaline coprecipitation method, covered by the aqueous core of the liposomes. In the case of liposome preparation, there is one more step with slight modification: reverse evaporation method, as nanoparticles MLs have spherical structure (Shah et al., 2016a,b).

- *Preparation by TFH method:*

Different molar ratios of egg-phosphatidyl choline (egg-PC) and cholesterol can be utilized in the synthesis of MLs. An accurate amount of egg-PC and cholesterol is measured and dissolved in chloroform/methanol which acts as solvent. By then using a rotary evaporator the solvent mixture is evaporated at room temperature under vacuum, resulting in formation of a thin lipid film. Following this, is the addition of magnetic fluid to the flask for 30 min of hydration at 40°C. Sonication of the sample for 15 min is necessary to produce unilamellar MLs. For precipitating the unencapsulated MNPs by ionic interactions, addition of normal saline solution 0.9% NaCl solution to the sample is needed. After centrifugation for 10 min at temperature of 4°C, the solid precipitate is discarded and supernatant collected. The product is stored at a favorable condition of (2–4°C) (Pradhan et al., 2007).

- *Preparation by DE method:*

The desired amount of egg-PC and cholesterol is measured correctly, then the chloroform/methanol solvent mixture is dissolved. The primary emulsion was prepared by sonication in a sonicating bath and the inner aqueous phase added dropwise into the organic phase. The secondary emulsion is then obtained by a re-sonication step. Next, is using the rotary evaporator to eliminate the chloroform/methanol completely at room temperature under vacuum from the solution. A separation step of unencapsulated MNPs can be achieved as the one that was used in TFH method. After removal of the solvent, 20 min of centrifugation is carried out and the resulting supernatant is taken. Addition of 0.9% NaCl solution is needed for removal the free form of the drug. Centrifugation procedure is repeated but only for 10 min, and then the wet liposomes are collected after removal of the supernatant. Again, sufficient amount of the saline solution is added, re-dispersing the particles within the medium. If the used drug was DOX (chemotherapeutic agent) (Tacar et al., 2013) the resulting liposomes will be reddish in color (Pradhan et al., 2007; Shah et al., 2016a,b; Seth et al., 2012; Tacar et al., 2013).

b. Mechanism of action of MLs:

The advances in the development of targeted drug delivery by loading liposomes with a drug and a magnetic-responsive material have attracted scientists in recent years as a result of their potential applications in various fields. In the previously mentioned example: doxorubicin, intravenous administration of doxorubicin along with magnetic liposomes can be done. Under 1 h of application of magnetic field, the drug concentration is enhanced fourfold in the affected tissue. Furthermore, for improved accumulation in tumor vasculature, loading of MLs in combination with adriamycin is carried out. Inhibition of tumor growth was noted as a result (Kenhirao et al., 2003; Torchilin, 2005; Nobuto et al., 2004).

c. Advantages of MLs (Nawroth et al.; Shah et al., 2016a,b; Pradhan et al., 2007; Shah et al., 2016a,b; Seth et al., 2012; Tacar et al., 2013; Kenhirao et al., 2003; Torchilin, 2005):

1. biocompatible substances;
2. ability to deliver the therapeutic agents into cells and within cellular compartments;
3. magneto liposomes are immunotolerance and biodegradable material.

d. Disadvantages of MLs:

1. rapid elimination from the systemic circulation due to short half-life (Torchilin, 2005).
2. the bilipid layer of the liposomes often undergoes oxidation and hydrolysis reaction (Akbarzadeh et al., 2013);
3. high cost involved in preparation (Akbarzadeh et al., 2013).

e. Biomedical applications of MLs:

1. MLs that contain curcumin showed controlled release in treatment of cervical cancer (Nigam et al., 2013);
2. applied as negative contrast agents for imaging (MRI), neutron capture, and photodynamic therapy which give localized radiotherapy (Nawroth et al.; Shah et al., 2016a,b; Pradhan et al., 2007; Shah et al., 2016a,b; Seth et al., 2012; Tacar et al., 2013; Kenhirao et al., 2003; Torchilin, 2005; Nobuto et al., 2004; Akbarzadeh et al., 2013; Nigam et al., 2013; Martínez-González et al., 2016);
3. manganese ferrite-based preparation used in targeted thermo-chemotherapy of cancers (Pradhan et al., 2007);
4. colorectal cancer cells therapy by DOX and magnetic-responsive particles (Hardiansyah et al., 2014);
5. MLs formulations that consist of ferromagnetic particles in combination with muscle relaxant drugs, for local anesthetic effect applications (Kuznetsov et al., 2001);
6. ultra-magnetic liposomes can penetrate tumor cells transported to tumors and are able to mimic the tumor growth pressure in vivo (Béalle et al., 2012);
7. enhance drug bioavailability at cancerous areas (Gogoi et al., 2012).

12.2.1.1.4 Magnetic-responsive emulsions

An emulsion can be defined as a biphasic system made up of two or more immiscible liquids (aqueous phase and lipophilic phase) stabilized by reduction of interfacial tension, which is accomplished with the help of surface active agents known as emulsifying agents. These emulsions can be classified into two major classes: water-in-oil (aqueous droplets dispersed in the organic phase thereby function as the continuous phase), and vice versa for oil-in-water emulsion (Ahmad et al., 2013). In the case of magnetic emulsions, magnetic nanomaterials are dispersed in the internal phase (Brown et al., 2012a). This type of emulsion can be stabilized by using ionic liquid surfactants (magnetic-responsive surfactant stabilizers) (Brown et al., 2012b).

a. Preparation of magnetic-responsive emulsions:

Magnetic-responsive emulsions are prepared generally by using pure dodecane, commercial lubricant oil, water or can also be made in brine. Triton-X100 solution is made by using suitable aqueous solution of emulsifying agents. Before addition of dodecane or commercial-based oil, the surfactant mixture that contains MagSurf and triton-X100 undergoes vortexing for 5 min. The emulsion is vortexed for 5 min, then sonicated for 4 min five times. Between each sonification, the mixture is kept for nearly 30 s, for cooling (Brown et al., 2012a,b). A magnetic emulsion can also be prepared in a water bath at 37°C, by using ethyl oleate-based magnetic fluid as organic phase that consists of palmitic acid; the aqueous phase is casein solution. The resulting mixture is emulsified for 5 min by Ultra Turrax (Morimoto et al., 1983).

b. Mechanism of action of magnetic-responsive emulsions (in vivo model):

Magnetic emulsions are administered intravenously and 10 min after the injection, the tissue distribution can be noted. Rat is used as in vivo model. Without applying electromagnetic fields, small portions of the drug were found in the lungs. However, when applying electromagnet, the emulsion level is increased in the lungs, liver, and spleen (Morimoto et al., 1983).

c. Magnetic-responsive emulsion advantages:

1. preparation procedure is simple (Brown et al., 2012a);
2. monitoring physicochemical properties of magnetic ionic liquid surfactants (MILSs) is accomplishable and effortless (Brown et al., 2012a);
3. MILSs are nonvolatile, can be synthesized in various particle size range, and scalable (Brown et al., 2012b);
4. advantages over conventional emulsions include being highly stable and biocompatible (Hyndavi et al., 2011);
5. ease of application with reduced invasiveness, reduced toxicity, and cost effectiveness (Tang et al., 2015).

d. Magnetic-responsive emulsion disadvantages:

1. the major disadvantage is the irreversible changes in system composition, and the requirement of high energy inputs (Tang et al., 2015);

2. limited applications due to magneto-responsive emulsions which are only realized with Pickering emulsions (Brown et al., 2012a).
- e. Magnetic-responsive emulsion applications:**
1. nanomedicine aerosols and gels, and targeted drug delivery (Brown et al., 2012a);
 2. diagnostic agents and for therapeutic applications with minimal toxicity (Morimoto et al., 1983);
 3. in water-in-oil-in-water emulsion type, bleomycin, used as cancer chemotherapeutic agent, whereas mitomycin is used in o/w emulsion for the same aim (Hyndavi et al., 2011).

12.2.2 ULTRASOUND-RESPONSIVE SYSTEMS

One of the most effective physically dependent stimuli techniques in monitoring and facilitating an “on-demand” drug release is ultrasound-responsive system. Ultrasound-sensitive nanopreparations can be triggered through two ways, either thermal or mechanical stimulation, which is produced by cavitation or radiation forces (nonthermal effect) (Mura et al., 2013). These nanoformulations can target specific cells and tissues, and are able to preserve the biological activity throughout transportation process (Zhou et al., 2014). A variety of applications, mainly those relevant to biomedical fields, has attracted great interest such as: anticancer therapy, transdermal drug delivery, and cartilage tissue (Zhao et al., 2013).

12.2.2.1 Mechanism of action of ultrasonic responsive systems

Mechanisms of action of ultrasound nano-nanocarriers are two as previously mentioned. Based on the induced stimulation type, they are classified to nonthermal and thermal effect.

a. Nonthermal effects:

Nonthermal effects can be further subdivided into two other classes, namely: cavitation and mechanical effects. When activation is induced by physical forces of ultrasonic waves and occurs in a liquid-like material that consist of bubbles and pockets that can include gas or vapor, this phenomena is referred to cavitation. Application of accurate ultrasonic pressure results in oscillation of the bubbles, which further collapse. Cavitation can be done by the following processes which will have an impact on the biological functions: increasing the temperature, application of mechanical stress, and presence of free radicals. If the intensity of the applied ultrasound field is low, the cavitation state will be stable. If the ultrasonic field was with high intensity (>1 MPa, 1 MHz) it leads to unstable cavitation, and the bubble oscillation magnitude will be enhanced, which can lead to micro explosion. The temporary presences of pores around the vessel walls facilitate ultrasonic gene and drug delivery. Polyether block copolymers show well-organized drug releasing patterns for tumor therapy. Releases drug from micelles by means of resulting

shear stress and shockwaves of the bubbles. To summarize, ultrasound generates both “injures” and “recovery response” via increasing the protein synthesis (Zhou et al., 2014; Zhao et al., 2013; Johns, 2002; Dyson, 1982).

b. Thermal effects:

Thermal therapeutic effects can be produced in this type of ultrasound-mediated drug/gene delivery mechanism. This process involves increasing the tissue temperature above 43°C for 5 min which influences the phospholipid layer and the permeability of the membrane. As a result, hyperthermia can be seen and that can be defined as high anticancer drug uptake, which can be accomplished by high intensity focused ultrasound (HIFU). Hyperthermia can initiate a well-studied anticancer drug delivery to the affected tissues from thermo-responsive liposomes. In in vivo conditions, the tissue temperature can be estimated directly during treatment period (Zhou et al., 2014; Zhao et al., 2013; Johns, 2002; Dyson, 1982; Kujawska et al., 2014).

12.2.2.2 Types of ultrasound-responsive targeted drug delivery system

12.2.2.2.1 Ultrasonic microbubbles

Microbubbles are microspheres containing gas filled with ultrasound contrast agent that can be effective contrast agents, due to their ability to interact with the ultrasonic wave. They are utilized for diagnosis and therapeutic purposes. Moreover, the structure of an ultrasonic microbubble contains polymers and gas core that demonstrate the shell. A therapeutic agent can be incorporated, along with the heavy gas in the gas core (Zhao et al., 2013; Johns, 2002; Dyson, 1982; Kujawska et al., 2014; Harshavardhan and Sai Venkata Vedavyas Pisipati, 2012). Interestingly, microbubbles are one of the most popular ultrasonic contrasting agents. Generally, the size of gas bubbles can range from 1 to 10 μm with a shell that contains phospholipids, polymers, or sometimes proteins. Sonazoid is a type of widely used ultrasound agent for targeting tumors in the liver tissue. Sonazoid is contrast agent that is filled with perfluorobutane enhancing contrast in 10 min after injection administration. Table 12.2 demonstrates two different types of ultrasonic microbubbles (Rosendahl; Dayton et al., 2006).

12.2.2.2.2 Ultrasonic responsive liposomes

Ultrasonic liposomes are one example of ultrasound-responsive nanocarriers that are produced from the phospholipid of the cell membrane that includes the hydrophilic core. The presence of selective lipophilic physiological membranes separate the internal and the external components. Fabricating the liposome structure is carried out by auto-grouping. Depending on their grouping pattern, they are categorized into unilamellar or multilamellar vesicles, with different diameter sizes that range from 20 nm to less than 10 μm . By film hydration process, multilamellar vesicles are formed. With high forces of sonication that reach up to kHz frequencies, the large assemblies of multilamellar vesicles are broken into smaller ones, termed unilamellar vesicles. Upon application of greater forces via

Table 12.2 Types of Ultrasonic Microbubbles

Type of Microbubble	Made up From	Diameters Range	How Does It Work	Examples
First-generation contrast agents	Microspheres Filled with air	1–8 μm	They have the ability to pass across the capillaries of pulmonary, and eliminate from the circulating blood so fast because of two reasons, firstly due to reduced resistance to arterial pressure gradients and secondly tea air is highly blood soluble	Albunex Levovist
Second-generation contrast agents	High molecular weight lipophilic gases such as perfluorocarbons or sulfur hexafluoride filled-microbubbles	2–8 μm	Here the bubble shell is consisting of Surfactants, albumin, and also phospholipids, to enhance the stability of the bubble while travelling in the blood stream	Optison Definity Lumason

extrusion, further size modification can be achieved. Availability of very large number of lipids led to an infinite range of physicochemical features, including: high permeability and capability of penetration and incorporation of certain ligands. Incorporation of imaging agents or hydrophilic therapeutic agents for curative purposes can be done at the interior region. Echogenic types of liposomes are mainly utilized as imaging contrast agents, which include application of ultrasonic waves with powerful frequencies, and in epitope targeting. Ultrasound agents in the form of liposomes show significant merits, such as prolonged half-life that ranges to many days, suitable desired pharmacokinetics, and favorable small diameter 100 nm or less. All these properties grant ultrasound nanopreparations the preference over other types of formulations. Rapid elimination from systemic circulation due to a short half-life (as any other liposomes) is considered an undesired characteristic of ultrasonic liposomes. The biphasic layer of these liposomes undergoes oxidation and hydrolysis reaction, and one more drawback is that the preparation process is costly.

12.2.2.2.3 Ultrasonic nanobubbles (nanodroplets)

Although nanodroplets have less echogenic properties, they are highly stable, compared to microbubbles under pressure and mechanical stress. They have the

capability to accumulate in pathological tissues, as well as having the ability to carry a large drug payload, as they are with sizes smaller than 1 μm (Zhao et al., 2013). The similarity between nanodroplets and microbubbles is that both of them can be filled with liquid and are able to be encapsulated by a phospholipid shell. Using nanobubbles helps in overcoming the major limitation of microbubbles which have large diameter value (1–10 μm), and for this reason microbubbles should penetrate the epithelial cells to reach the targeted tissue (Hyndavi et al., 2011). Another point is the restriction of microbubbles to target cardiovascular and tumor endothelium targets after administration by an intravenous injection. The reason behind this restriction is that microbubbles entrapped in the lungs in which the occurrence of exchanging of gas will be there. Nanobubbles coalesced to result in microbubbles with larger size by ultrasonic or hyperthermia induction, which shows an effective chemotherapeutic treatment for local tumor capillary occlusion (Zhao et al., 2013; Johns, 2002; Dyson, 1982; Kujawska et al., 2014; Harshavardhan and Sai Venkata Vedavyas Pisipati, 2012; Rosendahl; Dayton et al., 2006; Rapoport et al., 2007). Recently, development of numerous types of novel ultrasonic nanobubbles has been achieved for different potential uses and several aims, mainly for ultrasound tumor imaging, delivery of interfering RNA, as well as ultrasound-targeted chemotherapy. One interesting example is ultrasound-responsive chitosan nanobubbles which function as novel carriers for targeted DNA delivery (Cavalli et al., 2012).

12.2.2.2.4 Ultrasonic-mediated polymeric micelles

A spontaneously occurring spherical monolayer in aqueous consists of amphiphilic molecules consisting of a lipophilic tail and lipophobic head. When critical micelle concentration is attained, self-assembly of a micelle then occurs, with a diameter size range of 10–100 nm. Transportation of drugs that are attached to the hydrophilic polymer or to the hydrophobic portion can be easily achieved using polymeric micelles. Current micelle usage is less than, but still scientists prefer micelles because of their advantageous properties such as: biocompatibility, simple preparation, drug loading, easy tissue penetration due to their small sizes, controlled drug release from the core, high stability, and the most essential one is that they enhance the solubilization of the drugs. Hydrophilic building blocks can be poly(ethylene oxide), whereas be poly(propylene oxide) and poly(lactic acid) are used as the lipophilic monomers (Martins et al., 2015). The release of the drug from micelles is initiated by high-frequency ultrasound. Fifteen to twenty seconds of exposure to an ultrasonic wave can increase the Pluronic micelles intracellular uptake. Continuous exposure of ultrasonic wave increases the drug uptake from Pluronic micelles; DOX is an example. Ultrasound density is the major factor that influence uptake of the drugs; if the power is increased, the drug uptake will also increase (Zhao et al., 2013; Johns, 2002; Dyson, 1982; Kujawska et al., 2014; Harshavardhan and Sai Venkata Vedavyas Pisipati, 2012; Rosendahl; Dayton et al., 2006; Rapoport et al., 2007; Cavalli et al., 2012; Martins et al., 2015; Abdel-Hafez and Hussein, 2015). Recent studies demonstrate that DOX

conjugation with polymeric micelles increases the activity of the anticancer drug (DOX). A notable disadvantage of polymeric micelles in vivo condition can arise, which is the stability of the micelles. Once injected into the systemic circulation, micelles may undergo fast dissolution, which leads to releasing of the encapsulated drug early and prior the desired time. Using highly concentrated polymers avoids this limitation. Likewise, concentrated polymers may result in toxicity, so monitoring the concentration is a very important issue (Martins et al., 2015).

12.2.2.2.5 Ultrasonic responsive microemulsions

When a liquid solution is a thermodynamically stable system and consists of both water and oil phases in the presence of surfactants that are in form of micelles to enhance and improve the stability, then this system is termed a microemulsions (Zhao et al., 2013). Capability to form spontaneously, noninvasiveness, stability, and their simple preparation process are some examples of unique characteristics of microemulsions. Introduction of ultrasound waves to ultrasonic-mediated microemulsion is one of the interesting ultrasound-responsive DDSs that have attractive roles in treating many types of tumors, such as ovarian, breast, and orthotopic pancreatic. This is by entrapping anticancer material within the structure of microemulsion droplets, with a size range of 20–35 nm (Buckin and Kumar Hallone, 2012). The synthesis of this type of microemulsion can be done by high pressure homogenization method (Martins et al., 2015). In animal experiments, a combination of tumor-focused therapeutic ultrasound with drug-loaded microemulsion in intravenous injection accomplished a very effective role in regression of numerous types of tumors (Zhao et al., 2013). In the study of ultrasound-mediated release of curcumin (also called diferuloylmethane), curcumin was released and produced aggregates of very low solubility. Furthermore, it demonstrated a different rate of release in different conditions. At acidic pH, the releasing rate was decreasing, while in saline solution of phosphate buffered, this rate was highly increased. In contrast to liposome cargo which were encapsulated, the cargo in microemulsions of curcumin is partitioned. As a result, microemulsions show the efficacy of ultrasonic DDSs. The preparation of curcumin solution is carried out by solubilization of curcumin in 5 mL of ethyl oleate at different loading concentrations. Then, to prepare stable microemulsions, add the curcumin solution in oil (ethyl oleate) to lecithin and Tween 80 (emulsifying agents) for stabilization purposes, heating to 50°C. After that, in a bath sonicator, add water along with the sample mixture at 50°C and carry out the sonication process until a transparent isotropic microemulsion is obtained. The resulting microemulsions can be stable nearly for 2 weeks (Lee et al., 2008).

a. Biomedical applications of ultrasound-responsive drug delivery systems:

1. increase vascular and cellular permeability which enhance the transportation of drug/gene (Zhou et al., 2014);
2. sonophoresis: ultrasound is used to deliver drugs in efficient way throughout the skin (Zhao et al., 2013);

3. targeted ultrasound-induced blood–brain barrier disruption may solve the problem of small and large-molecule drugs delivery to the brain (Zhao et al., 2013);
4. stimulate tissue repair and initiate healing in several inflammations and ulcers in less than 2 months (Dyson, 1982);
5. perfluorocarbon nanodroplets are applied as therapeutic vehicles for DOX and different therapeutic agents, and used as contrasting agent for imaging (Dayton et al., 2006)
6. targeted drug delivery and controlled release of anticancer therapeutic agents, that is, curcumin (Lee et al., 2008);
7. acoustic droplet vaporization in ultrasound therapy, for instance: vessel occlusion, and thermal ablation (Zhou, 2015).

12.2.3 ELECTRO-RESPONSIVE DRUG DELIVERY SYSTEMS

The diverse techniques for advanced targeted DDS under the effect of electricity field have drawn growing attention in biomedical applications. Stimulation through electric field is extremely precise, and demonstrates different drug release patterns, such as pulsed or sustained drug release of payloads within nanocarriers, even in application of weak electric fields (nearly 1 V) by several actuation methods (Mura et al., 2013). A conductive polymer such as polypyrrole, is utilized to monitor and predict release rates of vasopressin from and for other therapeutic agents, such as insulin, as well as proteins releases by applying electric current in iontophoresis and electroporation form. Under effect of the electric-field strength, the hydrogel matrix shows the higher release of the loaded drug. In electro-responsive systems, which are mainly applied for hydrogel polymers, the release of drug is due to electro-induced changes in the gels. Moreover, the drug release can be affected by many factors, for example: drug and gel nature, experimental set-up, and electric-field strength (Murdan, 2003). One major application of electro-sensitive DDS is delivery of nucleic acid against cancer (Mura et al., 2013). Better drug release was obtained in the presence of electric-field strength than the drug release when electric field is absence (Niamlang and Sirivat, 2007). Various multiparticulate formulations, such as liposomes and hydrogels, act differently to light, temperature, magnetic, ultrasonic and electrical fields or any other external stimuli. The ideal properties of the electro-responsive hydrogels permit them to be utilized as intravascular drug delivery with less invasive application. At body temperature, when a biodegradable hydrogel is administrated as subcutaneous injection, it should be liquid in form of gel and it should expand and contract many times. By this injection the need for surgical implantation will be removed, thus it will enhance patients' accessibility and their compliance (Verbrugghe et al., 2015).

a. Mechanism of action of electrical responsive systems

Several experiments have been done to study the responses of polyelectrolyte hydrogels when they undergo electrical field. The placement of the gel could be in electro-conducting vehicle including: saline solution, water, or it can be also placed in buffer. The gel in the container may touch the electrodes that are made out of platinum or carbon, and if there is no contact between the gel and the electrodes, then locate them in the conducting vehicle. In some cases, there will be no conducting medium. Experimental set-up may influence the gel response. In general, electro-sensitive gels shrink or de-swell after application of electric field. However, some types of gels erode. Frequently, the therapeutic agent can be inserted into polyelectrolyte hydrogels which monitor the releases of the drugs upon application of electrical fields and this releasing pattern can be ended or decreased by removing the electrical stimulus. The incorporation of the drug can be done either during or post-gel production by diffusing the drug molecules within the solution. Activation of drug release is based on stimulus response mechanisms, including: releasing pattern from the gel, interactions between the therapeutic agent and gel structure (Murdan, 2003).

b. Method of preparation of electric-field responsive hydrogels:

Electric-field responsive hydrogels are prepared by mixing of Pluronic bismethacrylate powder with methacrylic acid sodium salt. This mixture takes place in nitrogen gassed de-ionized water. After that, cool the resulting product for period of 8 h. Introduce 1 M of ammonium persulfate, which functions as an initiator that aids in obtaining free radical polymerization. 1 M tetramethylethylenediamine is an accelerator solution. Refrigerate the resulting mixture for 60 min after placing it in PVC mold. In the final step, at 37°C treat the produced hydrogel approximately for 3 h. Take the hydrogel to a phosphate buffered saline solution after polymerization is done. Refresh the solution three times a day for the three following days (Verbrugghe et al., 2015).

c. Applications of electric-field responsive hydrogels:

1. accurately control the drug release levels in the body (Murdan, 2003);
2. insulin released after electric-stimulation of implant, then the therapeutic effect obtained when insulin diffuses as drops in the blood (Verbrugghe et al., 2015);
3. stimulation of neurotransmitter release, which is incorporated with the nanoparticles, thus activating specific brain areas upon application of electric fields (Ge et al., 2012);
4. essential role in cardiovascular tissue engineering, by releasing the incorporated material to be at regular intervals with every pulse (Ge et al., 2012);
5. Generally, it can be used in diagnostic/therapeutic purposes (Liu et al., 2011).

12.3 OTHER CLASSES OF EXOGENOUS STIMULI-RESPONSIVE SYSTEMS

12.3.1 LIGHT-RESPONSIVE SYSTEMS (PHOTO-SENSITIVE)

The application of novel light-responsive biomaterials in various fields of biomedicine (drug delivery, cell biology, micro- and nanodevices) has seen a considerable growth in recent years. Typically, development of microdevices in light-responsive DDS had many attractive properties (noninvasiveness, safety in drug delivery, and high permeability) (Mura et al., 2013). One example of external stimulus is UV light irradiation, which can also be applied to trigger mesoporous silica nanoparticles to release drug. The drug molecules are entrapped within the mesoporous structure with the addition of different capping agents (Knežević et al., 2011).

a. Mechanism of action of photo-responsive systems:

By way of illustration, photo-sensitive azobenzene group or its derivatives (Mura et al., 2013), are aromatic robust molecules with small versatile moieties that demonstrate spectroscopic and photophysical characteristics. There are two conditions of existence of azobenzenes: they can exist as *cis* state or a more stable *trans* state (Barrett et al., 2007). The capability of absorption of the polarized light is one of the interesting properties of azobenzenes during their conversion on the azo dipole axis. Azo groups exhibit efficient and reversible photopolymerization upon light irradiation on UV (300–380 nm) (Zhao and Ikeda, 2009) into the more polar nonplanar meta-stable *cis* configuration. With new direction of the irradiating light in the visible area, it reverts back to the thermally stable nonpolar planar *trans* form (Yin et al., 2016), which enhances the hydrophilicity of the lipophilic regions. This helps to ensure the maintenance of drug release by photoregulation. Similarly, reverting the process can either be done by locating the azobenzenes in a dark place or application of UV light irradiation on 430–490 nm. Thus, in a reversible behavior the functionalities may be isolated with UV light and gathered again via visible light.

b. Preparation of photo-responsive polymers (azo block copolymers):

Azo materials can be incorporated in polymers to be in higher stability by covalent bonds. With the help of atom-transfer radical polymerization method, azo block copolymers can be prepared. The formulators use poly-(*tert*-butyl acrylate)PtBA-Br initiators, which have limited polydispersity to initiate various lengths of the other unit of azobenzenepoly(methacrylate) (PAzoMA). As a result, PAzoMA-*b*-PtBA copolymer polydispersity is enhanced to some extent. With a specific hydrolysis process this diblock copolymer will then increase more to produce amphiphilic PAzoMA-*b*-PAA diblock copolymer. In the analysis of the azo block copolymers, ^1H NMR spectroscopy was employed prior or following the hydrolysis reaction. ^1H (NMR) spectroscopy

can be used to eliminate the assemblies of *tert*-butyl from PtBA block to control hydrolysis process, in the identification of the component of the individual copolymer, to assess the amphiphilic nature in several solvents (Wang et al., 2004).

c. Biomedical applications of photo-responsive materials:

Development of photo-responsive block copolymer uses a delivery system for chemotherapeutic agent anticancer drugs. For instance, DOX delivery is triggered by infrared irradiation of the DNA at gold nanorods plane, to treat breast cancer (Paasonen et al., 2010).

A photo-responsive hydrogel structure almost identical to the living tissues configuration is used as a growth vehicle (matrix) for culturing the cells, illustrating the vast progress in monitoring stem cells relocation by photo-stimulation (Tomatsu et al., 2011).

12.3.2 THERMO-RESPONSIVE SYSTEMS

Among the other types of external stimuli-responsive systems, increasing attention has been paid to thermo-responsive systems, (also called temperature-responsive systems) in oncology, DDSs, and in other biomedical applications because certain diseases demonstrate temperature variation. In simple words, we can define it as: a sharp alteration occurs in a nanocarrier component with temperature, and by changing the temperature the drug will release as a response (Coelho et al., 2010). These intelligent polymeric systems are able to release the drug by local heating or cooling during a particular time period. These can be classified as lower critical solution temperature (LCST) and higher critical solution temperature. These materials can be liposomes, polymer micelles, or nanoparticles which exhibit a LCST mainly in water (Safari and Zarnegar, 2014). Thermo-responsive composites can be further categorized into different systems such as: thermosensitive hydrogels and thermosensitive dendrimers.

a. Mechanism of action of exogenous thermo-responsive drug delivery systems:

Typically, at human body temperature thermosensitive nanocarriers should quickly deliver the drug within a locally heated tumor ($\sim 40\text{--}42$) to counteract rapid blood-passage time and washout from the tumor. However, in different circumstances in cancer diseases for instance this value commonly deviates from the normal value 37°C . Thus thermo-responsive materials should retain their load at regular body temperature (Mura et al., 2013).

An attractive example are polymers, [poly-*N*-isopropyl acrylamide, (PNIPAM)] one of the most popular linear temperature-responsive polymers which used in biomedical application, the structure of these polymers shows amphiphilic balance with a little temperature changes around a critical solution temperature (CST) which can be defined as: temperature at in which two miscible liquid undergoes separation into two phases, result in reversible phase transition

from hydrophilic to hydrophobic so chains are collapsing or extending, as a response to the alteration of the hydrophobic and hydrophilic interactions between the polymer chains and the aqueous solution at LCST is equal to 32°C (You et al., 2010; Wander et al., 2010). Whereas, some other materials such as zwitterionic brushes own an upper critical solution temperature (UCST) and change their wetting behavior with temperature alteration. As LCST materials these polymers can also undergo reversible changes and the material can maintain several transitions backwards and forwards (Stuart et al., 2010).

12.3.2.1 Hydrogels that can act as external temperature-responsive material

Stimuli-responsive nanogels illustrate another type of interesting material for DDS as a category of thermo-responsive polymers makes them an ideal class of materials for biomedical applications. This phenomenon can be described as hydrophilic three-dimensional insoluble polymeric network can absorb large amount of water or physiological fluid (Sony and Yadav, 2014). Hydrogel nanoparticles (HNPs) have been investigated as carrier systems for controlled drug release that designed to allow change to the nanostructure in response to external change in temperature. Therefore, HNPs are ideal DDSs due to their versatile and unique features: hydrophilicity, flexibility, high stability and biocompatibility thus they possess perfect drug loading capacity (Lamberti et al., 2016). There are two major types depending upon their crosslinking mechanism: physical gels and covalently (chemical) crossed-linked gels (Ward and Theoni, 2011). The former (physical crosslinks) such as entangled chains, hydrogen bonding, hydrophobic interaction and crystallite formation. Although the physical crosslinks not permanent in nature they are sufficient to make hydrogels insoluble in an aqueous media. The latter (covalently gel) conversely, formed by covalent crosslinking of polymers and are permanent. Unfortunately inflexibility of these polymers is conceded as a limitation that processing properties of insolubility an infusibility. Some drawback can arise sometimes associated with crosslinked polymers is that they are relatively when it comes to their processing properties because they are insoluble and infusible (Maitra and Shukla, 2014). In covalently linked networks, two approaches can be taken for drug delivery: (1) the networks can be formed in situ as a response to chemical or photo stimulation or (2) the networks are formed before implantation to the body. Scientists prefer, the first approach due to its less invasive nature. In both of these systems, drugs are loaded into the hydrogel at room temperature, and while heating to body temperature, the gels shrink and trap the drug molecules which will reduce their release. Inclusion of hydrophobic polymers into the network which result in interactions between hydrophobic drugs to increases the overall strength of the gel. PNIPAAm is a well-studied example of these polymers as mentioned previously (Ward and Theoni, 2011). PNIPAAm can trigger the platelets upon contact with body fluids, and it's consisting of quaternary in its structure which limits its use (Table 12.3) demonstrate several types

Table 12.3 Different Types of Hydrogel Polymers and Their Biomedical Applications

Hydrogel Polymer	Application	References
Poly(methacryloisobutyl (POSS) (PMAPOSS)	Vehicle for controlled drug delivery application	Ata et al. (2016)
PLGA-PEG-PLGA thermogels	Barrier for reduction of epidural scarring	Liow et al. (2016)
PNIPAM	Tissue regeneration cardiac tissue engineering	Ashraf et al. (2016)
Am-AA IPNs	Create function gates for permeability control	Ma et al. (2010) Mah and Ghosh (2013)

Table 12.4 Polymers and Their Behavior as (LCST/ UCST) ([Gandhi et al., 2015](#))

Polymer	LCST/UCST Behavior
Poly(<i>N</i> -isopropylacrylamide)	LCST
Poly(<i>N,N</i> -diethylacrylamide)	
Poly(methyl vinyl ether) Poly(<i>N</i> -vinylcaprolactam)	
Block copolymer of poly(ethylene oxide) and poly(propylene oxide)s	
Poly(pentapeptide) of elastin	UCST
polyacrylic acid IPN	
Polyacrylamide	

of hydrogel polymers with their potential applications in medicine ([Table 12.4](#)) illustrate the polymer's behavior as (LCST/UCST).

12.3.2.2 *Novel dendrimers which can behave as thermo-responsive substances*

Dendrimers are synthetic macromolecules characterized by highly branched structures which contain pure tetra(ethylene glycol) chains act as nanoscopic drug carriers due to their unique features and properties, including: high structure controllability, surface properties, and size. Moreover these properties rely on the hydrophobicity and surface compactness of the functional groups, guest molecule binding, medium pH, and the concentration of dendrimers that has shown tremendous promise in many biomedical and pharmaceutical applications including carriers of imaging agents, drug delivery, therapeutically active compounds and tissue engineering contain polar enantiomerically. The drawback associated with

production of dendrimers includes some synthetic problems, hemolysis and cytotoxicity in biomedical terms. However, if we first consider low generation poly (amidoamine) dendrimers are less cytotoxic, the disadvantage is that there will be a difficulty to provide them with thermosensitive properties because they are surface terminal groups deficient. The discussed example demonstrates the fundamental progress in the area of novel drug-delivery systems (Zhao et al., 2012; El Malah et al., 2012).

12.4 EXTERNAL MULTISTIMULI-RESPONSIVE DRUG DELIVERY SYSTEMS

With the considerable progress in developments of stimuli-sensitive agents, current responsive targeting carriers are not only used as individual units, but they also can be utilized in combination together, aiming to greater efficiency in drug delivery and targeting pathogenic sites. Multistimuli-responsive materials refers to substances that show responses upon several stimulations (from two or more stimuli), dual stimuli-responsive systems are an example of a multistimuli-responsive system, for example, physical–physical dual stimuli-responsive. In addition, multistimuli-responsive preparations are similar to our biological system; instead of responding to one change, they can adapt to several ecological alterations. Therefore they are highly favorable over conventional DDSs and single stimuli-responsive systems.

12.4.1 EXTERNAL DUAL STIMULI-RESPONSIVE SYSTEMS

12.4.1.1 *Physical–physical dual stimuli-responsive materials*

Physical–physical dual stimuli-responsive macromolecules act as example of external stimuli system which includes many types, one of them is thermo-responsive and photo-responsive formulations. In temperature-responsive systems the material experience hydrophilic-hydrophobic phase transition in temperature deviation that refers to LCST, in which the temperature of the used material is close to human body temperature. The light sensitivity of different polymers has been studied along with thermo-responsive polymers. Azobenzene is a well-known example of a chromophore that shows reversible photoisomerization from trans-to-cis configurations when it undergoes irradiation, as discussed previously. This photoisomerization process results in rising in the dipole moment (0D to 3D) and subsequently in the polymer chain LCST. Fascinating scientific studies have been done on polyacrylamide copolymers when incorporating it with various ratios of azobenzene groups. The main important characteristic of these polymers is that their LCST influenced by azobenzene quantity. The LCST is directly proportional to the amount of azobenzene incorporated with the polymers and this increasing is because of the alteration of chromophore polarity. Other examples

of photo-responsive units that can transform undergo thermo-stimulations are at near-IR wavelengths: fulgimide, spiropyran, and dithienylethene moieties. Additionally tissue penetration depth is irradiation wavelength dependent and for the most cases this irradiation depth is extremely low. Likewise the used temperature should be lies within applicable physiological range (Zhuang et al., 2013; Huang et al., 2016).

12.5 GENERAL PHENOMENA IN DELIVERING ANTICANCER THERAPEUTIC AGENTS VIA STIMULI-RESPONSIVE DRUG DELIVERY SYSTEMS

As mentioned earlier, the most wide clinical application of smart stimuli sensitive nanopreparations (SSNs) is to deliver chemotherapeutic drugs absolutely to tumor and pathological tissues. Bypassing the other unaffected cells and targeting the pathological cell was the major challenge in delivering these types of drugs that physicians currently face. Construction, classification, and evaluation of various nanopreparations have been done to develop targeted drug delivery which is significant to cure many diseases, especially cancer with the help of different parameters (Padh and Sakhrani, 2013; Jhaveri et al., 2014). In the case of cancer, which is known as uncontrolled growth of cells and irregular cell division that have the ability to spread to the other tissues, the conventional ways that are used to treat it have notable undesirable side effects. These were not easily overcome, and often result in systemic toxicity due to deficiency in the ability of targeting specific pathological region (Lammers et al., 2012). Therefore, significant progress over the past few years has been made in the improvement of advanced drug delivery to treat cancerous cells via novel strategies by the mean of formulating smart nanocarriers that are mainly sensitive to different stimuli and releases their payload locally at the tumor tissue. There are bulky gaps in the solid tumor with average size of 100–600 nm among each endothelial cell, which is larger than normal tissues. Through this property of tumor vasculature, the molecules may exhibit extravasation to many extra nanometers, passive accumulation that results in lack of clearance, and less fluid drainage of these nanoparticles. In this situation, this technology of the extravasation is referred to enhanced permeability and retention (EPR) effect (Rajora et al., 2014). Several researches have reported evidence for the utilization of EPR technique and its impact for delivery of the loaded drug within nanocarriers to the solid tumors. Many studies have demonstrated the relationship between size, tumor type, and the magnitude of the EPR effect. If the size of carrier was small, drainage will be promoted, whereas a larger one will be removed easily by the reticuloendothelial system and unable to exhibit extravasation through the intracellular space of the tumors. Using

polymers to increase nanocarrier surfaces hydrophilicity to produce long-lasting circulation enables multiple EPR effects within the tumor areas. Polyethylene glycol (PEG) is one example of polymers that give prolonged effects. However, in some circumstances carriers that are coated by PEG are unable to release the drug simultaneously, which is unwanted for delivery of drug. Accordingly, the remedy of this issue is to remove PEG from the lipophilic chain (Fang et al., 2011; Sawant and Torchilin, 2012). There are two forms of targeting strategies for nano-preparations, namely: active and passive techniques. The former one less favored due to problems that arises during using it, absence of uniformity of the pore sizes as an example. Whereas smart delivery involves selective targeting of the pathogenic cell, tissue or organs, where the drug release is endorsed at the tumor site after physiological (intrinsic) stimuli or physical exogenous stimuli, including: photo stimulus, thermo stimulus, and ultrasonic-mediated drug release or magnetic stimulus, which offers less adverse reaction (Jhaveri et al., 2014; Lammers et al., 2012; Rajora et al., 2014; Fang et al., 2011; Sawant and Torchilin, 2012; Perche and Torchilin, 2013) (Tables 12.5–12.7).

Table 12.5 Some Examples of Drugs That Can be Used in Magnetic-Responsive Systems

Drug	Technology	Method Used	Indication	References
Bleomycin	Magnetite nanoparticles	Solvothermal method	Anticancer	Xu et al. (2013)
Cisplatin	Gold-coated iron oxide nanoparticles	o/w emulsion method	Treatment of solid tumors	Wagstaff et al. (2012) Yang et al. (2006)
Doxorubicin	Iron oxide nanoparticles	Precipitation and coating with polyvinyl alcohol (PVA)	Anticancer	Sun et al. (2008)
Indomethacin	Magnetic microspheres	Emulsion solvent evaporation technique	Antiinflammatory drug that reduce fever and pain	Chandna et al. (2013) Aw et al. (2012)
Taxol (TXA)	Magnetic microspheres	External sink method	Anticancer for breast and ovarian cancers	Timko et al. (2010)

Table 12.6 Marketed Products of Magnetic Microspheres (Chandna et al., 2013)

INCI Name	Product Name	Company
Ethylene/acrylic acid copolymer	EA-209	Kobo Products, Inc.
HDI/trimethylol hexyllactone crosspolymer (and) silica	BPD-800	
HDI/trimethylolhexylactyl cross polymer (AND silica)	BPD-500	
HDI/PPG/polycaprolactone cross polymer (AND silica)	BPD-500T	
Polymethyl methacrylate	BPA-500	
Methyl methacrylate cross polymer	BPA-500X	
Polymethyl methacrylate	MSP-822	
Methyl methacrylate cross polymer	MSP-825	
Methyl methacrylate cross polymer	MSP-930	
Methyl methacrylate cross polymer	SUNPMMA-H	
NYLON-6	TR-1	
NYLON-6	TR-2	
NYLON-6	POMP-605	
NYLON-6	POMP-610	
NYLON-12	SP-10	
NYLON-12	SP-10L	
NYLON-12	SP-500	
Polyethylene	CL-2080	
Polymethylsilsesquioxane	TOSPEARL 1110 A	
Polymethylsilsesquioxane	TOSPEARL 120 A	
Polymethylsilsesquioxane	TOSPEARL 145 A	
Polymethylsilsesquioxane	TOSPEARL 2000B	
Polymethylsilsesquioxane	TOSPEARL 3000 A	

Table 12.7 Marketed Products of Ultrasonic Responsive Nanopreparations

Product Name	Other Names	Company	Indication
Albunex	Albumin (human) alburx	Molecular Biosystems Inc.	Ultrasound contrast agent
Levovist	Levograf	Berlex Laboratories Inc.	Vascular sonography and echocardiography
Optison	Perflutren	GE Healthcare	Ultrasound imaging agent (echocardiogram)
Definity	Perflutren lipid microsphere	Jubilant HollisterStier (JHS) Ben Venue Laboratories (BVL)	Ultrasound imaging agent (echocardiogram)
Lumason	Sulfur hexafluoride	Bracco Diagnostics Inc.	Ultrasound contrast agent echocardiography and for liver ultrasonography

12.6 FUTURE DIRECTIONS FOR DRUG DELIVERY SYSTEMS THAT RESPONSE TO EXTERNAL STIMULI

With growing interest in relieving the undesirable drawbacks and alleviating the limitations of different therapeutic agents on healthy tissues, scientists did great work in creating such systems that can target the affected tissues without reaching the healthy tissues. They aimed to achieve the maximum therapeutic efficacy with minimum side effects. Many studies and researches have been done in stimuli-responsive DDSs which contribute notable progress in the past few decades. In spite of the fascinating progress that was achieved in external stimuli-responsive drug delivery studies, unfortunately most of the currently developed stimuli-responsive nanopreparations are facing a difficulty in reaching clinic, and require more adjustments in various aspects such as degradability and biocompatibility. There are many challenges and future aspirations that need to be taken in hand, including resolving the limitations which have been raised during formulation procedures as well as in the preclinical trials. Some of the limitations of external responsive DDSs are summarized in [Table 12.8](#). Additionally, the most important goals in the near future are to approach the approval of magnetic-responsive nanosystems for their novel properties over the conventional DDSs and focusing in providing clinically acceptable systems that are sensitive to specific stimuli. Exogenous stimuli-responsive materials have strong potential for future biomedical and healthcare applications, which demands extensive researches in medical fields among other drug releasing systems.

Table 12.8 Limitations of External Responsive Drug Delivery System

Type of External System	Limitations
Magnetic-responsive systems	1. Costly and requires specialized technical approach 2. Undesired localization of the drug in different organs
Ultrasonic responsive systems	1. Microbubbles and liposomes are rapidly eliminated due to their short half-life in circulation 2. Costly and requires specialized technical approach
Light-responsive systems	1. Wavelength of light and density issues 2. Issues in the degradation process of light-responsive contrast agent
Electrical responsive systems	1. Long time to response 2. Non linearity between drug release and current (Zhuang et al., 2013)

12.7 CONCLUSION AND PERSPECTIVES

A number of studies on intelligent nanocarriers, done for more than 40 years, have shown that selective and controlled drug delivery can provide significant advantages. However, more vigorous investigations are currently applied in both in academic and in industrial levels. Furthermore, with the novel improvement of the advanced smart DDSs, exogenous techniques for stimuli sensitive delivery illustrate the connection between therapeutic need and drug delivery. These site-specific DDSs can be used in combination to achieve greater efficiency in drug delivery and in specific targeting. In this regard, we believe that novel DDSs have emerged to enhance drug bioavailability, to prevent harmful side effects, and the main interesting part is to produce controlled targeted drug delivery. However, few of the reported dual and multistimuli-responsive systems were proved by different studies that they demonstrate low biodegradability with low drug loading capacity, leaving them to be not amenable to in vivo conditions. Therefore, future efforts shall be shifted toward improvement of dual and multistimuli-responsive polymeric materials, which are highly biodegradable and noncytotoxic, efficiently loaded, and retain drugs in systemic circulation. All these efforts will ensure the efficient improvements of targeted DDSs. The continuing advances in smart drug delivery technologies can be an extremely fertile field for researches and investigations for several diseases, especially cancer, tumor targeting and other diseases.

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Ligand-directed tumor targeting with hybrid viral phage nanoparticles

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CHAPTER OUTLINE

13.1 Introduction	484
13.1.1 Early Foundations of Gene Therapy	484
13.1.2 Clinical Applications of Gene Therapy	484
13.2 Vectors	485
13.2.1 Nonviral Vectors	485
13.2.2 Viral Vectors	486
13.3 Targeted Gene Therapy	490
13.4 Ligand-Directed Nanoparticles	490
13.5 Advantage of Targeted Gene Therapy With Bacteriophage	491
13.6 Hybrid Adeno-Associated Virus/Phage Vector	491
13.7 Construction of Targeted Adeno-Associated Virus/Phage Vectors for Eukaryotic Cell Transduction	495
13.8 Clinical Potential of Adeno-Associated Virus/Phage	496
13.9 Molecular-Genetic Pet Imaging Using RGD-AAVP-HSVtk	497
13.10 Targeting Tumor Vasculature With RGD-Targeted Adeno-Associated Virus/Phage Vector-Mediated Tumor Necrosis Factor-α	499
13.11 RGD-Targeted Adeno-Associated Virus/Phage Vector-Mediated Tumor Necrosis-α In Vitro Assay	500
13.12 Preclinical Trial in Small Animal Model With RGD-Adeno-Associated Virus/Phage-Tumor Necrosis Factor-α	501
13.13 Preclinical Trial in Large Animal Model With RGD-Adeno-Associated Virus/Phage-Tumor Necrosis Factor-α	502
13.14 Octreotide-Targeted Adeno-Associated Virus/Phage Vector-Mediated Tumor Necrosis-α In Vitro Assays	503

13.15 Preclinical Trial in Small Animal Model With Oct-Adeno-Associated
Virus/Phage-Tumor Necrosis Factor- α 503

13.16 Toxicity and Preclinical Safety of Adeno-Associated Virus/Phage-Tumor
Necrosis Factor- α 504

13.17 Combination Adeno-Associated Virus-Mediated Gene Therapy With Radiation
Therapy 505

13.18 Preclinical Evaluation of Adeno-Associated Virus/Phage-Tumor Necrosis
Factor With Radiation Therapy in Metastatic Melanoma Model 506

13.19 Future Directions 507

References 511

13.1 INTRODUCTION

13.1.1 EARLY FOUNDATIONS OF GENE THERAPY

In 1963, the Ciba Foundation published a collection of scientific discussions in a volume titled *Man and His Future* in which a chapter by Nobel Laureate Joshua Lederberg mused about the role of genetics and medical progress in a chapter entitled *Biological Future of Man* (Lederberg, 2008). In this chapter, Dr. Lederberg described the ultimate application of molecular genetics as direct control of human chromosomes with the capability of recognizing, selecting, and modifying genes of interest. The first clinical application of gene transfer was demonstrated in 1990 by Rosenberg et al., in which they demonstrated the safety and practical application of retroviral gene transfer, introducing the neomycin-resistance gene to tumor-infiltrating lymphocytes (TIL) harvested from patients with advanced melanoma. These modified TIL were then expanded in vitro and were subsequently re-infused to the patients with no side effects appreciated from the modified TIL (Rosenberg et al., 1990). This initial study served as the precedent upon which further clinical trials have been conducted. Indeed, from 1989 to 2012, over 1800 gene therapy clinical trials have been approved worldwide, many of which are ongoing or have been completed (Ginn et al., 2013).

13.1.2 CLINICAL APPLICATIONS OF GENE THERAPY

Monogenic disorders, characterized by a specific protein deficiency, were attractive candidates for gene therapy trials. Indeed, the first therapeutic application of gene therapy was performed on a 4-year-old with severe combined immunodeficiency, secondary to a genetic defect and leading to a deficiency of the enzyme adenosine deaminase. In this trial, Blaese et al. (1995) harvested T cells from the patient, expanded the cells in culture, transduced them with ADA retroviral vector and then re-infused the T cells into the patient. In addition to monogenic disorders, gene therapy has been used to treat a variety of disease categories, including: cancer, cardiovascular, infectious, inflammatory, neurologic, and ocular

disease. In fact, approximately 64% of all clinical trials in gene therapy are designed for the treatment of cancer, followed by monogenic disorders (8.7%) and cardiovascular disease (8.4%) (Ginn et al., 2013).

13.2 VECTORS

The transfer of genetic material into a cell is mediated by a vector. Gene therapy makes use of a wide variety of vector strategies. Ultimately, vectors can be broadly categorized as nonviral or viral. Please refer to [Table 13.1](#) for a summary of the advantages and disadvantages of each vector approach.

13.2.1 NONVIRAL VECTORS

The use of nonviral vectors in gene therapy has increased over the past decade, largely in part due to several key advantages over viral vectors. Firstly, nonviral vectors are generally less immunogenic than viral vectors, which greatly improves

Table 13.1 Advantages and Disadvantages of Various Gene Therapy Approaches

Gene Therapy Approach	Advantages	Disadvantages
Non-Viral Vectors	Safety. Easily mass produced. Low cytotoxicity.	Low transfection efficiency. Low delivery efficiency due to low penetration. Difficult to engineer.
Bacteriophages	Safety. Low immune response. Narrow potential for resistance. High titers. Large packaging capacity.	Poor ability to express transgenes inside eukaryotic cells. Narrow host range.
Retroviral Vectors	Safety. Gene integration into the chromosome. Low immunogenicity.	Low titer. Only effective in actively dividing cells. Ability to induce insertional mutagenesis.
Adenoviral Vectors	High titers. Efficiently transfer transgene into both dividing and quiescent cells.	Highly immunogenic. Short-term transgene expression.
Adeno-associated Viral Vectors	Integration into the chromosome. Broad range of hosts. Longevity. Low immunogenicity. Effects both actively dividing and quiescent cells.	Low packaging capacity. Possibly toxicity in stem cells.
Lentiviral Vector	Permanent integration. Low risk of insertional mutagenesis.	Only in quiescent cells.
AAV/P Hybrid Vector	No tropism for mammalian cells. Easily modified.	Concerns for immune mediated silencing, proteosomal degradation, inefficient transfer of gene product

treatment tolerance and allows for repeat administration of the compound without the immune system rejecting the therapy. In addition, nonviral vectors have the advantage of increased genetic-carrying capacity, thus allowing for multiple gene delivery and more complicated treatment designs (Mintzer and Simanek, 2008). Finally, nonviral vectors are generally easier to mass-produce, compared to viral vectors (Pack et al., 2005). Despite these advantages, nonviral vectors have a fundamental deficiency in delivery efficiency (Putnam, 2006; Yin et al., 2014). Viral vectors have evolved over hundreds of millions of years with the sole drive to deliver their genetic material into prokaryotic or eukaryotic cell types. Therefore, there is a significant engineering difficulty when designing nonviral vector systems to achieve comparable gene transfer efficiency. Nevertheless, researchers continue to approach this problem and develop novel synthetic vectors. For example, Abu-Rub et al. (2016) explored the use of siRNA as a means to silence XT-1, the rate-limiting enzyme involved in CSPG synthesis during spinal cord injuries. With the use of this siRNA, there was a 65% silencing rate in Neu7 astrocytes, which conferred a reduction of expression of CSPGs.

13.2.2 VIRAL VECTORS

Viral vectors used in gene therapy have been modified in order to bolster safety, increase uptake, and improve efficiency. They are derived from either RNA or DNA genomes and can be both integrating and nonintegrating vectors (Soofiyan et al., 2013). Ideally, viral vectors in gene therapy are able to harness the highly evolved viral infection pathway but avoid expression of viral genes that lead to replication and toxicity to the host cell (Bilsland et al., 2016). There are currently five main classes of clinically applicable viral vectors; they include: bacteriophage vectors, retroviral vectors, adenoviral vectors, adeno-associated viral vectors, and lentiviral vectors. These classes are categorized into two groups: integrative and nonintegrative. The former category includes lentiviruses and retroviruses, which integrate themselves into the cellular chromatin of host cells, whereas other classes of viral vectors primarily exist in the cellular nucleus as extrachromosomal episomes (Thomas et al., 2003).

13.2.2.1 Bacteriophage vector

Bacteriophages are a class of viruses, both RNA and DNA, which have a specific affinity for bacteria. They are named for the bacterial species, group, or strain for which they are associated. Bacteriophages represent a promising group of vectors for systemic gene delivery, due to their ability to home in to specific tumor receptors without the need to ablate any native tropisms. They accomplish this because the phage particles target through a phage capsid display ligand (Bakhshinejad et al., 2014; Tsafa et al., 2016). This unique feature allows bacteriophages to have an advantage in gene therapy; one of which is that, due to their lack of inherent

infectivity, they are less likely to induce adverse effects. These vectors also present other advantages, including an inherent ability to survive with dramatic physical stability under a variety of harsh conditions, capacity for packaging large DNA fragments, and able to be synthesized at a low-cost due to mass production and large-scale purification because they are produced at high titers in bacterial cultures (Bakhshinejad et al., 2014; Khalaj-Kondori et al., 2011; Manchester and Singh, 2006). Although bacteriophages have shown promise for targeted systemic gene delivery, they are considered poor vectors for gene transfer (Yata et al., 2015). Tsafa et al. showed that, with the addition of genistein, bacteriophages would be protected from proteasome degradation and would enhance vector genome accumulation in the nucleus. This, in turn, improved the phage-guided gene transfer efficacy and therefore its cancer killing ability as a gene therapy (Tsafa et al., 2016). Bocian et al. evaluated the effects of T4 and A3/R bacteriophages on the differentiation of human myeloid dendritic cells from monocytes. They found that, while bacteriophages do not substantially affect differentiation of DCs, some products of the phage-induced lysis of bacterial cells may influence the differentiation and potentially impact the function of DCs (Bocian et al., 2016). Chang et al. used a recombinant MS2 bacteriophage to deliver an RNA maternally expressed gene 3 in an effort to target EGFR in hepatocellular carcinoma. This bacteriophage vector was found to be fast, effective, and safe in its targeted effects, while not activating EGFR downstream pathways, attenuating tumor cell growth in both in vivo and in vitro experiments (Chang et al., 2016).

13.2.2.2 Retroviral vector

Retroviruses are a class of single-stranded RNA viruses that belong to the family *retroviridae*; they contain a capsid of viral glycoproteins to increase the infectivity spectrum of the vectors. One drawback to the use of retroviral vectors is their low titer volume, which decreases the efficacy of in vivo gene transfer. Retroviral vectors need to cross the nuclear membrane for integration and are only effective in infecting actively dividing cells (Verma and Weitzman, 2005). Advantages to this vector strategy include the integration of the gene of interest into the chromosome, stable gene expression, and no immunogenicity by virtue of using a complementary packaging cell line to generate infectious particles. These, however, feed the disadvantage that these vectors could facilitate insertional mutagenesis. Thomas et al. developed a GD2-targeting CAR retroviral cassette in the treatment of neuroblastoma. In the study, they found that by modifying the CAR design with the incorporation of an inducible suicide gene, there was an enhanced, normalized, and maintained transgene expression that could facilitate forward movement into clinical trials for neuroblastoma (Thomas et al., 2016). Tsai et al. developed a gammaretroviral vector from envelope-modified Moloney murine leukemia virus that carries IL-12 for the treatment of bladder cancer. The study demonstrated that the modified retroviruses carrying the IL-12 gene decreased the growth of tumors and prolonged

survival of tumor-bearing mice, compared to those treated with unmodified virus. This was accomplished through the increased expression of tumor necrosis factor α (TNF α) that induced apoptosis, and IL-1 β , which promoted inflammation (Tsai et al., 2010). Kai et al. demonstrated, through the use of retroviral vectors, that metastasis-associated protein 1 (MTA1) had angiogenic and invasive properties in prostate cancer cells of mouse xenografts. This functional link was determined to be due to the low levels of VEGF expressed in both in vitro and in vivo studies, after the integration of the retroviral vector that knocked out MTA1 activity (Kai et al., 2011). Rocha et al. used bicistronic retroviral vectors encoding both endostatin and interleukin-2 to evaluate its antitumor effects in metastatic renal cell carcinoma. The bicistronic gene transfer led to the secretion of functional ES and IL-2. This increased secretion was sufficient enough to inhibit tumor angiogenesis and tumor cell proliferation, as well as increase the infiltration of immune cells (Rocha et al., 2011).

13.2.2.3 Adenoviral vector

Adenoviruses are double-stranded DNA viruses that are encapsulated within a viral capsule and belong to the family of adenoviridae. As vectors, they bind initially to the target cell through a viral fiber protein and, with the use of a penton protein, the virus is internalized. Adenoviruses can be produced in high titers and efficiently transfer the transgene into both dividing and quiescent cells. The vector's infectivity and expression is dependent on the expression of adenovirus-specific receptors on target cells and the evocation of an immune response. In most cases, the serotype for this receptor is the Coxsackie-adenovirus receptor (Verma and Weitzman, 2005). The vectors currently in use are highly immunogenic; short-term transgene expression is observed. By eliciting an inflammatory and toxic response in the cell, the host may decrease gene delivery efficiency by omitting transduced cells and limiting the re-administrations by generation of neutralizing antibodies. This is the basis for the use of adenoviruses in cancer treatment. Su et al. (2012) synthesized and used an adenoviral-based vector, CDglyTK, for antiangiogenic therapy in breast cancer. Yao et al. used an adenovirus vector conjugated to polyethylene glycol with an additional tumor vascular homing peptide (CGKRRK) tail to target tumor tissue and vasculature after systemic administration. This systemic administration led to a 500-fold higher expression of transgene. Furthermore, the study determined that an Adv-PEG_{CGKRRK} vector has the potential to have suitable efficacy and safety for systemic cancer gene therapy in cases of both primary tumors and metastases (Yao et al., 2011). Adenoviral vectors also have promising indications in the prophylactic treatment of transplant patients, in an attempt to stop rejection due to viral illness. Dasari et al. used the novel adenoviral antigen presentation platform (Ad-MvP) as a tool for rapid generation of multivirus-specific T cells in a single step. Ad-MvP encodes for T cell epitopes from cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, and BK virus, which are all viruses that increase the potential for rejection. Dasari's experiment demonstrated that this vector was

successful in the in vitro expansion of the T cells from transplant recipients. They were also proven highly effective in controlling EBV tumor outgrowth and improving overall survival (Dasari et al., 2016).

13.2.2.4 Adeno-associated viral vector

Adeno-associated viruses (AAVs) are small, naked icosahedral viruses that contain single-stranded DNA. AAV vectors have been shown to transduce cells via both episomal transgene expression and through random chromosomal integration (Soofiyan et al., 2013). This vector has exhibited distinct tropisms for various tissues and organs in a variety of mouse models (Srivastava, 2016). Advantages to the use of AAV vectors include its broad range of hosts with a low level of immune response, and longevity of gene expression; this lack of immunogenicity and pathogenicity is also exhibited in humans. Eukaryotic AAVs are chimeric vectors that most often contain parvovirus and adenovirus. Due to its chimeric nature, the vector has the ability to actively transfect dividing cells and quiescent ones. Trepel et al. used AAV with a therapeutic suicide gene, delivered by a dual-targeted AAV vector, for the treatment of multifocal breast cancer in a murine model. This vector was shown to significantly inhibit tumor growth after a single administration, while avoiding side effects that compared with untargeted vectors (Trepel et al., 2015). Frisch et al. used a recombinant AAV vector containing chondrogenic TGF- β in peripheral blood aspirates, in an effort to develop a convenient tool in the treatment of articular cartilage lesions. This study confirmed that rAAV vectors are stable and safe vehicles for long-term transgene expression by mediating the overexpression of chondrogenic TGF- β . This enhanced the proliferative and metabolic properties of the peripheral blood aspirates, while also increasing the chondrogenic differentiation process in the samples (Frisch et al., 2017).

13.2.2.5 Lentiviral vector

Lentiviral vectors are the newest vector system that establishes permanent integration into the genome of nondividing cells. They are retroviruses that are based upon human immunodeficiency virus-1, simian immunodeficiency, or feline immunodeficiency viruses. Lentiviral vectors can transfer genetic material in very quiescent cells, which can be categorized as nondividing or terminally differentiated (Soofiyan et al., 2013). They have also been found to have a lower risk of insertional mutagenesis that is of concern with other retroviral vectors (Emeagi et al., 2013). Large-scale production of lentiviruses is one of the largest disadvantages of the vector. The major obstacles to up-scaling include complications surrounding the use of stable cell lines, their restrictive nature of adherent human cell lines used in transient infection, and difficulties implementing downstream processes that can efficiently handle large volumes of vector supernatant. Due to these processes, large-scale processing of LV vectors often comes at the cost of maintaining functionality (Segura et al., 2013). The safety of these vectors has been further increased by the development of self-inactivating vectors that

eliminate the transcription of the packaging system required for mobilization (Thomas et al., 2003). Huang et al. (2016) constructed a gamma-synuclein carrying lentiviral vector that established a connection between this protein and the growth and invasion capacity of SW1116 colorectal carcinoma cells. Zhou et al. use a lentiviral vector to silence angiopoietin-2 at both a transcriptional and translational level in a mouse model of pancreatic cell carcinoma. Inhibition of this protein showed down regulation in the expression of VEGF and CD34, therefore exerting an antiangiogenesis effect in vitro and in vivo (Zhang et al., 2013; Zhou et al., 2011).

13.3 TARGETED GENE THERAPY

The systemic administration of either nonviral or viral nanoparticles can result in nonspecific uptake by various organs and tissues within the body, which can ultimately results in systemic toxicity or long-term consequences due to transgene expression. One approach that addresses this problem is to deliver the transgene directly into the tissue of interest or by administering the transgene via isolated perfusion. Nevertheless, both of these approaches are more invasive than venipuncture and are limited by organ or tumor location. Consequently, it is of paramount importance that the transgene be delivered and expressed in the target tissue only. There are a number of strategies that have been developed over the years to address this issue, including modification of viral coat proteins, tumor-specific promoters, and selective biodistribution from conjugating nanoparticles with antibodies or polymers. A full description of all available techniques is beyond the scope of this chapter, and the focus will be on viral coat protein modifications as a method for ligand targeting.

13.4 LIGAND-DIRECTED NANOPARTICLES

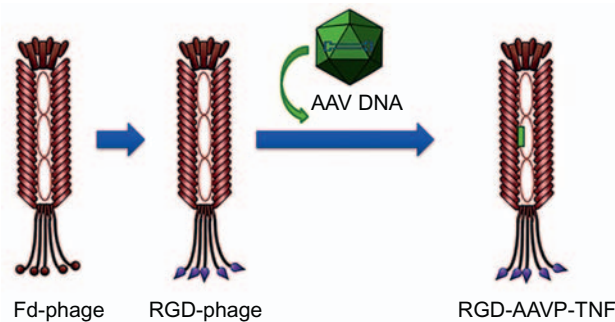
Eukaryotic viruses undoubtedly provided the highest level of transgene delivery and transduction (Kootstra and Verma, 2003); however, in order to modify viral vectors to display targeting ligands, the native tropism for mammalian cells must be ablated to prevent nonspecific uptake (Mizuguchi and Hayakawa, 2004). An alternative approach is to utilize bacteriophage-based nanoparticles, which have no tropism for mammalian cells. Unfortunately, phage particles are notoriously poor vehicles for mammalian cell transduction. Nevertheless, phage particles can be modified using phage display technology to allow for selection of ligand-directed homing peptides to receptors expressed on tumors or tissue of interest (Smith and Scott, 1993).

13.5 ADVANTAGE OF TARGETED GENE THERAPY WITH BACTERIOPHAGE

Bacteriophages (phage) are prokaryotic viruses that infect bacteria, represent a safer and alternative vector strategy for the targeted delivery of therapeutic transgenes (Barrow and Soothill, 1997; Ivanenkov et al., 1999; Larocca et al., 1999). Bacteriophages are known to have no natural tropism for mammalian cells, which reduces the risk of inducing an immune response in the host, and can be engineered to deliver genes directly to such cells. Importantly, unlike eukaryotic viruses, further context modification of the capsid is not required, as the targeted peptides are designed and isolated for specific cell-surface receptors via a phage display peptide library (Pranjol and Hajitou, 2015). Receptor-mediated internalization of bacteriophages by eukaryotic cells has been demonstrated by the display of different phage capsid ligands, such as integrin-binding ligands, transferrin, and fibroblast growth factors (Larocca et al., 1998). Systemic administration of bacteriophage particles has been used therapeutically in patients with antibiotic-resistant septicemia, has been used as antibiotic therapy during the preantibiotic era (Weber-Dbrowska et al., 2003), and can be safely administered to immunocompromised patients with minimal toxicity (Borysowski and Górski, 2008). Additionally, the US Food and Drug Administration (FDA) in 2006 approved the use of bacteriophage preparations as antibacterial food additives against *Listeria monocytogenes* for ready-to-eat poultry and meat products (Lang, 2006). Bacteriophages are, however, limited by their poor ability to efficiently express transgenes inside a eukaryotic cell, as they have evolved to selectively infect bacteria only. The development and design of a hybrid prokaryotic-eukaryotic viral vector that retains both the safety and targeting flexibility of bacteriophages and the transductional capacity of eukaryotic viral vectors, has addressed this limitation (Pranjol and Hajitou, 2015).

13.6 HYBRID ADENO-ASSOCIATED VIRUS/PHAGE VECTOR

The development of the adeno-associated virus/phage (AAVP) hybrid nanoparticle involves the incorporation of cis-elements, such as inverted terminal repeats, into a chimeric genome containing a CMV-driven transgene cassette. The addition of flanking inverted terminal repeats, derived from AAV serotype 2 and the genome of the M13 filamentous bacteriophage, has been shown to improve post-targeting transgene expression and transduction efficiency (Hajitou et al., 2006b, 2007). The phage genome encoding the capsid is modified, allowing for the incorporation of the target ligand within the pIII minor coat gene and subsequent expression of mature hybrid nanoparticles on the bacteriophage capsid, as shown in Fig. 13.1 (Hajitou et al., 2007). This vector has been shown to demonstrate superior gene delivery, when compared to a phage vector with long-term

**FIGURE 13.1**

The wild-type Fd-Phage particle undergoes a modification of its pIII minor coat gene to express the targeting ligand (e.g., RGD motif). In a subsequent step, the AAV DNA containing the gene of interest is incorporated into the modified RGD-Phage particle forming the hybrid AAVP-phage nanoparticle.

transgene expression *in vivo* following systemic delivery (Arap et al., 1998). A summary of the studies that have been published are included in Table 13.2.

Until recently, transgene expression by AAVP was driven by a CMV promoter in order to drive constitutive expression of transgenes. In one recent study, the introduction of a eukaryotic tumor-specific promoter of the glucose regulated protein 78 (Grp78) was studied to further improve AAVP efficacy at the genome level (Kia et al., 2013). Importantly, Grp78 is known to be selectively induced in tumors but has activity that is not detectable in normal tissues. It is a stress-inducible gene that supports cell survival, drug resistance; cancer cell chemoresistance increases with stress mediated by cell confluence. This same study found that histone deacetylation and DNA methylation play an important role in long-term gene expression silencing from the parental AAVP bacteriophage gene delivery vector. Although the exact mechanism of viral promoter silencing is unknown, both DNA methylation and histone acetylation play major roles in the regulation of gene expression by improving transcription factor accessibility to gene promoters. Imbalances of acetylated and deacetylated states of histones are found in many cancers, often due to alterations in histone acetyltransferase and histone deacetylase enzyme activity. Therefore, the combination of AAVP with inhibitors of histone deacetylation and DNA methylation, as well as the development of Grp78 guided gene expression, may provide novel strategies to enhance cancer gene therapy and to expand upon the AAVP-phage-derived vector (Rüegg et al., 1998).

Unlike eukaryotic viruses, bacteriophages have not developed any strategies to evade barriers presented by mammalian cells, such as proteasome degradation. Proteasomes are multisubunit enzymes that are responsible for the degradation of different cytosolic proteins and for processing foreign antigens prior to the activation of the cellular immune response (Tanaka et al., 2012). An investigation into

Table 13.2 AAVP Studies to Date

Study	Findings	Reference
A Hybrid Vector for Ligand-Directed Tumor Targeting and Molecular Imaging	AAVP provides superior tumor transduction over phage due to the incorporation of inverted terminal repeats. Positron emission tomography shown to monitor temporal dynamics and spatial heterogeneity of gene expression mediated by AAVP.	Hajitou et al. (2006b)
Design and construction of targeted AAVP vectors for mammalian cell transduction	Protocol that describes the design and construction of ligand-directed AAVP vectors, production of AAVP particles and the methodology to transduce mammalian cells in vitro and then to target tissues in vivo.	Hajitou et al. (2007)
A preclinical model for predicting drug response in soft-tissue sarcoma using AAVP molecular imaging	Reported drug-response monitoring and prediction in a nude rat model of human sarcoma with PET-based imaging of RGD-4C-AAVP delivered <i>HSV1K</i> . Observed specific localization to tumors and that systemic delivery of AAVP- <i>Luc</i> results in efficient and specific gene expression within human soft-tissue sarcoma xenografts.	Hajitou et al. (2008)
RGD-AAVP-TNF- α in vitro	RGD-AAVP-TNF- α vector effectively targeted M21 cells in vitro and induced expression of the cytotoxic TNF- α transgene. Control groups demonstrated no detectable level of TNF- α .	Tandle et al. (2009)
RGD-AAVP-TNF- α in small animal model	RGD-AAVP-TNF- α targets tumor vasculature, induces expression of TNF- α causing tumor cell apoptosis and a decrease in tumor volume. None of the control tissues demonstrated TNF- α expression and there was an increase in tumor volume.	Tandle et al. (2009)
RGD-AAVP-TNF- α in large animal model	Administration of a single dose was well tolerated and escalation of the dose demonstrated only one minor ADR at peak dose that resolved with minimal supportive care, indicating the safety and efficacy of RGD-AAVP-TNF- α therapy.	Paoloni et al. (2009)
Clathrin-mediated endocytosis and subsequent endo-lysosomal trafficking of AAVP	Investigation of the mechanism involved in gene entry and intracellular trafficking of AAVP. Using flow cytometry, confocal/electron microscopy techniques, RNAi and dominant negative mutants, and pharmacological agents, AAVP endocytosis was found to be both dynamin and clathrin-dependent	Stoneham et al. (2012)
Proteasome inhibition in cancer associated with enhanced tumor targeting by AAVP	Inhibition of proteasome improved targeted reporter gene delivery by AAVP in cancer cells in vitro and in tumors in vivo after intravenous vector administration to tumor-bearing mice	Przystal et al. (2013)

(Continued)

Table 13.2 AAVP Studies to Date *Continued*

Study	Findings	Reference
Blockade of IAPs in combination with tumor-targeted delivery of tumor necrosis factor- α	The combination of AAVP-TNF- α and LCL161 resulted in the inhibition of tumor growth and prolongation of survival in mice with melanoma xenografts with synergistic effect and without systemic toxicity. This combination therapy was significantly more effective than either agent alone.	Yuan et al. (2013)
Modulation of immune system with RGD-AAVP-TNF and RT in mouse model of melanoma	Combination therapy reduced intra-tumoral CD11c + cells, while increasing F4/80 + TAMs, suggesting TNF-mediated DC activation and macrophage-mediated ongoing phagocytosis intratumorally. In contrast, there was a reduction of suppressor Tregs in DLNs.	Quinn et al. (2014)
Modulation of extracellular matrix in cancer is associated with enhanced tumor cell targeting by AAVP	Collagenase and hyaluronidase-mediated degradation of tumor ECM were found to affect the composition of collagen, hyaluronic acid, and fibronectin. AAVP diffusion, internalization, and gene expression were enhanced after enzymatic treatment.	Yata et al. (2015)
Oct-AAVP-TNF in vitro	Oct-AAVP-TNF vector effectively targeted NET cell cultures and induced an increased expression of TNF over time. Cultures infected with control did not express TNF.	Smith et al. (2016)
Oct-AAVP-TNF in small animal	Oct-AAVP-TNF effectively targeted NET cells in mice with MEN1 gene mutation. TNF expression was observed in mice injected with targeted vector, but no TNF expression was observed in control mice or control organs.	Smith et al. (2016)
Genistein boosts bacteriophage-mediated cancer cell killing by improving phage-targeted tumor cell transduction	Combination treatment with genistein and AAVP increased targeted cancer cell killing by AAVP-HSVtk in 2D tissue cultures and sD tumor spheroids by protecting AAVP against proteasome degradation.	Tsafa et al. (2016)
Preclinical evaluation of radiation and systemic, RGD-targeted, AAV phage-TNF gene therapy in a mouse model of spontaneously metastatic melanoma.	Combination treatment with RGD-TNF-AAVP and RT results in modification of tumor microenvironment, reduction in metastatic potential, and prolongation of overall survival in a mouse model of melanoma.	Quinn et al. (2017)
Targeted molecular-genetic imaging and ligand-directed therapy in AVPC.	SNTRVAP-AAVP-HSVtk was able to deliver HSVtk to a preclinical model of AVPC using a xenograft model with MDA-PCa-118b cells for theranostic application.	Ferrara et al. (2016)
Toward a transcriptome-based theranostic platform for unfavorable breast cancer phenotypes.	WIFPWIQL-AAVP-HSVtk is able to target Grp78 overexpression in orthotopic IBC models in vivo, with deliver of HSVtk for theranostic utility.	Dobroff et al. (2016)

the efficacy of targeted gene expression and delivery by AAVP to cancer in the presence of the proteasome inhibitors MG132 and Calpain 1 inhibitor LLnL, showed significantly improved reporter gene expression both in vitro and in vivo, along with better tumor cell killing than with the vector alone (Przystal et al., 2013). However, these drugs are not usually clinically applicable and must be used at toxic pharmacological doses in order to be efficient. Other anticancer treatment strategies, such as conventional chemotherapy, have also shown to be efficacious when combined with RGD4C-AAVP gene therapy (Malvicini et al., 2015). In another study, combination treatment of AAVP with genistein, a natural nontoxic dietary plant product with anticancer properties, showed an increase in polyubiquitination of AAVP particles and accumulation of vector genome in the nucleus, which may indicate an advantage in gene expression without the toxic effects of conventional chemotherapy (Tsafa et al., 2016). In addition, treatment with genistein increased Luc reporter gene expression and tumor cell killing, consistent with previous reports that genistein increased AAV2-mediated gene transfer in the human HeLa cervical carcinoma cells. Given that both genistein and AAVP have been demonstrated to cross the blood-brain barrier, this particular combination treatment may have future clinical application for the treatment of brain tumors (Malinowska et al., 2010).

13.7 CONSTRUCTION OF TARGETED ADENO-ASSOCIATED VIRUS/PHAGE VECTORS FOR EUKARYOTIC CELL TRANSDUCTION

The construction of targeted AAVP nanoparticles is made via a two-step process (Hajitou et al., 2007). Briefly, the first step involves the development of a targeted backbone phage vector. Peptide ligands encoded by DNA oligonucleotide sequences are inserted into the SfiI site of the pIII minor coat protein gene of the M13 filamentous bacteriophage (Hajitou et al., 2006a; Parmley and Smith, 1988; Smith and Scott, 1993). This vector produces three to five copies of the specific ligand, such as RGD4C, which binds selectively to α_V integrins on tumor vasculature (Arap et al., 1998; Ellerby et al., 1999; Pasqualini et al., 1997).

In vivo phage display technology is a powerful tool that may be applied to isolate and identify phage clones with the potential to target tissues, diffuse, and achieve internalization in normal tissues after intravenous administration (Hajitou et al., 2006a). These phage clones displaying a specific peptide on its capsid can be used to generate a tissue-targeted AAVP vector for systemic gene therapy. Complex combinatorial peptide libraries contain proteins and ligands that bind selectively to predefined molecular targets. Phage display libraries are designed to consist of polypeptides expressed within the coat proteins of filamentous bacteriophage, such as the minor pIII coat protein which is known to contain as many as

10^9 unique peptide sequences (Hajitou et al., 2006a). Bacteriophages obtained from this in vivo phage display technology do not require any further capsid modification and can be used as targeting vectors for gene therapy.

A recombinant AAV carrying a eukaryotic transgene cassette is then inserted into an intergenomic region of the phage genome to generate the corresponding targeted AAVP vector (Hajitou et al., 2007). This also allows for the construction of nontargeted control vectors, either without ligands or displaying mutant versions of the ligands. In the protocol set by Hajitou, the transgene cassette is controlled by a CMV promoter, flanked by inverted terminal repeats from AAV serotype 2, inserted into the intergenomic region of an M13 bacteriophage clone displaying a cyclic RGD4C ligand on the minor pIII coat protein. Finally, the targeted AAVP nanoparticles are amplified, isolated, and purified from the culture supernatant of host bacteria, such as *Escherichia coli*, as reported (Barbas et al. 2001; Smith and Scott, 1993). Vector particles that remain in suspension are sterile-filtered through 0.45 μm filters and titrated via the infection of host bacteria for colony-counting on Luria-Bertani agar plates under double antibiotic selection and expressed as bacterial transducing units (TU).

13.8 CLINICAL POTENTIAL OF ADENO-ASSOCIATED VIRUS/PHAGE

The utility of AAVP is underscored by its ability to act as a receptor-mediated vector which transduces eukaryotic cells by the binding of a ligand displayed on the phage capsid to a specific receptor. Indeed, the ability to exchange the targeting ligand and transgene, depending on user-defined applications, has demonstrated great clinical promise. This system has potential biological and clinical applications which include molecular-genetic imaging and targeted gene therapy against tumor vasculature that overexpresses $\alpha_v\beta_3$ integrins on tumor angiogenic blood vessels. Several preclinical applications utilized an AAVP hybrid nanoparticle displaying the cyclic RGD4C peptide on its capsid, with TNF α as the transgene, in a human melanoma xenograft study (Tandle et al., 2009), as well as in domesticated dogs with soft-tissue sarcoma (Hajitou et al., 2008). Another promising approach to study the longevity of transgene expression in tumors after systemic administration of AAVP involves targeted molecular imaging. Noninvasive molecular-genetic imaging of spatial heterogeneity and temporal dynamics of transgene expression provides the ability to monitor the efficacy of genetic transfer, and to quantify the magnitude of such expression. In a previous study, targeted AAVP carrying transgenes such as HSVtk, which serve as dual reporter/therapeutic genes, could provide a clinic-ready AAVP-based candidate approach for translation in human cancer and lead to a more personalized cancer gene therapy (Hajitou et al., 2006b).

13.9 MOLECULAR-GENETIC PET IMAGING USING RGD-AAVP-HSVTK

The molecular imaging of marker genes has the potential to assess therapeutic levels of gene transfer and persistence of expression, as well as to quantify the magnitude and spatial distribution of transgene expression (Hajitou et al., 2006b). Currently, although bioluminescence imaging (BLI), which takes advantage of the light emitting luciferase enzymes as a reporter gene, appears to be effective in small animals, there is no evidence that BLI can be adapted to larger animal studies (Coleman and McGregor, 2015). The surrounding tissue significantly attenuates the light emitted from a deep source in classic fluorescence imaging. In theory, the larger the animal model, the more tissue between the light source and the detector, which results in a weaker signal (Contag and Bachmann, 2002). However, the HSVtk reporter gene, when combined with ganciclovir (GCV), can serve both as a “suicide” gene as well as a transgene for clinically applicable positron-emitting tomography (PET) imaging with HSVtk radiolabeled nucleoside analogs such as ^{124}I -FAIU, ^{18}F -FHBG, and ^{18}F -FEAU (Hajitou et al., 2006b). Positron-emitting isotopes, such as ^{18}F or ^{124}I , are incorporated into tracers that can be detected by a PET scanner. The utility of PET as a means to assess transgene expression upon in vivo delivery is underscored by its high sensitivity and quantification.

In theory, PET gives an indirect evaluation of the expression of a therapeutic gene of interest by directly measuring the expression of a PET reporter protein. Scanning of a single subject can be performed multiple times, which allows for the monitoring of transgene expression as well as efficacy of treatment. PET has been extensively studied in small animal models. Micro-PET is an example of a noninvasive system that allows for the acquisition of high-resolution three-dimensional images from mice or rats (Chatziioannou, 2002). HSVtk expression codes for an enzyme that phosphorylates specific radiolabeled nucleoside analogs, which become trapped inside the cell, and can be used for noninvasive localization of adenoviral vector-mediated HSVtk expression in animal models (Jacobs et al., 2001).

In one study, Hajitou used micro-PET in combination with the radiolabeled substrate ^{18}F -FEAU to monitor the spatial heterogeneity and temporal dynamics of HSVtk gene expression in tumor-bearing animals after intravenous administration of the AAVP-HSVtk vector (Hajitou et al., 2006b). DU145-derived tumor xenografted mice were given a single dose of AAVP vector intravenously, and then subsequently given ^{18}F -FEAU intravenously at differing time points in order to carry out the PET imaging. They reported a gradual increase of ^{18}F -FEAU during the first five days of administration, indicating the level of HSVtk transgene expression in tumors after administration of the targeted RGD-AAVP-HSVtk, and gradual stabilization by day 10 (Hajitou et al., 2006b). Importantly, mice that received control nontargeted vectors appeared to only show background levels of

^{18}F -FEAU. Mice were then treated daily with GCV when a plateau of HSVtk expression was achieved within the tumor. Meanwhile, tumor viability was monitored using an intravenous injection of ^{18}F -FDG with serial PET. The results showed that tumors were ultimately viable, and actively accumulated ^{18}F -FDG prior to GCV treatment, while experiencing significant regression and metabolic suppression (noted by a decrease in accumulation of ^{18}F -FDG) after GCV injection in mice that had received the targeted RGD-AAVP-HSVtk vector.

From the results, it is clear that ^{18}F -FEAU may be used in conjunction with PET in order to detect cells that express HSVtk after systemic delivery of targeted AAVP. This can be extrapolated to a molecular prediction of drug response in preclinical models of cancer using AAVP-mediated PET scan of *HSVtk* expression. Interestingly, similar results were obtained in another study using soft tissue sarcoma xenografts in nude rats (Hajitou et al., 2008). Vascular-targeted molecular-genetic imaging and its ability to monitor the specificity of HSVtk delivery and to predict tumor response after GCV treatment is an important application for translation to human cancer. The presented studies suggest that targeted AAVP can be integrated with PET imaging to predict tumor response to cancer therapies.

Aggressive variant prostate cancer (AVPC) is a clinically defined tumor with neuroendocrine or small-cell differentiation, visceral metastases, low prostate-specific antigen, androgen receptor insensitivity, and poor response to androgen-deprivation or platinum-based chemotherapy. These tumors are characterized by poor patient survival, for which limited diagnostic and treatment options are currently available. As a result of the increasing incidence of AVPC, the development of molecular imaging tools and specific therapeutic approaches tailored to AVPC are required. Altered glucose metabolism of cancer cells, as well as glucose starvation in poorly vascularized tumors, are associated with an increase in Grp78 expression and translocation to the cell surface. In one study, Grp78-targeting AAVP-HSVtk vectors were designed to bind to phage-display-selected ligands, such as the SNTRVAP motif (Ferrara et al., 2016). Here, the HSVtk gene was used to act as both a noninvasive molecular-genetic imaging sensor/receptor in the presence of a radiolabeled substrate, and a cell-suicide-inducing transgene in the presence of GCV. Daily treatments with either saline or GCV were started 5 days after intravenous administration of AAVP particles, which continued for 7 days. At 24 h after the last treatment, mice received ^{18}F -FDG to assess tumor viability as a function of glucose metabolism by PET-CT. Expression of the HSVtk transgene was evaluated after another 24 h by PET, immediately after intravenous administration of ^{18}F -FEAU. Quantification of ^{18}F -FEAU confirmed that HSVtk tumor expression in mice receiving Grp78-targeted AAVP particles was significantly higher than in mice receiving control AAVP particles ($P < 0.0001$). PET-CT scans demonstrated decreased tumor size in Grp78-targeted AAVP-administered mice treated with GCV, with an average 50% reduction in tumor size. This study ultimately demonstrated evidence of a successful

application in relevant preclinical models of untreatable and hard-to-diagnose aggressive tumor variants and potential translation into urologic oncology.

Inflammatory breast cancer (IBC) is one of the more lethal presentations of breast cancer and a major cause of death in women. It is characterized by diffuse erythema/edema involving the skin of the breast, a *peau d'orange* appearance, and tumor emboli within the dermal lymphatics. The presence of systemically accessible tumor and vascular endothelial cell-specific surface receptors allows for the use of targeted delivery of active imaging agents or therapeutic drugs. The possibility of enhancing drug efficacy and reducing side effects by concentrating an active agent at the tumor site is a promising alternative for aggressive breast cancer therapy; however, only a narrow group of tumor-specific receptors have been identified. Among these proteins, Grp78, which is classically involved in an endoplasmic reticulum-linked stress response mechanism that provides survival signals during environmental and physiologic stress, is a promising target. The same group that studied the use of Grp78 targeting AAVP-HSVtk vector for the imaging and diagnosis of AVPC has extended applications of that study to IBC (Dobroff et al., 2016). Their data showed a significant decrease in tumor growth for animals treated with Grp78-targeted AAVP with a mean reduction in tumor volume of 46%. To determine whether cell-surface associated Grp78 targeting could be exploited for in vivo imaging, Grp78-targing particles were conjugated to an infrared dye (RDye 800CW) and administered intravenously to orthotopic SUM190 IBC-bearing mice. Phage particles were imaged by whole-body NIR fluorescence and revealed specific localization of Grp78-targeted particles to established IBC xenografts. Quantification of the signal at the tumor site showed a significant threefold increase in fluorescence that persisted up to 72 h. Collectively, these results confirm the reliability of in vivo imaging and efficacy as a GCV-based targeted therapy in a preclinical IBC model.

13.10 TARGETING TUMOR VASCULATURE WITH RGD-TARGETED ADENO-ASSOCIATED VIRUS/PHAGE VECTOR-MEDIATED TUMOR NECROSIS FACTOR- α

Tumor angiogenesis is defined as the proliferation of a network of blood vessels which supplies a tumor with a supportive microenvironment rich with oxygen and nutrients to sustain optimal growth. The development of new vessels via angiogenesis is characterized by a unique set of integrins that differs from the integrins present on quiescent blood vessels (e.g. $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$) (Pasqualini et al., 1997). The inherent molecular diversity between abnormal and normal tumor vasculature allows for the specific targeting of neoangiogenic blood vessels, which represents an important target for the delivery of antivascular therapies. In addition, it has been shown that up to 100 tumor cells are supplied by a single

endothelial cell (Folkman, 1997). Vascular cells are readily accessible through systemic circulation of the vector, and the use of an AAVP vector engineered to selectively target $\alpha_v\beta_3$ -integrin on tumor-associated vasculature using an RGD (arginine-glycine-aspartic acid) amino acid motif (RGD4C) coupled to TNF- α is of great interest.

The inflammatory cytokine TNF α is known to have both antitumor and anti-vascular effects (ten Hagen and Eggermont, 2003). TNF α is able to induce the production of tissue factor, increase vascular permeability, and has a pro-coagulant effect on endothelial cells (Brett et al., 1989; Friedl et al., 2002; Scarpati and Sadler, 1989). Its role in anticancer therapy involves two distinct and successive actions on tumor vasculature: (1) an increase in vascular permeability, which leads to more efficacious drug penetration within the tumor tissue (Edelman et al., 2006); and (2) the targeted killing of angiogenic endothelial cells associated with neoangiogenesis resulting in tumor vasculature destruction (Lienard et al., 1992). Additionally, TNF- α acts to disturb cell-to-cell adhesive junctions and inhibits $\alpha_v\beta_5$ integrin signaling in tumor-associated angiogenic blood vessels which leads to tumor vascular collapse (Rüegg et al., 2002; Rüegg et al., 1998).

Unfortunately, the systemic utility of TNF- α as an effective therapeutic modality is limited by its severe but predictable toxicity (e.g., hypotension and organ failure) (Lejeune et al., 2006). At maximum therapeutic doses, no meaningful antitumor activity was noted when TNF- α is delivered systemically. However, in isolated limb and organ perfusions where the effect of TNF- α is limited to that of the perfused tissue, TNF- α has been shown to be an effective antitumor treatment. In one study, isolated hepatic perfusion of TNF- α together with melphalan resulted in a significant reduction and regression of advanced refractory cancers confined to the liver (Alexander et al., 2000). Regardless, most tumors cannot be treated in an isolated perfusion setting, and thus the alternative antiangiogenesis gene therapy using the novel hybrid RGD-AAVP-TNF- α is an exciting and promising modality with great clinical potential.

13.11 RGD-TARGETED ADENO-ASSOCIATED VIRUS/PHAGE VECTOR-MEDIATED TUMOR NECROSIS- α IN VITRO ASSAY

Targeted gene therapy delivers antivasculature and antitumor agents directly to the cell type of interest through the expression of a surface ligand, which has affinity for a specific receptor present on the cell surface of the target tissue. The selectivity of gene therapy prevents systemic toxicity due to limited nonspecific uptake. The efficacy of the engineered RGD-AAVP-TNF- α vector was examined using a human melanoma cell line expressing α_v on its surface. Melanoma M21 cells were grown in vitro and subsequently infected with RGD-AAVP-TNF- α or one

of the controls (vehicle, RGD-AAVP-MCS or nontargeted fd-AAVP-TNF- α) (Tandle et al., 2009). After the M21 cells were infected, the production of TNF- α gene product was measured over the duration of 30 days using an enzyme-linked immunosorbent assay (ELISA) (Tandle et al., 2009). Only the RGD-AAVP-TNF- α infected cells had measurable production of TNF- α , whereas each of the control groups demonstrated no detectable level of TNF- α . Additionally, the amount of gene product produced by the RGD-AAVP-TNF- α infected cells increased over the thirty day period indicating the long-acting properties of the targeted AAVP.

13.12 PRECLINICAL TRIAL IN SMALL ANIMAL MODEL WITH RGD-ADENO-ASSOCIATED VIRUS/PHAGE-TUMOR NECROSIS FACTOR- α

Systemic administration of RGD-AAVP-TNF- α , a novel hybrid vector, permits selective targeting of tumor-associated blood vessels. Due to successful targeting observed during the in vitro study, the efficacy of the vector was subsequently examined within athymic nude mice. Human melanoma cells were inoculated into the right flank of each mouse and afterwards the tumor-bearing mice were administered RGD-AAVP-TNF- α or one of the controls (RGD-AAVP-MCS or fd-AAVP-TNF- α), intravenously (Tandle et al., 2009). The mice were euthanized at established time intervals; tumor tissue and normal tissue (liver, lung, kidney, brain, heart, spleen, and skeletal muscle) was resected and frozen for further analysis. The presence of viral particles within the resected tissue was detected using dual immunofluorescence (IF) staining. The mice that were injected with RGD-AAVP-TNF- α and RGD-AAVP-MCS were found to have vector trafficking to tumor vasculature but none of the mice injected with the nontargeted vector (fd-AAVP-TNF- α) showed presence of AAVP within tumor vasculature (Tandle et al., 2009). Results demonstrated the efficacy of the RGD ligand to target the vector to the tumor vasculature. Analysis of the normal tissue specimens revealed the presence of AAVP particles within the liver and spleen; however, the AAVP particles did not colocalize with blood vessels within these tissues. Additionally, no TNF- α secretion was detected in these normal tissues.

Targeted therapy with RGD-AAVP-TNF- α stimulates the production and secretion of TNF- α , which induces apoptosis in tumor endothelial cells and consequently limits tumor growth. Mice that were administered RGD-AAVP-TNF- α were found to have significant expression of TNF- α within tumor cells, whereas mice treated with either RGD-AAVP-MCS or fd-AAVP-TNF- α had no tumor expression of TNF- α . The presence of apoptotic marker, caspase 3, was observed via IF staining in tissue samples treated with each of the three vectors. Tissue from mice treated with RGD-AAVP-TNF- α was positive for caspase-3 staining within the tumor-associated blood vessels as well as in the local tumor tissue, conversely the tissue samples treated with either RGD-AAVP-MCS or

fd-AAVP-TNF- α were negative for caspase-3 staining. Additionally, the effect of RGD-AAVP-TNF- α on tumor growth was analyzed and the reduction in tumor volume within this cohort became statistically significant compared to the cohorts treated with either RGD-AAVP-MCS or fd-AAVP-TNF- α , on day 20 of the observation (Tandle et al., 2009). This result indicates both the tumor suppressive capability of the RGD-AAVP-TNF- α vector therapy, as well as the long-lasting effects of tumor suppression.

13.13 PRECLINICAL TRIAL IN LARGE ANIMAL MODEL WITH RGD-ADENO-ASSOCIATED VIRUS/PHAGE-TUMOR NECROSIS FACTOR- α

In order to assess both the safety and efficacy of targeted gene therapy using the RGD-AAVP-TNF- α vector, a trial in large animals had to be performed. Evaluating the effects in large animals with intact immune systems (compared to the nude mice) is a necessary step before clinical trials can move into the realm of treating human patients. Additionally, this study examined both the toxicity and efficacy (therapeutic index) of the vector therapy within companion dogs with spontaneous tumors, providing an opportunity to significantly enhance the comparative value to human subjects (Paoloni et al., 2009).

The multicenter study consisted of two phases: the first demonstrated the results of dose escalation and single-dose safety, while the second demonstrated the targeting and delivery efficacy of the vector (Paoloni et al., 2009). In the first phase of the trial the dogs received one of five different RGD-AAVP-TNF- α doses ranging from 4×10^{11} to 1×10^{13} TUs, followed by surgical resection 4 days after administration. An additional six dogs were treated with either the lowest dose or the highest dose, followed by tumor resection within 4 h of vector administration. Single-dose administration of RGD-AAVP-TNF- α was well-tolerated, except for one significant adverse reaction that occurred during the intravenous infusion of the highest dose cohort. The event involved transient nausea, hypotension, and tachycardia that subsequently resolved with minimal supportive care.

The second phase of the trial involved staining and analysis of tissue specimens taken from tumor and normal tissue, both before and after the administration of the RGD-AAVP-TNF- α vector (Paoloni et al., 2009). The results of the second phase of the trial demonstrated clear colocalization of RGD-AAVP-TNF- α particles within the tumor vascular endothelium and no particles observed within blood vessels of normal tissues; these results were observed in specimens from both 4 h and 4 days posttreatment. The localization of the RGD-AAVP-TNF- α particles was determined via an IF staining mechanism. The specificity of the IF staining assay was supported by the lack of staining observed in any of the pretreatment biopsies. The results of both phase one and phase two of this study demonstrate the safety and efficacy of the RGD-AAVP-TNF- α vector-mediated antiangiogenic cancer therapy.

13.14 OCTREOTIDE-TARGETED ADENO-ASSOCIATED VIRUS/PHAGE VECTOR-MEDIATED TUMOR NECROSIS- α IN VITRO ASSAYS

Patients with inoperable, unresectable, or metastatic pancreatic neuroendocrine tumors (PNETs) have few treatment options available. However, it has been discovered that PNETs often overexpress receptors for somatostatin and its analogs, which could be utilized for a ligand-directed therapy involving an AAVP vector (Smith et al., 2016). Although the half-life for native somatostatin inhibits its therapeutic potential, the synthetic analog octreotide has been developed offering a more promising therapeutic outlook.

AAVP functionality necessitates an initial ligand-receptor binding step, followed by internalization of genetic material, and finally genomic integration and expression of the transgene (Smith et al., 2016). The first step was analyzed by culturing human PNET-derived cells and infecting them with either the ligand-directed Oct-AAVP-TNF vector or the untargeted control AAVP-TNF. Using biopanning and rapid analysis of selective interactive ligands (BRASIL) methodology, a fivefold increase in binding of Oct-AAVP-TNF to the PNET cells was observed compared to AAVP-TNF.

Subsequently, the ability of Oct-AAVP-TNF to internalize within target cells was studied. Both Oct-AAVP-TNF and the untargeted control AAVP-TNF were cultured with the PNET cell populations and were afterwards stained using IF to determine cellular internalization. Only the Oct-AAVP-TNF demonstrated an intracellular IF staining pattern at both the 2 and 24 h time points, while the AAVP-TNF demonstrated a lack of IF, indicating no internalization at either time period studied (Smith et al., 2016).

Finally, the ability of the Oct-AAVP-TNF to integrate and express the transgene within the PNET target cell was examined. The tumor cell culture medium was collected every other day over a 10 day period and the collected specimen was analyzed for TNF expression using an ELISA. Results of this study demonstrated a marked increase in the expression of TNF over time in tumor cells treated with Oct-AAVP-TNF particles; however, no expression was observed within the untargeted control groups. Indicating the ability of Oct-AAVP-TNF to efficiently target PNET cells, internalize and integrate the transgene into the cellular genome to be effectively expressed within tumor vasculature.

13.15 PRECLINICAL TRIAL IN SMALL ANIMAL MODEL WITH OCT-ADENO-ASSOCIATED VIRUS/PHAGE-TUMOR NECROSIS FACTOR- α

Since patients diagnosed with both hereditary and sporadic pancreatic NETs often bear an *MEN1* gene mutation, a mouse model was studied in which the multiple

endocrine neoplasia syndrome type 1 was induced in the mice examined (Jiao et al., 2011). This syndrome, associated with pancreatic NETs, is characterized by insulin-secreting tumors and by inducing this syndrome in the mice used in the trial, the tumor microenvironment closely resembled that of pancreatic NETs within human patients (Smith et al., 2016).

To evaluate the transduction capacity of Oct-AAVP-TNF in the mouse model, transgene expression was quantified in recovered tissue by analysis of both the protein and RNA. TNF expression was analyzed using an ELISA and was solely observed in the MEN1 transgenic mice, no expression was observed in the control mice or in the control organs from either experimental group (Smith et al., 2016).

Additionally, the intermediate results of Oct-AAVP-TNF vector therapy were examined in the preclinical setting. A single dose of either Oct-AAVP-TNF or untargeted control AAVP-TNF was administered in the Men1 mouse model. Imaging of the tumors was performed using MRI, both before treatment and 28 days after treatment administration. All five tumors in the Oct-AAVP-TNF treatment cohort decreased significantly in size (by $73 \pm 21\%$; $P < 0.01$), whereas all three tumors in the untargeted control cohort more than doubled in size, by as much as $116\% \pm 29\%$ ($P < 0.05$) (Smith et al., 2016). Therefore, the targeted Oct-AAVP-TNF vector was able to efficiently target in vivo PNET cells as well as induce tumor regression over a sustained period of time.

Subsequently, the long-term results of Oct-AAVP-TNF vector therapy were examined. Both MEN1 and control cohorts of mice were injected weekly with either Oct-AAVP-TNF or the control AAVP-TNF over a 2-month period. Mice injected with Oct-AAVP-TNF showed a substantial decrease in serum insulin levels produced by the PNET compared to mice injected with AAVP-TNF. These results demonstrate the ability of the Oct-AAVP-TNF therapy to reduce tumor-associated metabolic burden, indicating the significant clinical relevance of the targeted therapy. The efficacy of repetitive AAVP vector administration is likely the result of additional transduction of the transgene into new cells as well as a compounding the effect of cytotoxic TNF expressing cells on neighboring cells (Smith et al., 2016). Overall, these preclinical studies demonstrate the necessity for further research in order to determine the optimal dosing schedule so that this promising antitumor therapy can be translated for use in human patients suffering from PNETs with few treatment options available.

13.16 TOXICITY AND PRECLINICAL SAFETY OF ADENO-ASSOCIATED VIRUS/PHAGE-TUMOR NECROSIS FACTOR- α

Both the targeted RGD-AAVP-TNF- α and the Oct-AAVP-TNF vectors resulted in direct delivery to target tissue with sustained expression of TNF- α , induction of apoptosis in tumor vascular endothelium and significant inhibition of tumor

growth. There was no evidence of systemic toxicity or viral trafficking to normal tissues observed in either the small or large animal multidose studies. No significant adverse reactions or safety concerns were observed within the small animal study involving Oct-AAVP-TNF vector therapy (Smith et al., 2016). However, there is a need for future studies to examine possible safety concerns with the Oct-AAVP-TNF vector using a large animal model, which more closely resembles the conditions present within the human population.

The only significant adverse reaction noted within the RGD-AAVP-TNF- α vector therapy was a grade 3 hypersensitivity reaction among the highest dose cohort in the large animal study; however, all reactions were transient and resolved after a temporary pause (<30 min) and minimal supportive care was administered. Additionally, 36% of the large animals in the study developed a grade 3 or 4 nonneutropenic fever that also resolved with symptomatic care. The ability to reinstate administration of the vector at a slower intravenous administration rate without further complications indicates the transient nature of the reaction and the minimal safety concern associated with the reaction (Paoloni et al., 2009).

13.17 COMBINATION ADENO-ASSOCIATED VIRUS-MEDIATED GENE THERAPY WITH RADIATION THERAPY

Gene therapy has rapidly advanced to the forefront of modern medicine and is a promising modality through which to treat a variety of diseases, including cancers that are refractory to existing therapeutic approaches. As previously discussed, AAV vectors have been shown to successfully deliver a variety of targeted therapeutic genes to cancer cells, and are superior to other vectors as vehicles for cancer gene therapy. However, there are various challenges to this type of gene therapy, such as barriers that prevent efficient transduction of the vector that limit its use and efficacy as monotherapy (Russell et al., 1994). As such, research efforts have been focused on developing multimodal treatment solutions that include AAV gene therapy. Notably, combination radiation therapy (RT) and AAV-mediated gene therapy has been of paramount interest, and has been shown to exhibit complementary, synergistic effects between the two modalities.

AAV vectors preferentially transduce cells that are in the S phase of the cell cycle, for reasons that remain undefined (Russell et al., 1994). Outside of the S phase, nondividing cells can still be transduced, but AAV vector transduction occurs at a 200-fold lower frequency. In order to improve the transduction efficiency of AAV vectors, DNA-damaging agents can be utilized to increase the efficiency up to 750-fold (Alexander et al., 1994). This suggests that combination RT and AAV-mediated gene therapy has the potential to enable gene transfer into nondividing cells and amplify the utility of gene therapy. This also serves to highlight the fundamentally synergistic relationship between AAV-mediated gene

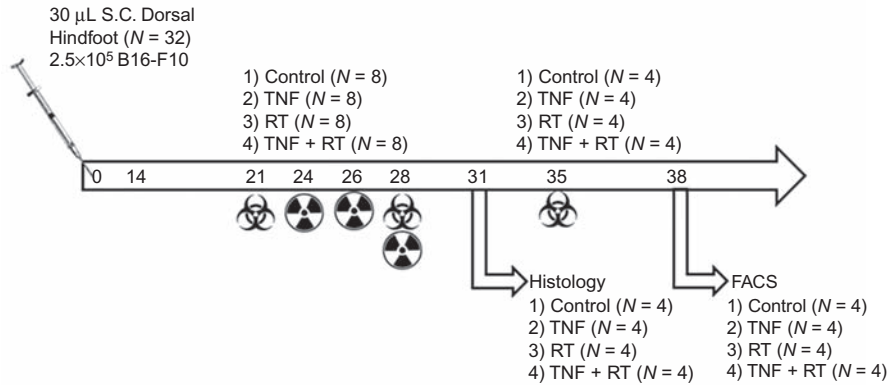
therapy and RT and the utility of this combination therapy as an antitumor modality.

Utilizing radiation-responsive promoters enables a degree of control over the expression of therapeutic genes, including those involved in early tumor cell response to radiation and of which can interact with genes involved in the inflammatory process, cell survival, and tissue recovery (Kaliberov and Buchsbaum, 2012). Indeed, the radiation-inducible promoter region of the early growth response-1 (Egr-1) gene can be linked to a complementary sequence encoding TNF to produce a construct that, when combined with RT, has demonstrated increased tumor cures compared to radiation alone or the construct alone (Weichselbaum et al., 1994). Notably, one study utilized AAV-mediated gene therapy to transduce a radiation-sensitive p21 gene promoter into breast cancer cells (MCF-7), and demonstrated that the transduced cells became sensitized to low dose ionizing radiation (Nenoi et al., 2006). When compared to p21 gene promoters transiently transfected by electroporation, the p21 gene promoters transduced via the AAV vectors were more radiation responsive (evaluated via luciferase expression). These findings suggest that, when compared to other gene delivery modalities, the use of AAV-mediated gene delivery with RT enhances the downstream effects of gene therapy.

13.18 PRECLINICAL EVALUATION OF ADENO-ASSOCIATED VIRUS/PHAGE-TUMOR NECROSIS FACTOR WITH RADIATION THERAPY IN METASTATIC MELANOMA MODEL

The age-adjusted incidence of melanoma in the United States is rising, with an associated increase in overall number of metastatic cases (Howlader N, 2013). Furthermore, metastatic melanoma has a poor prognosis, with more than 95% of patients with intracranial metastases succumbing to disease with 4–6 months of diagnosis (Okwan-Duodu et al., 2015). It is apparent that novel therapeutic interventions are needed. To that end, preclinical research evaluating the combination of RT and AAVP-mediated gene therapy has yielded promising results.

In a study evaluating the efficacy of image-guided radiation therapy (IGRT) combined with targeted TNF gene therapy on spontaneously metastatic melanoma, the addition of RT to RGD-AAVP-TNF vector therapy resulted in a synergistic effect on tumor growth, and further, seemed to prevent the development of distant metastases (Quinn et al., 2014, 2017). B16-F10 murine melanoma cells were grown and injected into mice, which were subsequently randomly divided into four treatment groups ($n = 4$; control, RGD-AAVP-TNF only, IGRT only, Combination RGD-AAVP-TNF plus IGRT) (Fig. 13.2). By day 20 of follow up, all treatment groups showed evidence of tumor growth. Within the RT alone and combination group, there was a delay in tumor growth with primary treatment

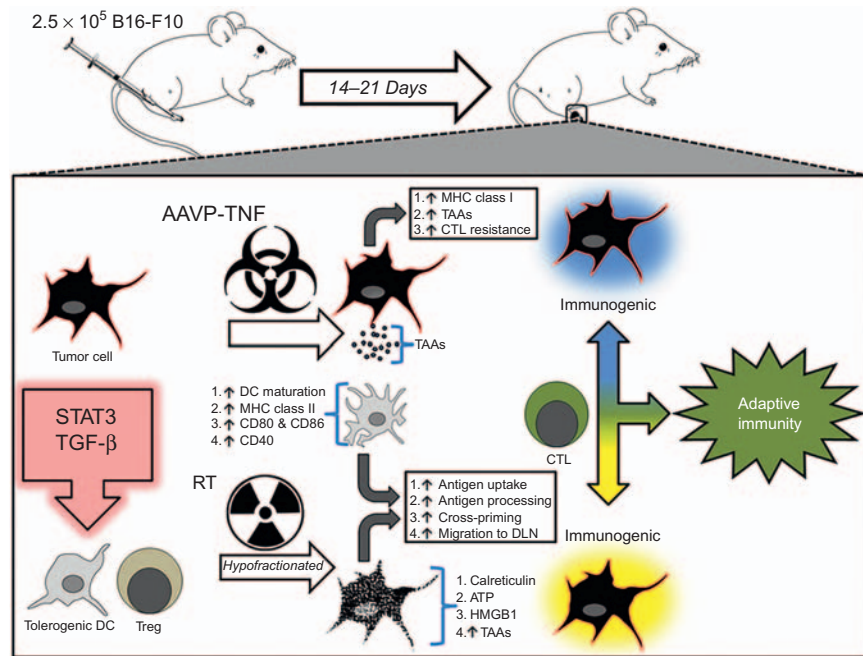
**FIGURE 13.2**

This schema summarizes an experiment in which the effect of combined RGD-AAVP-TNF plus RT was used to investigate changes in the tumor microenvironment. Briefly, mice were treated with either nontargeted Fd-AAVP-TNF alone, targeted RGD-AAVP-TNF alone, RT alone or RGD-AAVP-TNF + RT. The hybrid AAVP particles were administered weekly for up to 3 weeks. RT was delivered every other day for 3 days. Mice were sacrificed at indicated time points to assess for trafficking of nanoparticles and immunomodulation.

failure in the RT alone group. In comparison, the combination therapy group demonstrated sustained control of the primary tumor for at least 120 days. Further, the combination therapy group was the only group to show no evidence of local or distant metastases and no mortality for at least 120 days (Quinn et al., 2017, in press). Comparatively, all other treatment groups showed some amount of both local and distant metastasis, and high mortality rates that suggest that combination therapy significantly ($P < 0.01$) prolonged overall and disease free survival in the mice. Additionally, the amount of fractional tumor growth inhibition within mice treated with combination therapy was greater than the predicted combined effect of the monotherapies, suggesting a synergistic effect of the two therapies. The combination therapy also demonstrated the ability to improve the immune response within the tumor microenvironment (Fig. 13.3).

13.19 FUTURE DIRECTIONS

As previously discussed, the development of AAVP vectors has rapidly expanded the potential utility of gene therapy in the treatment of several diseases. There are still a variety of barriers that challenge the application of AAVP and present more avenues for further research and exploration of AAVP as a treatment modality. One such challenge involves inefficient gene transduction into tumor cells. One study hypothesized that a major contributory factor to this inefficiency is the

**FIGURE 13.3**

RGD-AAVP-TNF is able to target to murine melanoma where it expressed TNF in the tumor and tumor-associated vasculature. TNF acts locally in conjunction with RT to facilitate DC maturation, increase MHC II expression, and activate DCs by increasing CD80/CD86 and CD40. In addition, damage to the tumor cells results in release of tumor-associated antigens, which can be cross-presented by DC to CTL leading to an adaptive immune response.

extracellular matrix of the tumor (Yata et al., 2015). Utilizing a number of tumor cell lines from different species and enzymes collagenase and hyaluronidase, the study degraded the tumor ECM and found that AAVP diffusion, internalization, gene expression, and tumor killing were enhanced. By degrading this tumor barrier, vector transduction was increased, and further exploration with different ECM-degrading enzymes may prove to be fruitful in creating a clinically translatable strategy to improve AAVP-mediated cancer gene therapy.

Further, Stoneham et al. (2012) also sought to understand key transduction obstacles and studied the processes involved in the uptake and trafficking of AAVP. They followed the entry and intracellular trafficking of RGD-AAVP, observing targeted uptake in cells with α_v integrin and endocytosis mediated by both dynamin and clathrin. Human Embryonic Kidney (HEK293) cells either transfected with mutant dynamin or treated with dynasore, an inhibitor of dynamin, resulted in a substantial decrease in RGD-AAVP uptake. This suggests that

dynamin is a requirement for RGD-AAVP endocytosis. Furthermore, siRNAs directed against clathrin heavy chain resulted in an inhibition of AAVP uptake, suggesting that clathrin is also necessary for AAVP vector endocytosis. Further, electron microscope analysis of RGD-AAVP uptake demonstrated sequestration of the vector by the endo-lysosomal degradative pathway, which also decreased transgene expression. Chloroquine, a lysosomotropic agent that has been shown to cause destabilization of late endosomes and lysosomes at high concentrations, was used to disrupt the lysosomes. Consequently, chloroquine treatment was found to substantially increase AAVP transduction efficiency in HEK293 cells, and notably, induce expression in previously nonpermissive DU145 and HeLa cells. Overall, these findings demonstrate another set of barriers—endocytosis and intracellular trafficking through the endosomal-lysosomal pathway—to the otherwise promising use of AAVP vectors, and offer potential solutions to address these barriers. Further exploration into the design of AAVP-mediated gene therapy is necessary, and these findings may help to act as a guide for the design of the next generation of AAVP-mediated gene therapies.

Another study explored strategies to improve vector entry and gene transfer, namely by combining AAVP with various chemical transfection reagents (Donnelly et al., 2015). AAVP vectors were engineered to encode the CMV promoter-driven transgene and the RGD4C tumor targeting peptide. Three classically used transduction reagents, cationic lipids, cationic polymer polyethylenimine (PEI), and calcium phosphate (CaPi), were added to chemically modify the phage capsid. The transduction reagents were added to the AAVP vectors to allow for complex formation before being transduced into HEK293 cell lines. Efficiency of transduction was determined via green fluorescent protein or firefly luciferase reporter genes carried by the vectors. Analysis of luciferase expression demonstrated that gene expression significantly improved with increased concentration of reagents when compared to the vector alone. Further, hybrid vectors modified by PEI in particular were shown to induce higher efficiency of vector transduction, compared to those modified by cationic lipids and CaPi. Complexes of AAVP and CaPi demonstrated a 1300-fold increase in luciferase expression compared to AAVP alone and complexes of AAVP and PEI demonstrated up to a 1500-fold increase compared to AAVP alone.

Finally, after establishing that the use of RGD4C-AAVP vectors and PEI transfection reagent can improve gene transfer, Donnelly et al. demonstrated that this hybrid vector system supported transgene expression that could be sustained for the generation of stable cell lines. HEK293 and human breast cancer MCF-7 cells were transfected with the RGD4C-AAVP vector alone or the hybrid RGD4C-AAVP-PEI complex. Prior to transfection, the RGD4C-AAVP vectors were modified to carry puromycin-resistant gene. Cells were grown on media containing antibiotic puromycin, with successfully transduced cells producing clones. After 14 days, it was found that the RGD4C-AAVP-PEI complex transduced cells grew a significantly higher number of puromycin-resistant clones than those cells transfected with RGD-AAVP alone (18 ± 1 clones per well for hybrid

RGD4C-AAVP-PEI versus 3 ± 1 clones per well for RGD4C-AAVP alone). The resistant clones were then isolated, incubated without antibiotics, and passaged for at least 20 passages. Clones demonstrated maintenance of GFP expression through all passages. This study's findings demonstrate another way in which to improve upon the next generation of AAVP-mediated therapies, especially in its use as a suitable system for the generation of stable cell lines, and suggest that the use of these hybrid phage and transduction reagent complexes should be further explored.

Another study sought to further improve AAVP vector transgene expression, as RGD4C-AAVP vectors driven by the CMV promoter are not specific and undergo silencing by mammalian host cells (Brooks et al., 2004; Grassi et al., 2003). Azadeh et al. inserted the Grp78 eukaryotic tumor-specific promoter, which is selectively induced in tumors, and found that it provides persistent gene expression in cancer cells (Kia et al., 2012). A follow up study then found that DNA methylation and histone deacetylation contribute to long-term gene silencing from the AAVP vector, and consequently, inhibitors of histone deacetylation enhance gene expression in cells (Kia et al., 2013). Together, these studies present novel strategies through which to improve AAVP-mediated cancer gene therapy, perhaps a new multimodal treatment with Grp78-mediated gene expression and histone deacetylation inhibitors, and of which may be a viable candidate for evaluation in clinical trials.

Another limitation in AAVP-mediated gene therapy is its susceptibility to degradation by proteasomes, as bacteriophages likely do not have inherent abilities to evade the obstacles that mammalian cells usually present to foreign agents. Studies have shown that proteasomes have served as barriers against viral vectors, and furthermore, proteasomal activity can be increased in cancers (Monahan et al., 2010; Wu et al., 2010). As such, the efficacy of AAVP-mediated gene therapy in combination with proteasome inhibitors was evaluated and found to be significantly improved both in vitro and in vivo, with increased gene expression and better tumor killing than AAVP therapy alone (Przystal et al., 2013). These findings suggest that another potential barrier to AAVP gene therapy efficacy is proteasomal activity, and that the use of antiproteasomal drugs should be further explored for use in combination therapy alongside AAVP-mediated cancer gene therapy.

Finally, another area of interest in the development of AAVP-mediated gene therapy is in the enhancement of current AAVP-mediated therapies, namely by the RGD-AAVP-TNF vector. As previously discussed, systemically administered RGD-AAVP-TNF has been demonstrated to deliver targeted gene therapy and prevent systemic toxicity, but the effect on tumor growth and survival was relatively modest (Paoloni et al., 2009; Tandle et al., 2009). One study sought to augment the pro-apoptotic effects of TNF, and combined AAVP-TNF with LCL161, an orally bioavailable mimetic of Smac (pro-apoptotic protein) that induces proteasomal degradation of cellular inhibitors of apoptosis (IAPs) proteins (Yuan et al., 2013). LCL161 and TNF were observed to have a synergistic effect,

enhancing tumor growth inhibition and delaying the development of treatment resistance. Importantly, the combination of AACP-TNF plus LCL161 showed the highest levels of activated caspases 3 and 9, and led to an increased proportion of apoptotic cells in tumor tissues compared to either treatment alone with 41% on day 7 and 83% on day 21. Survival rate was also significantly improved ($P < 0.0001$), with 100% in the AAVP-TNF plus LCL161 group, 25% in AAVP-only group, and 0% for both the LCL161 alone group and control group. Notably, the combination therapy did not increase systemic toxicity in the treatment group. The study findings suggest that further studies on the potential clinical use of combination AAVP-TNF and LCL161 therapy are warranted.

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Delivering miRNA modulators for cancer treatment

14

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CHAPTER OUTLINE

14.1 Introduction	517
14.2 The Therapeutic Potential of miRNA Modulators in Cancer	525
14.3 MicroRNA Modulators Delivery.....	526
14.3.1 Principal Barriers	526
14.3.2 Viral Vectors.....	527
14.3.3 Nonviral Vectors	533
14.4 Clinical Trials and Future Perspectives	548
14.5 Conclusions.....	549
References	550

14.1 INTRODUCTION

It is well known that cancer results from genetic and/or epigenetic alterations in protein coding genes, oncogenes, tumor-suppressor genes, genes involved in programmed cell death, and genes involved in DNA repair. These alterations, particularly in solid tumors, usually result from somatic genetic events occurring over long time periods. Additionally, germ-line mutations may predispose a person to heritable or familial cancer (Croce, 2008; Taberlay et al., 2016).

In 1993, the group of V. Ambros found a gene (Lin 4) which affects the development of the worm *Caenorhabditis elegans* and which codes for a small noncoding RNA, considered the first microRNA (miRNA or miR) to be identified

(Lee et al., 1993). Other miRNA molecules have been discovered since then and currently more than one hundred have been identified in the mammalian genome (Price and Chen, 2014). The miRNAs are single-stranded small noncoding RNAs, with 21–23 nucleotides, which are involved in gene expression by causing either translation interruption or post-transcriptional degradation of targeted mRNAs (Bartel, 2004). Fig. 14.1 is a schematic representation of the miRNA processing.

Since their discovery, the knowledge about miRNAs in eukaryotic organisms has grown exponentially and it is currently well known that these molecules play an important role in the regulation of gene expression. Dysregulation of miRNAs expression has been found in several diseases [revised in Piletic and Kunej (2016)] and some miRNAs were found aberrantly expressed in some cancers (McManus, 2003; Calin et al., 2002; Michael et al., 2003). Indeed, the mapping

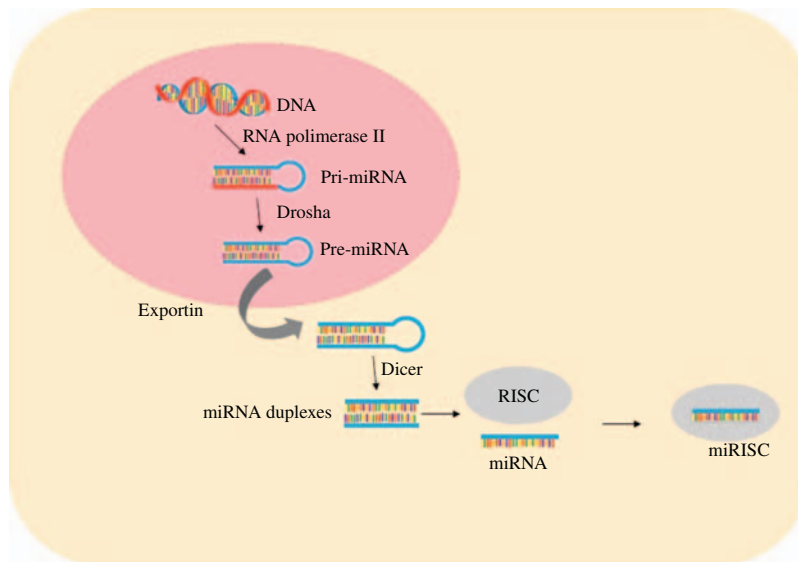


FIGURE 14.1 Representation of miRNA processing.

Micro-RNAs are firstly processed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II. These pri-miRNAs originate the precursor miRNAs (pre-miRNAs) by the action of Droscha, a nuclear RNase III enzyme. The pre-miRNAs are then transported from the nucleus to the cytoplasm, where they are processed by Dicer, an RNase III-familial endonuclease, into miRNA duplexes. The mature miRNAs are then loaded into the RNA-induced silencing complex (RISC). The miRNA-RISC complexes (miRISC) are able to regulate gene expression by binding to their target mRNA transcripts, typically to their 3' UTR or other genic regions (5' UTR, promoter or coding sequences) (Wahid et al., 2010; Carthew and Sontheimer, 2009). The attachment of miRISC complex to the targeted mRNA results in transcript degradation (if perfect complementarity exists) or translational repression (if only partial complementarity exists). These mechanisms are carried out by Argonaute proteins and result in gene silencing (Bartel, 2009; Meister et al., 2004).

of the chromosomal location of miRNA genes showed that many miRNA genes are in chromosomal regions frequently involved in chromosomal alterations (such as deletions and amplifications) found in many types of cancer (Croce, 2009; Rutnam and Yang, 2012). Furthermore, the expression of miRNAs may be affected by genetic changes (such as deletions, gene amplifications or mutations), by transcription factors and by epigenetic changes such as methylation of their promoter's CpG islands (Croce, 2009).

Defects in components of the miRNA biogenesis pathway may also cause expression changes in many miRNAs. Indeed, the enzymes involved in miRNA processing, as is the case of Droscha and Dicer, may be either increased or decreased in various types of cancer, causing abnormal levels of miRNAs (Hata and Kashima, 2016).

It has been proven that miRNAs may play an important role in the initiation and development of numerous cancers, having oncogenic potential (Price and Chen, 2014). These miRNAs, which act as oncogenes to promote tumorigenesis and tumor progression, by downregulating tumor-suppressor proteins, are called oncomiRNAs (Rutnam and Yang, 2012) (Table 14.1). Examples of oncomiRNAs are miRNAs that regulate the expression of enzymes involved in the methylation of tumor-suppressor genes, resulting in their silencing and contributing to malignant cellular transformation (Kaur et al., 2016; Wilting et al., 2016).

Other miRNAs have potent antitumor properties, by having a critical tumor-suppressor role and their overexpression is related to good prognosis. These are called tumor-suppressor miRNAs (Table 14.2). For example, miR-29 targets and downregulates the expression of two DNA methyltransferases, DNMT3A and 3B (DNA methyltransferase 3 α and 3 β), whose overexpression is a poor prognostic indicator. Conversely, addition of miR-29 to lung cancer cell lines promoted demethylation of CpG islands with a consequent loss of tumorigenicity (Fabbri et al., 2007).

p53 mediates tumor suppression by activation of a large number of downstream targets, promoting multiple cellular processes to collectively suppress tumor formation (Lane and Levine, 2010). Micro-RNAs have been recognized as important components of the p53 pathway (Lin et al., 2012). One of the best studied miRNAs involved in p53 action is miRNA-34 (a tumor-suppressor miRNA which is a direct p53 target). The overexpression of miRNA-34 stimulates p53 downstream effects including cell-cycle arrest, apoptosis, senescence, and suppression of the EMT (Kim et al., 2011; Chang et al., 2007; He et al., 2007). These effects are mediated by post-transcriptional repression of miRNA-34 targets such as cyclin D1, cyclin E2, cyclin-dependent kinase 4 (Cdk4), c-Met, c-Myc, B-cell lymphoma 2 (Bcl2), and Sirt1, which promote multiple oncogenic process (Hermeking, 2010). Consequently, low expression of miRNA-34 was found in multiple human cancers, which suggest that miRNA-34 acts as an important component of the tumor suppression system (Okada et al., 2014).

The double role of miRNAs in cancer (as oncomiRNAs or as tumor-suppressor miRNAs) is partly attributed to the ability of miRNAs to target different genes in different tissues (Hemmatzadeh et al., 2016). Indeed, the same miRNA may play different roles in different tissues. For example, miRNA-181

Table 14.1 Examples of OncomiRNAs Overexpressed in Cancer

miRNA	Cancer	Function	References
miRNA-9	Laryngocarcinoma	Downregulates phosphatase and tensin homolog (PTEN) gene, a tumor-suppressor gene, accelerating cell growth and metastasis	Lu et al. (2016)
	Prostate	Targets E-cadherin and suppressor of cytokine signaling 5 (SOCS5)	Seashols-Williams et al. (2016)
miRNA-10b	Osteosarcoma	Downregulates Kruppel-like factor 4 (KLF4), promoting proliferation of osteosarcoma cells	Wang et al., (2016c)
	Breast	Inhibits the expression of the transcription factor T – box-5 (TBX5), leading to repression of the tumor-suppressor genes dual-specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) and PTEN	Kim et al. (2016)
miRNA-21	Cervical, gastric, hepatocellular carcinoma (HCC), intrahepatic cholangiosarcoma, colorectal, chronic myeloid leukemia	Downregulates the tumor-suppressor gene PTEN to promote cell proliferation and downregulates the Sprouty 2 (Spry2) (in the case of colorectal cancer)	Peralta-Zaragoza et al. (2016) , Zhang et al. (2012) , Meng et al. (2007) , Wang et al. (2015e) , Zhang et al. (2010a) , Feng et al. (2011) , Bai et al. (2011)
miRNA-30	Gastric	Inhibits apoptosis through p53/ROS-mediated regulation of the mitochondrial apoptotic pathway	Wang et al. (2016b)
miRNA-93	Gastric	Downregulates expression of programmed cell death 4 (PDCD4), a tumor-suppressor gene, suppressing apoptosis of cancer cells	Liang et al. (2016)
miRNA-181a	Ovarian	Increases epithelial-mesenchymal transition (EMT) and decreases paclitaxel sensitivity	Li et al. (2016)

(Continued)

Table 14.1 Examples of OncomiRNAs Overexpressed in Cancer *Continued*

miRNA	Cancer	Function	References
miRNA-181b	Colorectal	Downregulates PDCD4, promoting cell proliferation and migration and suppresses apoptosis	Liu et al. (2016c)
miRNA-223-5p	Vulvar carcinoma	Targets TP63, a protein involved in vulvar cancer, promoting tumorigenesis	de Melo Maia et al. (2016)
miRNA-382-5p	Breast	Targets and repress the RAS-related and oestrogen-regulated growth inhibitor (RERG), that acts as a tumor suppressor	Ho et al. (2016)
miRNA-421	Gastric, biliary tract cancer (BTC), pancreatic, neuroblastoma	Promotes gastric cancer cell growth and prevents apoptosis through inhibition of Caspase-3 expression In BTC, downregulates farnesoid X receptor (FXR), a tumor suppressor Downregulates SMAD family member 4 (DPC4/Smad4), a critical tumor suppressor involved in the progression of pancreatic cancer In neuroblastoma cells downregulates the tumor-suppressor menin	Wu et al. (2014a) , Zhong et al. (2012) , Hao et al. (2011) Li et al. (2014)
miRNA-592	Colorectal	Targets Forkhead Box O3A (FoxO3A), promoting cancer progression and metastasis	Fu et al. (2016)

acts as an oncomiRNA in solid tumors, such as breast, liver and colon cancer, but in acute myeloid leukemia acts as a tumor suppressor ([Neel and Lebrun, 2013](#); [Wang et al., 2015a, 2010](#); [Pichler et al., 2014](#); [Marcucci et al., 2008](#)).

Regarding the oncomiRNAs, besides enhancing tumor progression, they can also be associated with tumor metastasis ([Ma and Weinberg, 2008](#)). For example, miRNA-10b was found to be upregulated in breast cancer tissues as well as in cell lines; this abnormal expression was shown to be closely correlated with the

Table 14.2 Examples of Tumor-Suppressor miRNAs Downregulated in Cancer

miRNA	Cancer	Function	References
miRNA-29b	Multiple myeloma	miRNA-29b expression with a miRNA-29b expression vector (Ad5F11p-miR-29b) induced apoptosis and elevated caspase-3 activation in human multiple myeloma cell lines	Zhang et al. (2011)
miRNA-144-3p	Renal cell carcinoma (RCC)	Downregulates mitogen-activated protein kinase 8 (MAP3K8), an oncogene upregulated in RCC, and suppresses EMT, a key process in the initiation of the metastasis cascade, increasing the expression of the epithelial marker E-cadherin and reducing the expression of the mesenchymal marker Vimentin	Liu et al. (2016a)
miRNA-145-5p and 3p	Lung squamous cell carcinoma	Directly targets metadherin (MTDH) gene and several antiapoptosis and pro-proliferation genes involved in pathways downstream from MTDH	Mataki et al. (2016)
miRNA-192	Osteosarcoma	Regulates T cell-specific transcription factor 7 (TCF7), which promotes oncogenic signaling and is associated with cancer development and progression	Wang et al. (2016f)
miRNA-214	Breast	Downregulates the expression of ring finger and WD domain 2 (RFWD2), a ligase that targets the tumor-suppressor p53 for proteasomal degradation	Zhang et al. (2016d)
miRNA-339 and miRNA-766	Colorectal	Targets the DNA methyltransferase 3 beta (DNMT3B) gene which could promote silencing of tumor-suppressor genes such as secreted frizzled related protein 1 (SFRP1), secreted frizzled related protein 2 (SFRP2), WNT inhibitory factor 1 (WIF1) and dickkopf WNT signaling pathway inhibitor 2 (DKK2)	Afgar et al. (2016)
miRNA-421	Breast	Inhibits metastasis associated 1(MTA1) protein expression, which plays an important role in invasion and metastasis	Pan et al. (2016)
miRNA-451	Acute myeloid leukemia	Promotes apoptosis and inhibits uncontrolled proliferation via direct targeting of the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta/AKT serine/threonine kinase (YWHAZ/AKT) signaling	Su et al. (2016)

(Continued)

Table 14.2 Examples of Tumor-Suppressor miRNAs Downregulated in Cancer
Continued

miRNA	Cancer	Function	References
miRNA-489	Gastric	Targets prospero homeobox 1 (PROX1), a critical modulator of malignant progression of cancer	Zhang et al. (2016a)
miRNA-613	Breast	Negatively regulates vascular endothelial growth factor A (VEGFA) and inhibits proliferation and invasion of breast cancer cells	Wu et al. (2016)
miRNA-634	HCC	Inhibits expression of Ras-related protein Rab-1A (Rab1A), a small GTPase that controls vesicle trafficking from the endoplasmic reticulum to the Golgi apparatus playing an important role in human cancer. Also inhibits DEAH (Asp-Glu-Ala-His) box polypeptide 33 (DHX33), an ATP-dependent RNA helicase essential for RNA metabolism, which is increased in HCC and associated to tumor progression	Zhang et al. (2016b)
miRNA-3622b	Prostate	Suppresses epidermal growth factor receptor (EGFR) Restoration of expression in PCa cell caused reduced cellular viability, proliferation, invasiveness and migration, together with an increase in apoptosis. Overexpression in vivo induced regression of established prostate tumor xenografts	Bucay et al. (2016)
miRNA-9501	Nonsmall cell lung cancer (NSCLC)	Upregulation suppressed cell growth and downregulation significantly suppressed apoptosis	Xi et al. (2016)

aggressiveness of the cancer. In the work of Han and coworkers, miRNA-10b was identified as a target gene of transforming growth factor- β (TFG- β), which is a multifunctional cytokine that induces EMT in multiple cell types, including breast. EMT is a key process in the tumor metastatic cascade, characterized by the loss of cell junctions and polarity, allowing the cells to migrate and invade other tissues. It was found that the inhibition of miRNA-10b reversed the EMT induced by transforming growth factor- β 1 (TFG- β 1), thereby decreasing the invasion and proliferation ability of breast cancer cells ([Han et al., 2014](#)). In a recent study, the antimetastatic effect of miRNA-125b and miRNA-100 was described in HCC, by negatively regulating angiopoietin 2 (Angpt2). Angiopoietin 2 levels are elevated in HCC, being essential for the formation of vessels that encapsulate the tumor cluster (VETC), a prevalent vascular pattern in HCC which facilitates the

entry of the whole tumor cluster into the bloodstream. In the mentioned study, it was observed that restoration of miRNA-125b and miRNA-100 levels in mouse models of human and murine VETC⁺ hepatoma cells significantly reduced the formation of VETC and attenuated metastasis formation (Zhou et al., 2016).

In addition to their important role in tumor initiation and progression, recent studies have documented that some miRNAs may influence response to chemotherapy (Wang et al., 2015c). The resistance to conventional chemotherapy and molecularly targeted drugs is a serious problem, since patients are subjected to an aggressive therapy in terms of adverse effects, without any clinical improvement. The resistance to chemotherapy may be intrinsic, based on genetic characteristics of cancer cells that exist from the time of diagnosis (i.e., before treatment), or can be acquired after treatment with a chemotherapeutic agent (Seca et al., 2014). The most common causes of acquired drug resistance include the expression of energy-dependent transporters (drug efflux pumps) such as P-glycoprotein, deregulation of apoptosis mechanisms (e.g., by overexpression of antiapoptotic proteins), enhanced drug metabolism, increased DNA repair or mutations in drug targets (Chang, 2010; Fojo, 2007; Borst, 2012). Most (if not all) of these pathways may be regulated by miRNAs. For instance, there are various miRNAs which target apoptosis-related proteins and have been described to be associated with drug response. For example, miRNA-15a, miRNA-15b, miRNA-16, miRNA-34a, miRNA-129, miRNA-181b, miRNA-200bc/429cluster, miRNA-497, and miRNA-1915, are miRNAs that downregulate Bcl-2 expression, an antiapoptotic protein, leading to decreased resistance to drugs such as tamoxifen, cisplatin, doxorubicin, etoposide, docetaxel, vincristine, gemcitabine, and 5-fluorouracil, in solid tumors such as colon, gastric, breast, and lung cancer (Bozok Cetintas et al., 2016; Xia et al., 2008; Kojima et al., 2010; Karaayvaz et al., 2013; Zhu et al., 2010, 2012a,b; Xu et al., 2013). In these cases, the overexpression of these miRNAs may be advantageous to promoting drug sensitivity.

There are many cancers in which reduced sensitivity to drugs results from downregulation of certain miRNAs. For example, miRNA-129-5p was significantly reduced in resistant gastric cancer cells due to hypermethylation; in agreement with this, the increase in the levels of this miRNA effectively restored chemosensitivity (Wu et al., 2014b). In a similar way, miRNA-375 was also found to be downregulated in breast cancer cell lines, due to promoter hypermethylation, and the overexpression of this miRNA reverted resistance to adriamycin and paclitaxel (Liu et al., 2016b).

Conversely, in many other cases, the overexpression of some miRNAs leads to chemoresistance; in these cases, the administration of complementary anti-miRs promotes sensitivity to cancer drugs. For example, it was shown that treatment of leukemic cells expressing miRNA-21 with anti-miR-21 increased cellular sensitivity to etoposide and doxorubicin, by induction of autophagy (Seca et al., 2013). The same miRNA was also shown to be responsible for chemoresistance in NSCLC and silencing its expression reversed multidrug resistance by promoting apoptosis (Ren et al., 2016). Another example was shown in a more recent

study, in which the inhibition of miRNA-9 with anti-miR-9 increased the sensitivity of resistant ovarian tumor cells to cisplatin (Zhao et al., 2015).

Interestingly, it is possible that miRNA expression may be changed by cancer therapy and that these changes may be associated with drug response (Price and Chen, 2014). This means that miRNAs may modulate response to chemotherapy and may also be themselves modulated by chemotherapy (Price and Chen, 2014).

14.2 THE THERAPEUTIC POTENTIAL OF MIRNA MODULATORS IN CANCER

The discovery of RNA interference (RNAi) was one of the most important discoveries in molecular biology, representing a significant breakthrough in the understanding of the function and regulation of genes, granting its discoverers the Nobel Prize of Medicine in 2006 (Fire et al., 1998). The capacity of RNAi-related molecules (such as siRNAs, shRNAs, and miRNAs) to selectively silence and suppress the expression of genes, made them important therapeutic weapons for treatment of human diseases caused by specific genes (Fujita et al., 2015). Oncology is one of the areas that may benefit from these therapeutic molecules, which allows modulation of expression of any gene involved in tumor initiation, growth, metastasis, and resistance to chemotherapy (Fujita et al., 2015). In fact, as mentioned previously, numerous miRNA genes are dysregulated in various cancers, which suggests that key pathways of tumorigenesis may have miRNAs as downstream targets (Volinia et al., 2006). If the dysregulation of a critical pathway results in loss of expression of a specific miRNA, then theoretically a miRNA mimic may be used as a therapeutic approach. Conversely, if it results in overexpression of specific miRNAs, then complementary (antisense) molecules may theoretically be used for treatment (Croce, 2009). With this purpose, researchers have been interested in designing inhibitors of oncogenic miRNAs and mimics of tumor-suppressor miRNAs, which may act alone or synergistically with current approved treatments.

One method to inhibit miRNAs may be the use of modified nucleic acids, known as anti-miRs, which can bind and inhibit a specific miRNA (Lennox and Behlke, 2011). Anti-miRs are complementary to the target miRNAs and contain chemical modifications to improve the base pairing affinity to the target miRNAs, resistance against nucleases, and pharmacokinetic properties (Crooke, 2007). Anti-miRs have been used to study the in vitro effects of miRNA's downregulation and also been developed for therapeutic applications (Stenvang et al., 2012).

The miRNA mimics may also have chemical modifications in their structure in order to turn them more stable. The miRNA mimics should possess higher affinity to a specific miRISC and consequently to the target mRNA (Merhautova et al., 2016). Fig. 14.2 summarizes the mechanism of action of miRNA mimics and anti-miRs.

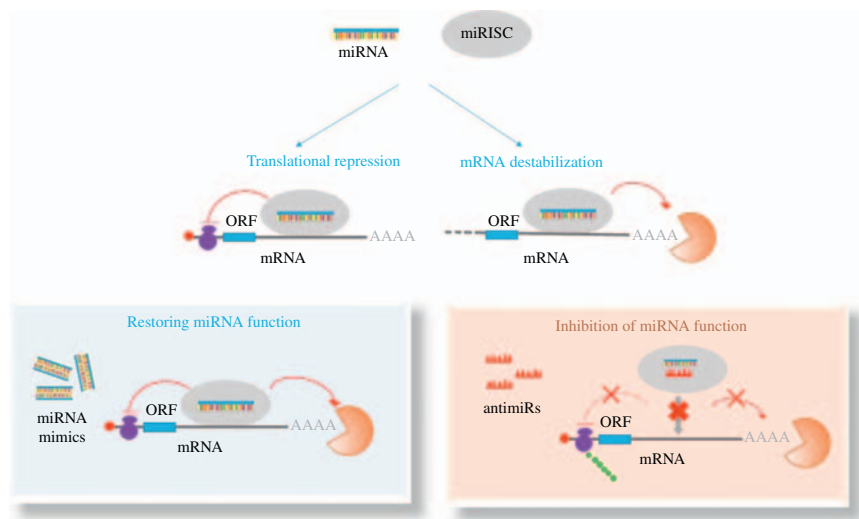


FIGURE 14.2 Mechanism of action of miRNA mimics and anti-miRs.

Micro-RNAs direct the miRISC to complementary sites in the target mRNAs, resulting in translational repression or mRNA destabilization and degradation. When a tumor-suppressor miRNA is downregulated, its activity may be restored by using miRNA mimics. In the case of oncogenic miRNAs overexpression, their inhibition is possible by using anti-miR oligonucleotides.

The miRNA-based therapeutics have been studied on animal models, following either local or systemic administration. For systemic delivery, the intravenous and intraperitoneal routes have been used, whereas for local administration, intra tumor injections in subcutaneous tumors has been performed [revised by [Merhautova et al. \(2016\)](#)].

Substances that can modulate the expression of miRNAs when these are aberrantly expressed in cancers may also become important therapeutic approaches. For example, it was found that matrine, an active component of a traditional Chinese medicine, upregulates the expression of miRNA-126 which leads to downregulation of its target gene, the vascular endothelial growth factor, in the A549 NSCLC cell line ([An et al., 2016](#)).

14.3 MICRORNA MODULATORS DELIVERY

14.3.1 PRINCIPAL BARRIERS

An active area of current research consists of optimizing the delivery of either miRNA mimics or miRNA inhibitors to the target site of action. These molecules

are easily degraded by nucleases and scarcely taken up into cells, if they are systemically administered without a vector (Kawabata et al., 1995). Additionally, they have high molecular weights and a poly-anionic nature, which limits their cell penetration and consequently their transfection efficiency (Aagaard and Rossi, 2007; Gavrillov and Saltzman, 2012). To perform their function, these molecules need to be internalized, escape from the endosomes, pass through the cytoplasm, and arrive into the cell nucleus (Wang et al., 2013).

A delivery system that promotes prolonged circulation time in the bloodstream and improved permeability through the cell membrane, enabling delivery to the target cells, is indispensable in order to achieve systemic therapy with miRNA modulators (Ando et al., 2013). One of the studied methods to achieve this was the use of engineered viruses. However, viral vectors have limited effectiveness in patients due to the possible occurrence of adverse effects such as immunogenicity, and the inability to specifically target the tumor, causing toxicity by delivering miRNAs to other tissues (Thomas et al., 2003b). Another possibility for delivery of miRNA-based therapies is the use of nanocarriers, which may overcome the disadvantages of viral vectors (Muthiah et al., 2013; Yin et al., 2014). All of these approaches will be discussed next.

14.3.2 VIRAL VECTORS

In recent years the use of viral vectors in gene therapy for the treatment of cancer has advanced significantly. The emergence of modern genetic engineering enabled viruses that are less toxic to humans and more specific to tumor cells. In Phase I and II trials these recombinant viruses have proven to be safe and tolerable (Carson et al., 2010). Furthermore, viral vectors are the preferred vectors for gene therapy because they can be modified to change their tropism, for transduction into any cell type (Cevher et al., 2012).

Although the preclinical use of viruses as gene carriers have demonstrated promising results, they have some drawbacks (e.g., immune responses and multi-gene disorders) that still need to be addressed before proceeding to clinical practice. Some of the strategies to overcome these disadvantages include efficient nuclear localization, preventing activation of oncogenes or inactivation of tumor-suppressor genes, and eliminating unwanted immunogenic responses in patients (Monroig and Calin, 2013).

The viral life cycle can be divided into two distinct phases: infection and replication. Viruses may become replication-incompetent by deleting a portion of the essential genetic sequence for viral propagation and replacing this sequence with a transgene (Phillips et al., 2007). The deletion of an essential genetic sequence enables a vector which maintains its replicative ability but is unable to infect the patient (Levesque, 2004).

In general, the ideal gene delivery vector should be specific; capable of efficiently delivering genes of the size needed for clinical application; unrecognized

by the immune system; safe; and purified in large amounts and at high concentrations (Misra, 2013).

In recent years, therapeutic viral vectors for cancer gene therapy have transitioned from being nonreplicative to being replication-competent, or oncolytic. Furthermore, replication-competent oncolytic viral vectors have been widely tested in preclinical studies and translated into human clinical trials (Wu and Zhou, 2011).

The use of viral vectors for encapsulation and delivery of miRNAs enables tests for modulating the expression of miRNAs *in vivo*, thereby facilitating the possible application of miRNAs in cancer therapy (Aravalli, 2013).

Viruses such as lentiviruses, adenoviruses, adeno-associated viruses (AAV), and herpes simplex viruses (HSVs) can encode miRNA mimics and express them in infected cells (Aravalli, 2013).

14.3.2.1 *Lentivirus*

Lentiviruses are a subcategory of the retrovirus family, which are able to infect nondividing cells. Although lentiviruses can integrate in the host genome, their integration sites are significantly more restricted than those of other retroviruses (Maurya et al., 2009). Due to their improved performance, lentivirus vectors were developed with the aim of becoming the substitutes of onco-retroviruses and have been successfully applied in gene therapy because they offer greater advantages than their retrovirus counterparts (Escors and Breckpot, 2010; Vigna and Naldini, 2000). These vectors have the advantages of long-term transgene expression and low immunogenicity (Cockrell and Kafri, 2007).

Lentiviral vectors have been successfully applied in gene therapy models, the generation of transgenic animals, and gene silencing by combination with siRNA- and miRNA-based technologies (Escors and Breckpot, 2010). Furthermore, lentiviral vector-based technology allowed long-term overexpression of miRNAs in almost all cell types and were considered important tools for this kind of application (Zöllner et al., 2014). However, the use of lentiviral vector-based miRNA inhibition for human therapy and functional genomic studies is limited because of the risk of insertion of mutagenesis and the requirements for *ex vivo* manipulation (Xie et al., 2012).

The studies of Zhang et al. demonstrated that miRNA-145 expressing episomal lentiviral vectors are a promising tool for gene therapy of esophageal carcinoma (Zhang et al., 2016c). Wang et al. also investigated the effect of a recombinant lentiviral vector encoding miRNA-145 on the biological features of esophageal cancer cells. The results obtained revealed that the overexpression of miRNA-145 inhibited oesophageal cell proliferation, increased apoptosis and influenced cell-cycle progression, suggesting a potential future role for miRNA-145 in the treatment of oesophageal cancer (Wang et al., 2015f). However, the studies of Cho et al. demonstrated that the miRNA-145 altered proliferation of lung cancer cells was not accompanied by changes in apoptosis (Cho et al., 2011b).

Bcl-2 is an antiapoptotic gene overexpressed in many tumors, including nasopharyngeal carcinoma. It has been described that miRNA-15a and miRNA-16-1 could act as Bcl-2 inhibitors. To investigate their effects on nasopharyngeal carcinoma, Zhang et al. used recombinant lentiviral vectors to allow the overexpression of miRNA-15a and miRNA-16-1 in nasopharyngeal carcinoma CNE-2Z cells. The results of this study demonstrated that miRNA-15a and miRNA-16-1 could inhibit cell proliferation and increase the apoptosis of CNE-2Z cells (Zhang et al., 2013).

The study described by Cao et al. showed that the expression of miRNA-1284 was reduced in gastric cancer and in vincristine-resistant (VCR) gastric cancer SGC7901 cells (SGC-7901/VCR) when compared to controls. Recombinant lentiviral vectors with miRNA-1284 caused overexpression of miRNA-1284 and reversed chemoresistance, accelerated drug-induced apoptosis and decreased migration and invasiveness of SGC-7901/VCR cells (Cao et al., 2016).

14.3.2.2 Adenovirus

Adenoviruses are nonenveloped, double-stranded DNA viruses (around 36 kb in size), which have several important features as cancer gene therapy vectors because of their broad tropism and high transduction efficiency (Wu and Zhou, 2011).

Unlike retroviruses, following cell infection, the adenoviral DNA becomes localized in the nucleus as an episomal element and does not integrate into the genome of the host cell, which makes these vectors safe (Ghosh et al., 2006; Phillips et al., 2007).

In order to be used as vectors for gene therapy, adenoviruses are attenuated by deletion of a genome fragment coding for early proteins. Several levels of attenuation can be achieved by removal of different numbers of genes: only one E1B gene (first generation vectors), the majority of early genes (second generation vectors), or full deletion of all genetic information of an adenovirus (the so-called gutless vectors). It is described that 1–2 kb can be inserted into early generation vectors, and up to 30 kb in gutless vectors (Lukashev and Zamyatnin, 2016). Although early generations of adenovirus had toxic side effects and caused immune responses, the second and third generation vectors, having many of the genes suppressed, demonstrated significant improvements (Lundstrom, 2003).

Adenoviruses type 2 and type 5 of subgroup C were the first to be used as viral vectors in gene therapy since these types are not associated with severe disease in humans. In addition to using adenoviral vectors as carriers for transgenes, these viruses themselves provide antitumor activity by targeting tumor cells with a defective p53 (Walter and Stein, 2000).

Adenoviral vectors are easy to purify at high concentrations and are able to infect dividing and nondividing cells. Another advantage of these viruses is their ability to infect target cells, which minimizes the risk of insertional mutagenesis (Walsh, 2007).

Recombinant adenoviruses are constructed by insertion of a transgene into the adenovirus genome, and the most advanced third generation adenoviruses retain only the cis-acting elements essential for packaging the virus (Huang and Kamihira, 2013).

Recombinant adenovirus vectors are commonly used in many studies because, as mentioned previously, they do not integrate into the host genome but replicate as episomes in the nucleus and express miRNAs in a stable manner (Aravalli, 2013).

Furthermore, oncolytic adenoviruses can be specifically engineered to target, replicate and eliminate cancer cells. For example, a potent preclinical anticancer strategy for pancreatic cancer consists of the treatment with oncolytic adenoviruses. Indeed, the study of Bofill-De Ros et al. demonstrated that pancreatic miRNA-selective adenoviruses could be suitable agents to fight pancreatic cancer without damaging normal tissues. This study provided preclinical evidence that miRNA-148a and miRNA-216a confer adenoviral selectivity and anticancer activity in pancreatic cancer models and proposed plasmids engineered by 8-miRNA148aT as a strategy for genetically-engineered therapies against pancreatic ductal adenocarcinoma (Bofill-De Ros et al., 2014).

In order to investigate the effect of miRNA-30b on laryngeal carcinoma, Li et al. stably overexpressed miRNA-30b in the laryngeal carcinoma cells HEp-2. These authors found that although there were no significant differences in cell viability between miRNA-30b overexpressed cells and control HEp-2 cells, p53 expression was enhanced in miRNA-30b overexpressed cells. Therefore, it was evaluated whether miRNA-30b could improve the antitumor effect of adenovirus-p53 (Ad-p53) in laryngeal carcinoma and other cancer cell lines. It was demonstrated that miRNA-30b overexpressed HEp-2 cells presented an increase in p53-mediated tumor cell apoptosis both in vitro and in vivo (Li and Wang, 2014).

As stated previously, one of the main disadvantages of viral vectors is that they can induce immune responses in transduced cells, thereby reducing gene transfer. The systemic administration of high doses of adenovirus can lead to an inflammatory response, which can be lethal in some extreme cases. Therefore, adenoviral vectors are currently less used in gene therapy due to their side effects, complexity of administration and large genome size (Lukashev and Zamyatnin, 2016). Accordingly, physical or chemical modification of adenoviral vectors using polymers has been described as a strategy to improve cancer therapy. In recent years, a variety of ligands such as peptides, growth factors or antibodies, have also been introduced to avoid unwanted transduction and improve the therapeutic efficacy and safety of adenoviral vectors (Kim et al., 2012).

14.3.2.3 Adeno-associated virus

AAV are small (4.7 kb), nonenveloped, linear single-stranded DNA viruses from the *Parvoviridae* family. These viruses are nonpathogenic, have low immunogenicity and sustain prolonged expression kinetics (Latronico and Condorelli, 2011). As these viruses are replication-defective they need a helper virus, such as

adenovirus or herpes virus, to replicate within a host cell nucleus (Kootstra and Verma, 2003; Daya and Berns, 2008).

The AAV genome consists of two genes (Rep and Cap), encoding for polypeptides essential for their replication and encapsidation. The Rep gene is involved in viral genome replication and encapsidation and the Cap gene provides structural proteins for the viral capsid which determines the tissue tropism and immune biology in viral infection. AAV can transduce dividing and nondividing cells without having known pathogenic consequences, making them an effective delivery strategy (Xie et al., 2012).

As mentioned previously, the AAV vectors are single-stranded adenoviruses devoid of critical genes involved in viral infection, making them relatively safe for use in gene therapy (Walter and Stein, 2000). The AAV-based vectors created so far are not capable of integrating into a genome and thus do not have a genotoxic effect. It should also be mentioned that the AAV capsid is less immunogenic than that of other viruses such as adenovirus or poxvirus (Lukashev and Zamyatnin, 2016). The entry of these viruses into the host cell occurs by binding to the cellular receptor and, after receptor-mediated endocytosis, vectors transport the genetic material into the nucleus (Walter and Stein, 2000; Phillips et al., 2007; Daya and Berns, 2008).

Recombinant adeno-associated virus (rAAV) are the most promising vectors obtained by bioengineering. These viruses are constructed by deletion of Rep and Cap genes and the replacement of these genomic sequences with therapeutic transgenes and internal promoters regulating transgene expression. Comparing with other gene delivery vectors, AAV have some advantages because they are nonpathogenic, nonimmunogenic, and are able to infect a wide variety of dividing and nondividing cells (Walter and Stein, 2000; Phillips et al., 2007; Daya and Berns, 2008). In addition, they do not appear to be associated with any known diseases in humans. The simplicity of AAV makes it possible to administer high doses of these vectors systemically without causing acute inflammatory responses or toxic side effects (Thomas et al., 2003a).

Although rAAV vectors have many advantages regarding in vivo gene delivery when compared to other viral vectors, their restricted tropism and relatively small capacity for insertion of transgenes (ranging from 4.1 to 4.9 kb) limits their application (Walter and Stein, 2000; Phillips et al., 2007; Daya and Berns, 2008).

As mentioned previously, AAV vectors are suitable in vivo gene delivery vehicles due to their tissue-specific tropism, long-term expression and high transduction efficacy. Thus, the combination of rAAV with optimized miRNA expression cassettes or inhibitors, will be promising tools for miRNA functional genomic studies and potential therapeutics (Xie et al., 2012).

One of the first notable advances in miRNA-based therapy was the use of the AAV vector to administer miRNA-26a in order to suppress liver cancer in an inducible mouse model-cMYC12 (Xie et al., 2012). Successful therapeutic delivery of a miRNA-26a-expressing AAV vector was also reported in a murine liver cancer model (Kota et al., 2009). In this study, Kota et al. intravenously

administered the adeno-associated virus 8 (AAV8), expressing miRNA-26a, through the tail veins of induced MYC mice containing liver tumors. These authors demonstrated that 3 weeks after infection with AAV8, a significant regression of the tumors in mice receiving miRNA-26a treatment was observed, when compared with mice receiving control treatment (Trang et al., 2008).

Kota et al. reported that systemic administration of miRNA-26 (a hepatocellular carcinoma suppressor miRNA), using AAV vectors in an animal model of hepatocellular carcinoma, allowed the inhibition of cell proliferation and tumor-specific apoptosis. This approach reduces toxicity since AAV vectors do not integrate into the host genome (Budhu et al., 2010).

14.3.2.4 Herpes simplex virus

HSV are a class of double-stranded DNA viruses, belonging to the *Herpesviridae* family, characterized by a large genome (152 kb) encoding 84 genes, which occur naturally in humans, having several advantages as a template for viral oncolysis vectors. HSV has two serotypes, HSV-1 and HSV-2, and its relatively large genome allows it to accept transgenes as large as 30 kb (Sun and Li, 2012). The HSV genome does not integrate into the host genome, thus, these viruses are generally nonmutagenic to their hosts (Carson et al., 2010).

HSV possesses a lipid envelope containing viral glycoproteins essential for virus binding to cellular receptors (Levesque, 2004; Huang and Kamihira, 2013). Once inside the cell, the capsid is transported to the nucleus, where viral DNA is expressed (Huang and Kamihira, 2013).

These viruses can infect dividing and nondividing cells, present a large packaging capacity for transgenes (allowing not only the inclusion of more than one transgene but also large transcriptional regulatory elements) and a natural tropism mainly toward neuronal cells (Walter and Stein, 2000).

HSV are suitable to be used as vectors for gene therapy, because they are highly infectious; their cellular receptors are widely expressed on the cell surface of numerous cell types; the nondividing cells may be efficiently infected and transduced by HSV; and the recombinant HSV vectors can be easily produced with high concentration and purity (Manservigi et al., 2010).

The major disadvantages of the HSV vectors are that they require much additional engineering to be efficient and that there is a relatively low transduction efficiency (Manservigi et al., 2010).

In the last decade, research on the development of HSV vectors has resulted in the engineering of recombinant vectors that are nontoxic and capable of expressing long-term transgenes (Goins et al., 2014).

HSV can infect and replicate in a broad scope of epithelial cells and neurons, including cancer cells. The attenuated herpes virus strains were adapted as oncolytic viral vectors against tumors, while reducing cytopathogenicity in normal cells (Wu and Zhou, 2011).

The genetically manipulated vectors that compose the oncolytic herpes simplex viruses (oHSVs) have demonstrated great safety and efficacy in clinical

trials. There has been a major focus on increasing the efficacy of oncolytic vectors, by adding exogenous transgenes to increase tumor destruction (Carson et al., 2010). Replication-competent (oncolytic) HSV vectors specifically replicate in actively dividing tumor cells and have been used in Phase I–III human trials in patients with brain tumors and also in malignant melanoma (Goins et al., 2014).

The first aim when developing therapeutic agents from HSV species was to eliminate or minimize the toxicity resulting from infection of noncancer tissues. Accordingly, the first generations of genetically modified oHSV vectors were constructed by deletion of selected HSV-1 genes whose function was thought to be essential for infection of normal tissues but redundant for the infection of cancer cells (Carson et al., 2010).

It has been demonstrated in human clinical trials that injections of oHSV into the blood vessels or into tumors caused stabilization and regression of the tumors. Indeed, the HSV oncolytic viruses replicate preferentially and persist at the tumor site for a long period of time after administration. In addition, the antiviral immune response may provide an additive effect on the effectiveness of oncolytic therapy (Wu and Zhou, 2011).

Studies by Mazzacurati et al. demonstrated that genetically modified miRNA-124 may eliminate off-target replication by nonattenuated oHSV vectors, without compromising their oncolytic activity, thus providing increased safety (Mazzacurati et al., 2015).

Castration-resistant prostate cancer is associated with high mortality, and has few treatment options. Micro-RNAs have provided a new opportunity for more stringent regulation of tumor -specific viral replication, in prostate cancer. Although miRNA-143 and miRNA-145 are highly expressed in normal tissues, they are significantly downregulated in prostate cancer cells. Lee et al. demonstrated a tumor -specific targeting capacity of miRNA-143–regulated and miRNA-145–regulated oHSV-1 viruses for killing prostate cancer cells both in vitro and in vivo. This study was the first to show that the inclusion of specific miRNA target sequences into the 3'-UTR of an essential HSV-1 gene is a promising strategy for restricting viral replication and oncolysis to cancer cells, including human prostate carcinoma cell lines, while preserving normal tissues (Lee et al., 2009).

14.3.3 NONVIRAL VECTORS

14.3.3.1 Liposomes and lipoplexes

Liposomes are lipid vesicles composed of an aqueous core surrounded by one or more concentric phospholipid bilayers (Mallick and Choi, 2014). The size and the number of bilayers depends on the production method and liposomes are classified as multilamellar vesicles (MLV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV) (Vemuri and Rhodes, 1995). The SUV type liposomes are those intended for drug delivery. Liposomes can be easily obtained

by hydration of the lipid film (formed after evaporation of the solvent used to dissolve the phospholipids) with water or other appropriate solution. After hydration, large liposomes with various phospholipid bilayers (MLVs) are obtained. In order to get SUV, these MLV should be submitted to extrusion, sonication or high pressure homogenization (HPH). Other methods described in the literature include pH-gradient drug loading, which is employed to encapsulate numerous anticancer drugs, including the marketed Doxil or Caelix (Barenholz, 2012).

The structure of liposomes makes these systems able to entrap hydrophilic and ionic molecules into the aqueous core, as well as lipophilic molecules into the lipid bilayer. Typically, liposomes can be obtained from one or more biodegradable lipids like phosphatidylcholine, cholesterol, sphingomyelin, and glycerophospholipids (Ashizawa and Cortes, 2015). They can be easily obtained and can be tailored with functional lipids, such as PEGylated lipids, in order to escape from the cells of the reticuloendothelial system (RES) thereby extending their circulation time, and/or with targeting moieties for tissue-specific delivery (Xue et al., 2015).

Cationic liposomes are considered the standard carriers for nucleic acids, because their positive charges can improve the incorporation efficiency of the nucleic acids (with negative charge) as a result of electrostatic interactions (Mallick and Choi, 2014). The complexes formed by interaction of a negatively charged nucleic acid with cationic liposomes are often referred as lipoplexes (Xue et al., 2015). Examples of cationic lipids include lipofectin, lipofectamine, *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*,-trimethylammonium chloride (DOTMA), dioleoyl phosphatidylethanolamine (DOPE), 2,3-dioleoyloxy-*N*-(2(sperminecarboxamido)ethyl)-*N,N*-dimethyl-1-propanaminiumtrifluoroacetate (DOPSA), 1,2-dioleoyl-3-trimethylammonium propane chloride (DOTAP), and dioctadecylamidoglycylspermine (DOGS). Lipofectin consists of DOTMA and DOPE, which functions as a helper lipid since it helps the nucleic acids gaining access to the cytoplasm. Lipofectamine consists of DOTMA with DOPE (Ashizawa and Cortes, 2015). Both DOPSA and DOGS are multivalent cationic lipids due to the presence of different ammonium groups, which contribute to a more efficient packing and binding of nucleic acids, compared to monovalent cationic lipids (Balazs and Godbey, 2011). Among all the cationic lipids, DOTAP is the most used to obtain cationic liposomes to form a complex with oligonucleotides (Lee et al., 2016).

Wu and coworkers developed lipoplexes carrying pre-miRNA-133b to directly target myeloid cell leukemia 1 (MCL-1), an antiapoptotic member of the Bcl-2 family which is overexpressed in NSCLC. The empty cationic liposomes were first prepared with DOTMA, cholesterol and D- α -tocopheryl poly(ethylene glycol) 1000 succinate (TPGS) and then mixed with pre-miRNA-133b. TPGS was added to increase the stability and the circulation time. The developed lipoplexes exhibited higher cellular uptake in an NSCLC cell line and caused an increase in miRNA-133 expression both in vitro and in vivo (Wu et al., 2011).

The major problems of cationic lipids include poor serum stability and toxicity due to interaction with the cell membranes and serum proteins, including

complement activation, induction of pro-inflammatory cytokines, leukopenia, and thrombocytopenia (Ozpolat et al., 2014). Currently, less toxic cationic lipids have been synthesized, by altering the structure of the phospholipid in one or more regions (Lv et al., 2006; Zhi et al., 2010). Conversely, the negatively charged liposomes are rapidly cleared from circulation (Ozpolat et al., 2014).

Anionic liposomes have also been studied for DNA and RNA delivery, due to their high safety and stability, being able to overcome the cytotoxicity associated to cationic liposomes (Patil et al., 2004; Kapoor and Burgess, 2012). However, with anionic liposomes, low encapsulation efficiencies of oligonucleotides can be found and to enhance this important parameter, helper cationic molecules such as calcium chloride, poly (amino acid), or some arginine-rich oligomers, are usually used to complex with the nucleotides encapsulated (Lee and Huang, 1996). Most recently, the use of octa-ammonium polyhedral oligomeric silsesquioxane was reported, a molecule with eight ammonium groups that functions as a cationic helper molecule with lower cytotoxicity (Nasreen et al., 2015). However, to our knowledge, to date there are no reports using these liposomes to deliver miRNA modulators.

In a work developed by Zhang et al., negatively charged liposomes were developed to carry anti-miR-221. However, in spite of being electrostatically conjugated with the lipid, the DNA molecule was encapsulated into the inner core of the liposome. The liposomes were prepared with hydrogenated soybean phospholipid (HSPC)/cholesterol (CHOL)/1,2-distearoylsn-glycero-3-phosphoethanolamine (DSPE)-methoxy (polyethylene glycol) (mPEG), by lipid film hydration with a phosphate buffer solution containing the anti-miR-221. The obtained liposomes had a nanometric size of 122.5 nm, a negative zeta potential, and an encapsulation efficiency of 70%. Moreover, they presented higher miRNA-221 silencing efficacy, both in HepG2 cells and in a xenografted mice model (Zhang et al., 2015b).

14.3.3.2 Stable nucleic acid lipid particles

Stable nucleic acid lipid particles (SNALPs) were first developed in 2001, by Semple and colleagues, and are considered an evolution of the cationic liposomes for delivery of nucleic acids (Semple et al., 2001). Typically, SNALPs are composed of a high transition temperature phospholipid, a PEGylated lipid, and an ionizable cationic phospholipid, such as 1,2-dioleoyloxy-*N,N*-dimethyl-3-aminopropane (DODMA) and 1,2-dioleoyl-3-dimethylammonium propane (DODAP). The use of the ionizable cationic phospholipid promotes high encapsulation efficiency, but the net charge exposed on the surface can be neutralized after the nucleic acid encapsulation, which consequently makes these vesicles more stable in biological fluids than the conventional cationic liposomes (Semple et al., 2001, Semple et al., 2010). Following the first reports about the use of SNALPs, more ionizable cationic phospholipids have been synthesized, with different degrees of saturation and improved fusogenic properties (Heyes et al., 2005).

One example of the applicability of SNALPs to deliver miRNA modulators is the work of Di Martino and colleagues. In this work, SNALPs encapsulating miRNA-34a were developed using disteoylphosphatidylcholine since it has high transition temperature, DODAP, and *N*-palmitoyl-sphingosine-1-succinyl[methoxy (polyethylene glycol)2000] as a pegylated phospholipid, by quickly adding the previously warmed lipid solution to the miRNA solution, while stirring. The developed SNALPs exhibited a mean size of approximately 150 nm, negative zeta potential, and an encapsulation efficiency of about 83%. The SNALP-miRNA-34a complexes were in vitro evaluated in a multiple myeloma cell line (SKMM-1 cell line), demonstrating significant inhibition of cell growth following 48 h of treatment. When tested in a mice model, tumor growth inhibition and induction of apoptosis was verified, while not causing loss of weight in the tested mice (Di Martino et al., 2014).

14.3.3.3 Extracellular vesicles

The vesicles naturally secreted by cells, generally named extracellular vesicles (Evs), can be mainly categorized as exosomes, microvesicles, or apoptotic vesicles, according to their size, pathway of origin, and constituent molecules (Théry et al., 2009). The term exosomes refers to vesicles ranging from 40 to 100 nm, released as a consequence of multivesicular endosome fusion with the plasma membrane. Exosomes have in their constitution various components from the donor cells, for example, heat shock proteins, glycoproteins from the tetraspan proteins superfamily (CD9, CD63, and CD91) and components of the endosomal sorting complex required for transport (Kalra et al., 2016). They can be isolated from the cell culture medium or from body fluids, using techniques such as sucrose gradients or ultracentrifugation (Théry et al., 2006). Microvesicles, also called shedding vesicles, ectosomes or microparticles, are shed from the plasma membrane and exhibit a diameter between 100 and 1000 nm. These vesicles are enriched in phosphatidylserine, integrins, selectins, and CD40 ligands (Cocucci et al., 2009). Apoptotic vesicles, derived from apoptotic cells, differ from exosomes since they contain very abundant histones associated with membranes and are very heterogeneous in size and morphology (Théry et al., 2001). Since the methods used to isolate and purify these vesicles differ substantially between studies, it is common to collectively call them EVs (Ohno et al., 2016; Tominaga et al., 2015).

Evs may carry genetic materials such as mRNAs and miRNAs, which means that they are natural vehicles for genetic messages, protecting the encapsulated molecules from degradative enzymes, and becoming potential natural drug delivery systems for cells (Bobrie et al., 2011; Ohno et al., 2016). Due to their biological origin, it is expected that they have low adverse effects if used as drug delivery systems. Additionally, EVs may mediate tumor selective drug delivery (Tominaga et al., 2015) since they represent an important intercellular communication method and therefore are more able to be accumulated in target organs.

In comparison with liposomes, EVs could be advantageous as drug delivery systems, since their membranes are structurally similar to the other membranous cellular structures, containing transmembrane associated proteins, receptors, adhesion molecules and a natural corona. Conversely, these features must be artificially engineered in liposomes in order to obtain a suitable drug delivery system (Ohno et al., 2016). The schematic comparison between liposomes and EVs is represented in Fig. 14.3.

The incorporation of miRNA mimics or anti-miRs into EVs may be achieved by transfection of EVs (secreted by donor cells) with the desired miRNA mimics or anti-miRs, using optimized transfection protocols. Alternatively, the donor cells may be incubated with the desired miRNA mimics or anti-miRs for some time (e.g., 14–18 h) and then further incubated (e.g., for 24 h) with normal culture medium to secrete EVs having incorporated miRNA mimics or anti-miRs, which are then collected (Akao et al., 2011; Pan et al., 2014).

The incorporation of various anticancer drugs, such as paclitaxel, doxorubicin, or curcumin in EVs has also been achieved, by treating/incubating the EVs or the donor cells with the drugs of interest in fetal bovine serum (FBS) for a certain time period (Yang et al., 2015; Kalani et al., 2014; Pascucci et al., 2014) or via electroporation (Tian et al., 2014).

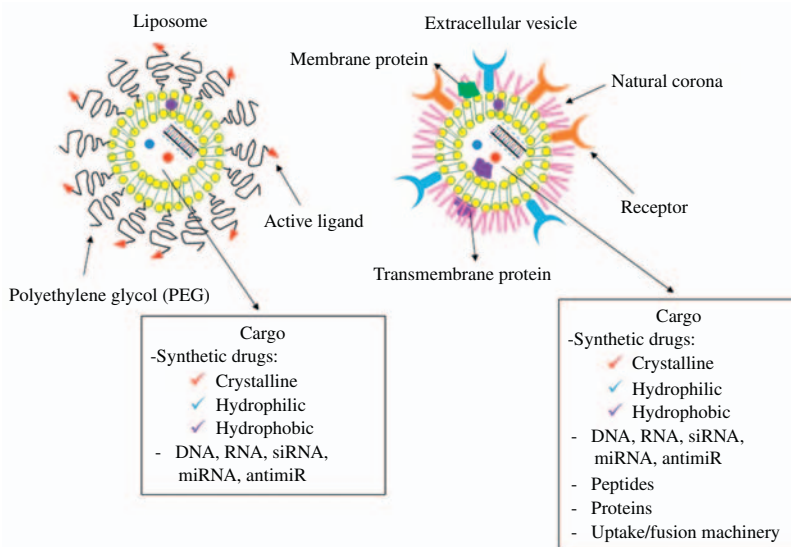


FIGURE 14.3 Schematic representation of a comparison between liposomes and EVs.

In relation to liposomes, the EVs membrane not only consists of lipids but also contains transmembrane and membrane proteins, receptors, adhesion molecules, as well as a natural corona. Conversely, these features need to be artificially engineered into liposomes (Ohno et al., 2016).

In a work performed by Wang's group, exosomes secreted from macrophages were used to transfect anti-miR-21 into a human gastric cancer cell line (BGC-823), since it had been reported that miRNA-21 is significantly overexpressed in gastric cancer tissue when compared to adjacent tissues, being associated with cancer pathogenesis (Sekar et al., 2016; Cui et al., 2013). In this study, the exosomes were isolated by ultracentrifugation and the anti-miR-21 incorporated by electroporation. The expression of miRNA-21 was significantly reduced in BGC-823 cells following incubation with exosomes carrying anti-miR-21, which suggests that exosomes were a good delivery system carrying the active molecules into the cells. Additionally, the antisense inhibition of miRNA-21 suppressed cell migration, promoted apoptosis, inhibited cell growth and upregulated PDCD4 tumor-suppressor gene which is a target of miRNA-21 (Wang et al., 2015d).

14.3.3.4 Polymeric micelles

Micelles are structures with size ranging from 5 to 100 nm formed by self-assembly of amphiphilic block copolymers at a critical micelle concentration (CMC), when these are dispersed in an aqueous medium. The outer shell of the micelle is formed by the hydrophilic block while the inner core is formed by the hydrophobic part and can be used to solubilize hydrophobic drugs (Kowalczyk et al., 2014; van Elk et al., 2016). In comparison with micelles formed by surfactants, polymeric micelles are formed at low polymer concentration, being more stable and less susceptible to dilution when compared to surfactant micelles (Kataoka et al., 2001; Deng et al., 2012). Polymeric micelles offer the capacity to condense and protect the nucleic acids segments, while showing high colloidal stability, prolonged circulation time, improved cell association and internalization, enhanced transfection efficiency as well as lower toxicity, when compared with cationic homopolymers (Navarro et al., 2015; Zheng et al., 2012). Micelleplexes consist of the complex formed by the electrostatic interaction of the nucleic acids with micelles previously obtained (Zhang et al., 2016e).

The physical as well as biological properties of micelles can be adjusted using multiple copolymers with different shell-forming blocks to form co-assembled micellar structures. To enhance the in vivo efficiency of micelles as drug delivery systems, some aspects must be taken into account: (1) a polycation such as poly-ethylenimine (PEI) should be used to promote condensation with the nucleic acid; (2) hydrophobic segments enable the formation of the micellar structure; (3) repetitive units serve as weak links in the hydrophobic block for intracellular degradation; and (4) general concepts such as the addition of PEG to reduce cytotoxicity and increase serum stability (Wang et al., 2016e). Micelle-based drug delivery is compromised by stability features both before and after systemic administration (Kim et al., 2010). The stability of polymeric micelles in the bloodstream can be improved by cross-linking their inner core (Rijcken et al., 2007).

For this purpose, various copolymers have been designed for nucleic acids delivery, including miRNA modulators: polyethylenimine-*graft*-polycaprolactone-*block*-poly(ethylene glycol); hyaluronic acid-*g*-poly(L-histidine) (PHis) and D- α -tocopheryl polyethylene glycol 2000; poly (lactide-*co*-glycolide)-*graft*-polyethylenimine; poly (ethylene glycol)-*block*-poly(aminolated glycidyl methacrylate)-*block*-poly(2-(diisopropyl amino) ethyl methacrylate) (PEG-*b*-PAGA-*b*-PDPA) triblock copolymer; poly (ethyleneglycol)-*block*-poly(2-methyl-2-carboxyl-propylenecarbonate-*graft*-dodecanol-*graft*-cationic ligands) (mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-CAT); polylactic acid-*block*-polydimethylaminoethyl methacrylate (PDMAEMA)PLA-*b*-PDMAEMA (Zheng et al., 2012; Chen et al., 2016a; Gwak et al., 2016; Yu et al., 2016; Mittal et al., 2014; Qian et al., 2014).

Micelles have been extensively studied for the delivery of various drugs, oligonucleotides, proteins, and peptides (Cabral and Kataoka, 2014). In the meantime, micelles have also been widely applied in the co-delivery of drugs and gene molecules, including miRNAs. In a work developed by Mittal and coworkers, gemcitabine was conjugated with a cationic copolymer, a poly(ethyleneglycol)-*block*-poly(2-methyl-2-carboxyl-propylenecarbonate-*graft*-dodecanol-*graft*-cationic ligand), where polyethyleneglycol forms the hydrophilic segment and polypropylene carbonate forms the hydrophobic segment. MicroRNA-205 was then complexed by hydration of the polymeric film using a HEPES buffer solution containing the miRNA. The micelleplexes developed in this work could revert chemoresistance to gemcitabine in a human pancreatic cancer cell line and reduce tumor growth rate and tumor weight in vivo (Mittal et al., 2014). In general, the formation of micelleplexes is possible by stirring an appropriate amount of the polymers in a solvent or solution to form micelles that are then mixed with the nucleic acid molecule. In the cases where it is intended to incorporate another drug, this is mixed previously with the polymers (Zheng et al., 2012; Yu et al., 2016; Chen et al., 2016a).

Amphiphilic star copolymers serve as a good delivery system for drugs and genes. Star polymers are three-dimensional globular or branched polymers containing multiple arms connected to a central core (Cho et al., 2011a). They differ from linear polymers due to their higher molecular weights, while maintaining similar solubility and viscosity (Sulistio et al., 2011). The cross-linked star copolymers possess lower CMC, avoiding the problem of micelle dissociation as a result of dilution. Since the inner core of star polymers is biodegradable, the resultant micelles release the drug through degradation of the polymer, in the site of action (Gao, 2012). In a work developed by Qian and coworkers, micelles were developed using an amphiphilic star-branched copolymer of polylactic acid (PLA) and polydimethylaminoethyl methacrylate (PDMAEMA), entrapping the hydrophobic drug doxorubicin and binding a miRNA-21 inhibitor. The developed micelles mediated the escape of the miRNA-21 inhibitor from lysosome degradation and the release of doxorubicin into the nucleus, resulting in a significant synergistic inhibition of tumor proliferation by regulating Bcl-2 expression in a glioma cell line (Qian et al., 2014).

14.3.3.5 Polymeric nanoparticles and polyplexes

Polymeric carriers have been extensively studied for gene delivery due to their outstanding features (Lee et al., 2016). Polymeric nanoparticles are colloidal systems obtained by nonbiodegradable or biodegradable polymers. However, biodegradable polymeric nanoparticles are highly preferred since, in addition to promoting controlled/sustained release, they are biocompatible with tissues and cells (Panyam and Labhasetwar, 2003). Additionally, these nanoparticles are stable in the blood, are nontoxic, nonthrombogenic, nonimmunogenic, noninflammatory and do not activate neutrophils, are biodegradable, avoid the RES, and are applicable to various molecules such as drugs, proteins, peptides or nucleic acids (des Rieux et al., 2006). The biodegradable polymers most used to obtain biodegradable polymeric nanoparticles include poly(lactic acid co-glycolic acid) (PLGA), PLA, poly- ϵ -caprolactone (PCL), poly-alkyl-cyanoacrylates (PACA), chitosan and gelatine (Kumari et al., 2010).

Polymeric nanoparticles can be obtained by several methods, the most commonly used being: emulsification, solvent evaporation/extraction, nanoprecipitation (solvent displacement), supercritical fluid technology, and salting out. The first method is mainly used for lipophilic drugs and consists of dissolving the polymer and the drug in a nonpolar solvent (chloroform, dichloromethane, ethyl acetate) to subsequently emulsify this organic solution in an aqueous phase containing a surfactant, by high speed homogenization (HSH). The solvent is then evaporated or extracted and the polymer precipitates forming the nanoparticles. In the case of the nanoprecipitation method, the polymer is dissolved in a water miscible solvent (acetone or ethanol), so that when this solution is added dropwise into an aqueous phase, the solvent diffuses to the water phase and the polymer precipitates. This method can only be used with lipophilic drugs, but presents important advantages such as high drug incorporation efficiency, narrow size distribution, no need for homogenization, and easy scale-up. In the supercritical fluid technology, a solution containing the polymer is sprayed in the form of tiny droplets into a high pressure vessel containing a liquid antisolvent, such as CO₂. This method avoids the utilization of organic solvents and is useful to incorporate proteins and peptides. In the salting-out method, the organic solution is added to an aqueous solution, containing a high concentration of a salt. The disadvantage of this method is the need for an extensive washing to remove the salt (Masood, 2016; Rao and Geckeler, 2011). Depending on the production conditions, it is possible to obtain nanospheres, which consist of a polymeric matrix system, or nanocapsules, with a reservoir system containing an oily or aqueous core surrounded by a polymeric membrane (Estanqueiro et al., 2015; Kumari et al., 2010). The nucleic acids can be encapsulated into the aqueous core of the nanospheres or adsorbed at the nanoparticles surface (either in the case of nanospheres or nanocapsules), as represented in Fig. 14.4.

PLGA is a water insoluble polymer, approved by FDA for drug delivery in humans and widely studied for biomedical applications in the last century (Lu

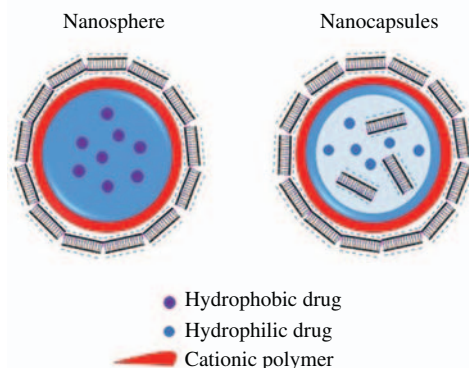


FIGURE 14.4 Schematic representation of polymeric nanospheres and nanocapsules.

In both cases, anionic miRNAs or anti-miRs can be attached by electrostatic interaction if a cationic polymer is used. In the nanospheres it is possible to carry a hydrophobic drug, incorporated into the polymeric matrix, while in the nanocapsules with an aqueous core, the hydrophilic molecules can also be encapsulated as well as hydrophilic drugs.

et al., 2009; Kulkarni et al., 1971). PLGA polymers can form micro- and nanoparticles, entrapping biologically active molecules. They can be useful for therapy with miRNA modulators since they can protect nucleic acids from degradation, promote high loading capacity and allow surface modifications to improve pharmacodynamics (Uchegbu, 2006). In a recent study, PLGA nanoparticles co-delivering anti-miR-21 and anti-miR-10b were developed in order to downregulate the expression of these oncomiRNAs (miRNA-21 and miRNA-10b) in glioblastoma cells, thereby improving response to therapy with temozolomide. It is known that the overexpression of miRNA-21 leads to downregulation of PDCD4 and PTEN, promoting cell proliferation, apoptosis inhibition, and chemoresistance. It is also known that the overexpression of miRNA-10B downregulates Homeobox 10 (HOXD10), benefitting cell proliferation, invasion and migration. The developed nanostructures carrying the antisense miRNAs (for miRNA-21 and miRNA-10b) promoted in vitro reduction of the viable glioblastoma cells and increased cell-cycle arrest at the G2/M phase upon temozolomide treatment (Ananta et al., 2016). The same anti-miRs had also been co-incorporated into PLGA-PEG nanoparticles, in order to reduce breast cancer cell proliferation in vitro as well in vivo (Devulapally et al., 2015).

The cationic polymer PEI is the most widely used polymer for the delivery of DNA and RNA molecules. Polymeric nanocarriers containing PEI in their composition usually exhibit high incorporation efficiency of negatively charged molecules and high in vitro transfection due to the positive charge of polymer (Patnaik and Gupta, 2013). However, it is important to be aware that the high positive charge of PEI is also cytotoxic. In order to be used effectively in vivo, it may be copolymerized with neutral and biodegradable polymers such as PEG, PLGA, or

biopolymers which will minimize the cytotoxicity and the enzymatic attack (Su et al., 2012; Kafil and Omid, 2011). Cationic polyplexes formed by complexation of PEI with nucleic acids typically retain a positive charge, promoting interaction with negatively charged polysaccharides on the cell surface. The polyplexes escape from the endosomes through the so-called proton sponge effect, where PEI causes an influx of protons and water and endosome swelling and eventually endosome disruption to release the polyplexes to cytoplasm (Boussif et al., 1995).

Chitosan and hyaluronic acid (HA) can be also used as polymeric drug carriers. However, their application is less explored for gene delivery purposes due to limited incorporation efficiency of nucleic acids and low transfection efficiency. Conversely, these polymers can be incorporated into PEI-based systems in order to reduce their cytotoxicity and increase their stability (Ganesh et al., 2013; Lee et al., 2016).

In a recent work, a new type of amphiphilic cetylated PEI (cetPEI) was synthesized and self-assembled with PLGA and HA, to form PLGA/cetPEI/HA nanoparticles for gene delivery. This system was tested for transfecting anti-miR-221 into a liver hepatocellular carcinoma cell line (HepG2). The introduction of cetPEI in PLGA nanoparticles is susceptible to increase the loading capacity of anti-miRs and their cellular uptake. Coating the nanoparticles surface with HA may increase tumor-cell targeting. The developed nanoparticles exhibited positive charge, size around 200 nm and higher gene transfection efficiency than PEI itself and other commercial transfection agents (Zhu et al., 2016).

Using a similar approach, Wang and coworkers developed HA-coated PLGA/PEI nanoparticles for co-delivery of doxorubicin and miRNA-542-3p, for triple negative breast cancer therapy. The developed system presented an average size of 131.7 nm, stability in serum and cytotoxic effect in breast cancer cell lines, since intracellular restoration of miRNA-542-3p levels promoted apoptosis by activating p53 and inhibiting survivin expression (Wang et al., 2016d).

14.3.3.6 Dendrimers

Dendrimers are three-dimensional molecules consisting in a central core from which highly branched tree-like polymer chains emanate, in a highly ordered and symmetric structure resultant from a stepwise synthesis (Lee et al., 2016). Additionally, these molecules also exhibit a unique and well-defined molecular weight, with low polydispersity index and a well-defined number of peripheral groups (Gillies and Frechet, 2005). Due to this structure and high ratio of surface moieties-to-molecular volume, dendrimers have called the attention of researchers working in gene delivery (Dutta et al., 2010). The drug molecules can be either physically entrapped or chemically conjugated with the dendrimer, during or after the synthesis of the dendritic structure (Thanki et al., 2013).

Dendrimers with cationic groups have been used to complex with nucleotides via electrostatic interactions, promoting nucleic acids delivery, and protection (Lee et al., 2016). Poly(amidoamine) (PAMAM) or poly(propyleneimine) are the dendrimers most used for nucleic acids delivery, as a consequence of the higher number of amine and amide groups on their chains (Pack et al., 2005). Additionally, the

degradability of amide linkage in PAMAM dendrimers enhances its transfection effectiveness (Lee et al., 2016). PAMAM dendrimers also exhibit relatively low genotoxicity and cytotoxicity, being the most widely used ones (Choi et al., 2010).

Dendrimers are synthesized in a layer-by-layer sequence around the core and each layer is called a generation. For example, a dendrimer with three layers is a dendrimer with three generations (branching points) or G3 (Svenson, 2009). Since the different generations of the dendrimer contain various amounts of amine functional groups, the binding affinity of the nucleic acids is highly dependent on the dendrimer generation (Zhou et al., 2007). This means that a higher number of generations leads to the formation of tighter complexes with the nucleic acids. However, higher transfection efficiencies can be obtained with lower generation dendrimers (Ottaviani et al., 2000).

Since the cationic dendrimers are also associated with cytotoxicity and low bioavailability, the dendrimers can be acetylated, PEGylated or glycosylated to prolong circulation time and decrease cytotoxicity by masking the cationic charges. Moreover, dendrimers can also be functionalized to promote active targeting (Kesharwani et al., 2014; Cheng et al., 2011). To promote active targeting to a specific cancer cell, targeting moieties can be attached to dendrimers. For example, Wang and coworkers attached an aptamer selective to the A549 lung cancer cells to a PAMAM-PEG dendrimer. Aptamers are short, single-stranded oligonucleotides, selected from systematic evolution of ligands by an exponential enrichment process. In comparison to antibodies, the aptamers present the advantage of convenient synthesis and modification, high affinity and specificity to the target molecules, low immunogenicity, less toxicity, higher stability and rapid tissue penetration (Tan et al., 2011; Song et al., 2012). In this work, a PAMAM with five generations was functionalized with the aptamer S6 and this complex was then mixed with miRNA-34a. The miRNA-34a is often found downregulated in NSCLC and its upregulation inhibits tumor growth and metastasis formation. The developed complex, with a diameter of 100–200 nm and a positive charge, exhibited higher cellular uptake and transfection efficiency as well as induction of apoptosis and antiproliferative effect, when compared to the nontargeted complexes, in the A549 cell line (Wang et al., 2015b).

Dendrimers can also be used to promote the co-delivery of miRNA modulators and conventional anticancer drugs, as was demonstrated by Ren et al. These authors used a G5 PAMAM dendrimer to co-deliver anti-miR-21 and 5-fluoracil to glioblastoma cells and enhanced the cytotoxic effect of the latter. The miRNA-21 is usually overexpressed in glioblastoma, and its downregulation stops cell growth, increases cellular apoptosis and cell-cycle arrest, which improves the chemotherapy efficacy. To obtain this complex, a determined amount of 5-fluoracil was firstly incubated with a dendrimer solution for 1 day. Then, after removing the nonincorporated drug, the anti-miR-21 was incubated with the 5-fluoracil-loaded PAMAM solution for 30 min. Dendrimers with high drug loading were obtained, which was attributed to the fact that a dendrimer with five generations has more cavities for drug loading. The dendrimers exhibited a size smaller than 100 nm, which

benefited uptake by tumor cells. The combination treatment with anti-miR-21 and 5-fluoracil exhibited synergistic antiproliferative and apoptotic effect in a glioma cell line (U251) and suppressed tumor cell proliferation (Ren et al., 2016).

14.3.3.7 Nanofibers

Nanofibers are ultrafine fibers obtained by electrostatic forces with micro- to nanometer range diameters and controlled surface morphology. These fibers can be used for multiple applications including for tissue regeneration and drug delivery, which is one of the most promising areas (Sill and von Recum, 2008; Pillay et al., 2013; Pham et al., 2006). Nanofibers can be interesting nano-delivery systems for cancer therapy due to their large surface and porosity, high drug encapsulation efficiency, possibility to combine diverse therapies and surface functionalization, and low cost (Chakraborty et al., 2009).

Nanofibers are mainly obtained by electrospinning, because this is an easy, reliable and versatile production technique to prepare ultralong fibers at a micro- or nanoscale. Indeed, this is the preferred technique to obtain nanofibers, allowing the ability to spin a broad range of polymers and potential to scale-up. The electrospinning technique requires a spinneret, a high voltage power supply, and a grounded collector (Sill and von Recum, 2008). Briefly, to obtain nanofibers, the spinneret is filled with a liquid polymer (in solution or melted) and then an electric field is applied. Consequently, electrical charges accumulate on the surface of the liquid droplet at the tip of the capillary. When the repulsive force among the charges overcomes the surface tension of the polymer, a conical jet of the polymer is ejected from the capillary tip of the spinneret at a fixed flow rate. The nanofibers will be formed on the oppositely charged grounded collector, while the solvent is evaporated (Rutledge and Fridrikh, 2007). Being an electrohydrodynamic technique, the morphological structures of electrospun nanofibers can be easily manipulated by adjusting the electrospinning parameters (Ebara et al., 2014). Electrospinning allow direct incorporation of hydrophobic and hydrophilic drugs and biomacromolecules, such as proteins and DNA (Zamani et al., 2013).

Examples of polymers that have been electrospun, include collagen, gelatine, chitosan, silk fibroin, and HA as natural polymers and PLA, PCL, polyethylene oxide, and copolymers, such as poly(L-lactide-*co*-caprolactone) and PLGA (Zamani et al., 2013).

Nanofibers differ from other systems because they cannot be injected into the blood stream. They are normally used in the form of mats or sheets to be implanted (Chen et al., 2016b) allowing local drug delivery which can decrease the minimum required dose of drug, reducing side effects (Zamani et al., 2013). Some studies described the development of implantable nanofibers carrying conventional anticancer drugs, demonstrating its efficacy in colorectal and ovarian cancer cell lines as well in an animal model of hepatic carcinoma. The polymers used in these studies were a mixture of PCL and poly(glycerolmonostearate-*co*- ϵ -caprolactone) in a ratio of 90:10, polyvinyl alcohol/chitosan, poly-lactic acid (Yohe et al., 2012; Yan et al., 2014; Liu et al., 2013).

Nanofibers are thus viewed as promising drug carriers for biomedical applications, especially for post-operative local chemotherapy (Hu et al., 2014).

Che and coworkers developed an interesting system consisting of paclitaxel loaded in PCL nanofibers, produced by electrospinning technique, together with nanoparticles containing disulfide cross-linked PEI carrying miRNA-145 immobilized over the nanofibers, to promote apoptosis in a hepatocellular carcinoma cell line (HuH-7). The developed nanofibers exhibited sustained release of paclitaxel and miRNA-145 and a higher cell growth suppression, when compared with nanofibers containing paclitaxel only, which suggests a synergistic effect of the developed system. Additionally, an increase in miRNA-145 expression levels was confirmed in the treated cells (Che et al., 2015).

14.3.3.8 Lipid nanoparticles

Lipid nanoparticles are mainly distinguished as solid lipid nanoparticles (SLN) or nanostructured lipid carriers (NLC). The SLNs were firstly developed in the early nineties, in order to improve some disadvantages of the liposomes and nanoemulsions, such as problems related with stability. SLNs are composed of approximately 0.1%–30% of a solid lipid dispersed in an aqueous phase with the help of surfactants, in a concentration between 0.5% and 5% (Naseri et al., 2015). For gene delivery purposes, the surface of the nanoparticles should be positively charged to bind the nucleic acids electrostatically (del Pozo-Rodriguez et al., 2007). NLCs were developed as an alternative to SLNs to improve drug loading and avoid drug expulsion during storage. NLCs differ from SLNs since they are composed of a mixture of a solid lipid with a liquid lipid (oils) in a proportion that guarantees that nanoparticles remain solid at room and body temperature (Han et al., 2016). Fig. 14.5 is a schematic representation of a SLN and a NLC,

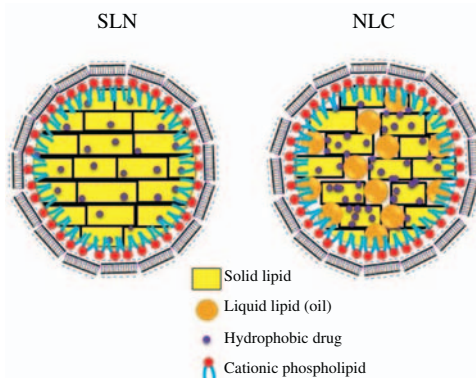


FIGURE 14.5

Schematic representation of a SLN and a NLC carrying electrostatically attached negatively charged nucleic acids, such as miRNAs or anti-miRs. Positively charged molecules, such as cationic phospholipids, should be used in order to confer a positive charge on the surface of the lipid nanoparticles.

carrying negative nucleic acids such as miRNAs and anti-miRs, attached by electrostatic interactions to positively charged nanoparticles.

Lipid nanoparticles present some advantages when compared to other nonviral vectors, such as low or absence of toxicity due to their composition in excipients “Generally Recognized as Safe”, often approved for pharmaceutical preparations for human use and the possibility to be produced by economic and solvent free techniques (Campani et al., 2016; Battaglia et al., 2016). The lipid nanoparticles can be produced by several techniques such as HPH, ultrasonication or HSH, solvent emulsification/evaporation techniques and supercritical fluid extraction. However, the HPH and the HSH are the most used ones, the former being susceptible of scaling up (ALHaj et al., 2008; Estanqueiro et al., 2014; Kushwaha et al., 2013; Chattopadhyay et al., 2007).

Lipid nanoparticles, both SLN and NLC, are entirely solid and lipophilic; therefore, when compared with liposomes, are more difficult to encapsulate the hydrophilic miRNA and anti-miR molecules. However, it is possible to coat the nanoparticles surface with positive charged molecules (Xue et al., 2015). Additionally, lipid nanoparticles can also be functionalized to overcome common hurdles in the transfection process (del Pozo-Rodriguez et al., 2016).

One interesting work that illustrates the development of lipid nanoparticles to deliver miRNA modulators, is the work of Shi and coworkers, who developed SLN co-delivering paclitaxel and miRNA-34a to obtain synergistic effect on cancer cell death. Firstly, the SLN containing glyceryl monostearate (solid lipid), cholesterol, soy phosphatidylcholine (stabilizer/surfactant), and dimethyldioctadecylammonium (cationic lipid) together with paclitaxel were obtained by sonication. After this, the colloidal dispersion containing the SLN was incubated with a solution containing the miRNA, to promote self-assemble by electrostatic interaction. The obtained complex exhibited an average particle size of 218.2 nm, a positive charge, high entrapment efficiencies for paclitaxel as well as for miRNA-34a, and was stable in FBS and mouse serum. Additionally, a higher cellular uptake of miRNA-34a and paclitaxel was possible by B16F10-CD44⁺ melanoma cells when using the SLN in comparison with the administration of free miRNA-34a and paclitaxel. The SLN co-delivering miRNA34a and paclitaxel caused a higher decrease in cell viability and increase in apoptosis induction. Following in vivo administration, a higher selectivity to lung tissues was observed, together with prolonged survival time and attenuated expression of CD44 (Shi et al., 2014). CD44 plays a key role in determining the tumorigenic and metastatic capacity in many tumor cells and can be repressed by miRNA-34a (Joshua et al., 2012, Liu et al., 2011).

14.3.3.9 Inorganic nanoparticles

The inorganic nanoparticles that have been reported to be used for gene delivery include gold nanoparticles, iron oxide nanoparticles and quantum dots. There are three approaches to modify inorganic nanoparticles to promote gene delivery, such as the use of positively charged inorganic nanoparticles, direct conjugation

of the inorganic nanoparticle with a responsive linker and the use of a cationic polymer for coating the inorganic nanoparticle (Loh et al., 2016).

Gold nanoparticles can offer several advantages such as high stability, specificity, drug carrying capacity, controlled release and capacity to transport both hydrophobic and hydrophilic drugs (Pietro et al., 2016). In order to achieve gene delivery using gold nanoparticles, the typical methodology applied is surface functionalization with positively charged molecules. Accordingly, with this purpose, the coating of gold nanoparticles with PEI has been described, as well as the entrapping of gold nanoparticles into dendrimers or the conjugation of gold nanoparticles with cationic polypeptides (Cebrián et al., 2011; Shan et al., 2012; Yan et al., 2012). Fan and coworkers developed a sequential drug delivery system consisting of gold nanoparticles functionalized to attach miRNA-218 mimics on nanoparticle surface and chitosan-folate nanogel loading temozolomide. Temozolomide is an anticancer drug with efficacy against malignant glioma and miRNA-218 overexpression in glioma cells suppresses the motility, invasion and proliferation of these cells (Tu et al., 2013; Nieder et al., 2006). Using the developed nanocarrier, since folic acid is considered a specific tumor tissue targeting ligand, the uptake of gold nanoparticles with miRNA-218 was enhanced. The temozolomide was released by diffusion due to the nanogel swelling, followed by release of miRNA-218 in tumor cells. This sequential release caused a synergistic effect in U87MG glioblastoma cells (Fan et al., 2015).

Magnetic iron oxide nanoparticles have been widely used as a magnetic resonance contrast agent, due to their safety and biocompatibility (Huang et al., 2011). More recently, their potential as a drug delivery system has called the attention of researchers. An interesting example is the work developed by Sun and coworkers, who developed a miRNA delivery probe using PEG-coated iron oxide nanoparticles as both carrier and as contrast agent. The delivery efficiency and therapeutic effect of miRNA-16 was assessed in both in vitro [a drug resistant gastric cancer cell line (SGC7901)] and in vivo models. Since the magnetic nanoparticles exhibited a positive charge, miRNA-16 was linked by electrostatic interactions with the nanoparticles surface. The developed nanoparticles showed tumor accumulation (visualized in vivo by fluorescence imaging), allowing cellular miRNA-16 uptake, together with Bcl-2 downregulation and remarkable resensitization of cells to adriamycin (Sun et al., 2014).

The use of quantum dots is an efficient method to achieve gene delivery because they are luminous and can be used as fluorescence probes for living cells and in vivo cancer targeting and diagnosis (Gao et al., 2004, 2005; Jamieson et al., 2007). Quantum dots are made from hundreds to thousands of atoms from the group II and IV elements (e.g., CdSe and CdTe) or from the group III and V elements (e.g., InP and InAs). The quantum dots can be polymer-encapsulated, in order to provide clinical application avoiding cytotoxicity. Consequently, polymer-coated quantum dots can be actively targeted by binding ligands having affinity to target cells and also pegylated to avoid opsonization (Gao et al., 2005). Dong et al. developed a multifunctional nanocomposite of PLA and PEG-grafted

graphene quantum dots, for simultaneous intracellular miRNAs imaging analysis and combined gene delivery to enhance therapeutic efficacy. The developed nanocomposite showed physiologic stability, biocompatibility, low cytotoxicity, and effectively delivered the miRNA probe for intracellular miRNA imaging analysis and regulation (Dong et al., 2015).

Additionally, other inorganic materials have shown to have potential for the delivery of miRNA modulators such as silica, calcium phosphate, and graphene oxide (GO) (Bertucci et al., 2015; Zhang et al., 2015a; Wang et al., 2016a)

GO is a good vector for gene and drug delivery due to its excellent physicochemical, electrical and optical properties (Yang et al., 2013). DNA, RNA and drugs can be effectively loaded into GO sheets through electrostatic, $\pi-\pi$, or other molecular interactions (Zhang et al., 2010b; Yin et al., 2016; Lu et al., 2010). In a recent work, nano-GO was modified with PEG and low molecular weight PAMAM dendrimer to deliver anti-miR-21. The developed system presented markedly higher transfection efficiency and lower cytotoxicity than bare dendrimers and a commercial transfection reagent. Additionally, nano-GO-PEG-dendrimer/anti-miR-21 effectively inhibited A549 cells migration. An activatable luciferase reporter, containing three perfectly complementary sequences against miRNA-21 in the 3' untranslated region (3' UTR) of the reporter, for monitoring nano-GO-PEG-dendrimer nanocarrier-based anti-miR delivery in a xenograft model was also developed, allowing to conclude that the developed system could potentially be applied for theranostic purposes (i.e., concomitantly promoting treatment and diagnosis) (Wang et al., 2016a).

To summarize, in addition to the advantages exhibited by other nanocarriers, inorganic nanoparticles can also act as imaging agents, allowing theranostics effects (Kievit and Zhang, 2011).

14.4 CLINICAL TRIALS AND FUTURE PERSPECTIVES

The first miRNA-based therapy entering clinical trials was MRX34 in 2013, which was patented by Mirna Therapeutics. This consisted of miRNA-34a mimics delivered in liposomes containing amphoteric lipids, which offered the possibility of being positively charged at acid pH and anionic at higher pH (SMARTICLES). Accordingly, it was possible to achieve high miRNA-34a incorporation at a lower pH, while at physiologic pH the anionic charge present on the surface of the vectors prevented vesicle aggregation. The MRX34 was studied as a single agent in a multicenter Phase I clinical trial, including patients with primary liver cancer, other solid tumors, and hematological malignancies (lymphomas and leukemia). The objective was to replace miRNA-34a expression levels since it acts as tumor suppressor and has been found downregulated in a variety of cancers. In

September 2016, the clinical trial was voluntary halted by Mirna Therapeutics, due to multiple immune-related severe effects observed in patients over the course of the trial ([MirnaTherapeutics, 2016](#)).

Although currently there are no active clinical trials with miRNA modulators, there are a growing number of studies reporting the therapeutic potential of miRNAs in cancer. Conversely, there are various clinical trials ongoing for siRNAs delivery, mainly with liposomes. Accordingly, it is expected that new products based on miRNA mimics and anti-miRs will reach clinical trials in the near future.

14.5 CONCLUSIONS

It is currently well known that miRNAs play an important role in cancer pathogenesis. Depending on the cancer type, their upregulation or suppression constitutes a promising strategy for cancer treatment. Despite the numerous efforts portrayed in the scientific literature, demonstrating the effectiveness of these molecules in numerous cancer models, their possible future clinical translation into viable therapies is still very challenging. To allow their possible future efficient clinical application, a delivery system must be developed that is able to overcome the delivery barriers to the target tissue and cells, while protecting these labile molecules and avoiding triggering unwanted immune responses.

In this review, we have reported several carriers for the delivery of miRNA modulators, which have been demonstrated to be effective both *in vitro* and *in vivo*. Although the use of viral vectors has often shown greater efficiency than the nonviral systems, preclinical studies have shown that they can trigger strong inflammatory reactions. Therefore, to avoid these drawbacks, viral vectors have been replaced by nanotechnological carriers such as liposomes, micelles or nanoparticles, among others. From the various possible approaches, the most studied ones to deliver miRNA mimics or anti-miRs are the liposomal and polymeric nanoparticles. To date, there are few studies involving EVs, although these seem to be a promising approach since they are produced by human cells, which makes them less immunogenic and potentially less cytotoxic to nontumor cells than other nanotechnological delivery systems. To date, the only clinical trial carried out involving a miRNA mimic used a liposomal formulation as delivery system. Unfortunately, this trial was suspended due to immunologic adverse reactions.

In conclusion, although significant progress has been made in confirming the ability to deliver miRNAs and anti-miRs to tumor cells, further improvements will be necessary. The development of a suitable delivery system will be essential for attaining the aim of taking miRNA modulators to the clinic.

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Oral controlled and sustained drug delivery systems: Concepts, advances, preclinical, and clinical status

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CHAPTER OUTLINE

15.1 Introduction	568
15.1.1 Overview and Classification of Oral Controlled Release Dosage Forms.....	568
15.1.2 Overview of Oral Sustained Release Dosage Forms	569
15.2 Ideal Properties of Drugs for Oral Controlled and Sustained Drug Delivery	570
15.2.1 Physicochemical Properties.....	570
15.2.2 Biological Properties.....	572
15.3 Polymers for Fabrication of Oral Modified Drug Delivery Systems	573
15.3.1 Polymers for Oral Matrix Systems.....	574
15.3.2 Polymers for Oral Membrane Controlled Systems.....	576
15.3.3 Polymers for Bioadhesive Controlled Release Systems	577
15.3.4 Stimuli-Responsive Polymers.....	578
15.4 Oral Controlled Release Dosage Forms	581
15.4.1 Design and Concepts in Oral Controlled Release Dosage Forms	581
15.4.2 Biopharmaceutics and Preformulation Considerations in Oral Controlled Release Dosage Forms.....	584
15.4.3 Coating Systems for Oral Controlled Release Systems	588
15.4.4 Challenges and New Technologies in Oral Controlled Release Drug Delivery	590
15.4.5 Quality by Design Approach in Oral Controlled Release Drug Delivery	597
15.4.6 Advantages and Disadvantages of Controlled Release Drug Delivery Systems	599

15.5 Oral Sustained Release Dosage Forms	600
15.5.1 Design and Fabrication of Oral Sustained Release Systems	600
15.5.2 Potential Advantages and Limitations of Oral Sustained Release Dosage Forms	604
15.5.3 Key Formulation Considerations in Oral Sustained Release Dosage Forms	605
15.5.4 Coating Systems for Oral Sustained Release Dosage Forms	607
15.6 Preclinical and Clinical Status of Oral Controlled and Sustained Release Dosage Forms	616
15.7 Conclusion	616
References	616
Further Reading	626

15.1 INTRODUCTION

15.1.1 OVERVIEW AND CLASSIFICATION OF ORAL CONTROLLED RELEASE DOSAGE FORMS

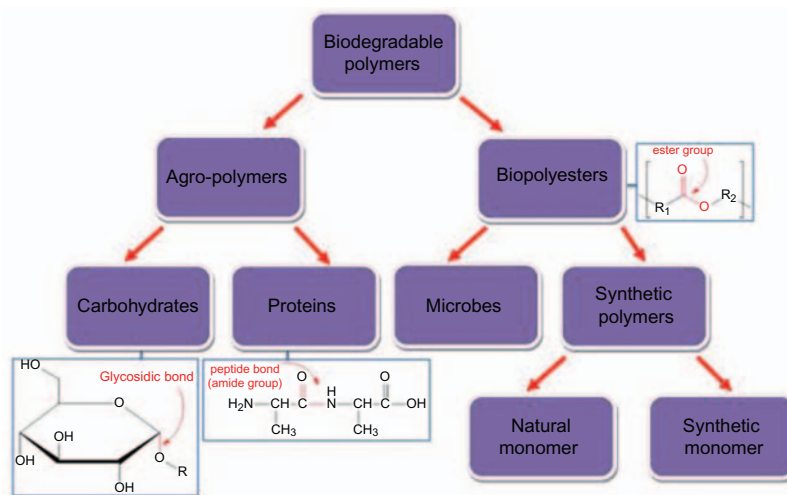
The oral route is the main acceptable route for drug administration owing to ease of administration and gastrointestinal physiology offers extra flexibility in dosage form design in comparison to other routes. Moreover, the oral route is associated with high patient compliance and cost-effective manufacturing processes. The sustained release, prolonged release, modified release, extended release, or depot formulations are categorized under drug delivery systems that are customized to improve therapeutic effect by continuously releasing medicament over an extensive time phase following administration of a solitary dose. The controlled release system delivers a constant supply of the drug, usually at a zero-order rate for a definite period of time (Fig. 15.1). Moreover, an ideal controlled drug delivery system delivers the drug at a predetermined rate either locally or systematically, for a definite period of time. Collectively, these dosage forms are termed modified release dosage forms (Abdul and Poddar, 2004). Consequently, modified release drug delivery systems can be classified in the following categories.

15.1.1.1 *Controlled release dosage forms*

These dosage forms incorporate a group of pharmaceuticals or other biologically dynamic products of which a drug is released in a predictable, planned, and slower than-normal manner for a prolonged period of time. Furthermore, controlled release dosage forms can be sub-classified as:

15.1.1.1.1 Sustained release

These are intended to attain extended therapeutic effect by releasing the medicament over a prolonged period of time after administration of a single dose of drug (Fig. 15.1).

**FIGURE 15.1**

Schematic representation of plasma concentration-time profile curves of conventional oral dosage form, controlled release oral dosage form, and sustained release oral dosage form.

15.1.1.1.2 Extended release

Extended release dosage forms are pharmaceutical dosage forms that liberate the drug at predetermined rate slower than the normal rate and essentially decrease the dosage frequency by twofolds.

15.1.1.1.3 Prolonged release system

These systems are designed to offer slow and constant release of drug over an extensive time period. Moreover, these systems also prevent quick absorption of the drug, which may result in exceedingly high peak plasma drug concentration.

15.1.1.2 Delayed release

Delayed release systems constitute repetitive and discontinuous dosing of a drug from one or more immediate release units built into a single dosage form.

15.1.2 OVERVIEW OF ORAL SUSTAINED RELEASE DOSAGE FORMS

The most convenient route of administration of drugs is oral drug delivery system. Conventional dosage form—related factors such as fluctuation in plasma drug concentration, undesirable tissue distribution, toxicity, poor efficiency, repetitive dosing, and unpredictable absorption lead to the idea of oral sustained release drug delivery systems. Sustained release dosage forms offer advantages over conventional drug delivery systems (tablet and capsules) by augmenting physico-chemical traits, pharmacokinetic (PK) and pharmacodynamic (PD) properties, and

reducing the dosing frequency. Thus, once daily dosage form maintains uniform plasma concentration and thereby augments the utility of drug in addition to reduction in local and systemic side effects. This also assures high patient compliance. A sustained release system generally tries to mimic zero-order release by providing drug in a slow first-order.

15.2 IDEAL PROPERTIES OF DRUGS FOR ORAL CONTROLLED AND SUSTAINED DRUG DELIVERY

The design of a controlled release system depends on various physicochemical and biological properties of the drug.

15.2.1 PHYSICOCHEMICAL PROPERTIES

15.2.1.1 *Aqueous solubility*

Drugs with low aqueous solubility seem to be poor candidates for oral controlled release formulations. Controlled release formulations for such drugs aim at making their dissolution more uniform rather than lowering the dissolution rate. Drugs that have good solubility in the stomach are also a poor choice for controlled or sustained oral dosage forms (Shokri et al., 2008; Jacob et al., 2005). The water solubility limits the loading efficiency of drug into a variety of carrier systems such as liposomes and microparticles because highly water soluble drugs tend to leach rapidly from the carrier. The pH-dependent solubility of drug, particularly in the physiological pH range, would be another trouble for controlled release dosage form because of the difference in pH throughout the gastrointestinal tract and distinction in the dissolution rate. The biopharmaceutical classification system (BCS) allows estimation of contribution of three major factors: solubility, dissolution, and intestinal permeability that eventually influence oral absorption. Class III (high solubility-low permeability) and Class IV (low solubility-low permeability) category of drugs are poor candidates for translating into a controlled release dosage form.

15.2.1.2 *Partition-coefficient*

The partition-coefficient can be defined as the partition of a neutral noncharged species in to oil or water phase. Drugs with high partition-coefficient value can easily permeate through biological membrane. The diffusion of drug molecules across rate-controlling membrane or through the matrix system essentially relies on the partition-coefficient. Drugs having lower partition-coefficient value are not suitable for translating in to oral controlled release formulations and drugs that have higher partition-coefficient are also poor candidates for oral controlled formulations. In contrast, ionic drugs depending on the pH partition into charged and noncharged species, and can be measured at various pH values. The distribution

constant at pH ~ 7.4 is most relevant to biological systems (Allen et al., 2004). For oral absorption, the pH partition profile over a pH range of 1–8 is pertinent to the GI absorption window. For controlled release, a pH partition over pH 1–8 is more valuable, since a wide absorption window utilizing the entire GI tract is needed. Drugs depicting high partition coefficient values, i.e., highly lipid-soluble or highly water soluble, exhibit either low flux into the tissues or rapid flux followed by accretion in tissues. Although both cases are undesirable for sustained release systems.

15.2.1.3 Stability of the drug

Drug in a solid-state undergoes degradation at a much slower rate than a drug formulated as suspension or solution. Consequently, drug unstable in gastric pH can be developed as a slow release dosage form and the drug release can be delayed until the dosage form reaches the intestine. Drugs that become metabolized in the gut-wall and show instability in small intestine are not suitable for oral-controlled drug delivery systems. As most oral controlled release systems intend to release their contents throughout the GI tract, drugs that are unstable in the environment of intestine are difficult to translate into prolonged release system (Vyas and Khar, 2000).

15.2.1.4 Size of the dose

A controlled release dosage form may require a large quantity of drug, as in the case of those drugs exhibiting elimination half-life less than 2 h and administered in large dosages.

15.2.1.5 Molecular size and diffusivity

Diffusivity can be defined as the ability of a drug to diffuse through the membrane and is inversely related to the molecular size. Diffusivity also depends on size and shape of the cavities of the membrane. About 95% of drugs are absorbed by passive diffusion. The upper limit of drug molecular size for passive diffusion is 600 Da. Few drugs which are difficult to transform in to controlled release dosage forms are peptides and proteins. In many sustained release systems, drugs ought to diffuse through a rate controlling membrane or matrix, apart from diffusion through a variety of biological membranes. The ability of a drug to pass through the membrane is a function of its molecular size or molecular mass. In polymers, the molecular size of the diffusing species have a vital effect on the value of diffusivity “D.” Accordingly, the D value is associated with size and shape of the cavities, and size and shape of the drugs. Generally, the value of diffusion coefficient for intermediate molecular weight drugs (150–400 Da) through flexible polymers ranges from 10^{-6} to 10^{-9} cm²/s and 10^{-8} cm²/s is most common. Therefore, in sustained release systems, very slow release kinetics is displayed by high molecular weight drug when released by diffusion through the polymeric membrane or matrix (Allen et al., 2004; Vyas and Khar, 2000).

15.2.1.6 Drug pKa and ionization of the drug

For controlled release formulation, the pH-dependent solubility of the drug is more significant because absorption of the drug will take place through the entire GI tract, having a pH gradient from 1 to 8. Consequently, dissolution behavior continuously changes with change in pH value. Achieving pH-independent dissolution for a drug having pH-dependent solubility is the major challenge in the development of a controlled release dosage form. The ionized form of a drug is a poor candidate for oral controlled release drug delivery system, as the absorption rate of ionized drug is 3–4 times smaller than that of unionized form. The pKa of acidic drugs in the range of 3.0–7.5 and 7.0–11.0 for basic drugs is ideal for optimum positive absorption.

15.2.2 BIOLOGICAL PROPERTIES

15.2.2.1 Absorption

Slowly absorbed drugs or drug with variable absorption rates are poor candidates for a controlled release system. Potent drugs, although water soluble but poorly absorbed (i.e., absorbed by carrier-mediated transport processes or through absorption window) are poor candidates for controlled release systems.

15.2.2.2 Metabolism

The outcome of drug metabolism is either inactivation of an active drug or conversion of an inactive drug to an active metabolite. It takes place mainly in liver, as it contains a variety of enzymes. Therefore the route of drug administration also depends upon the drug metabolic pattern. There are two major factors associated with metabolism that significantly govern the design of controlled release product. First, it would be difficult to maintain uniform plasma levels of drug capable of either inducing or inhibiting enzyme synthesis, upon chronic administration. Second, if the drug undergoes intestinal (or other tissue) metabolism or hepatic first pass metabolism, this will also result in fluctuating drug plasma level. Controlled release products for extensively metabolized drugs can be generated successfully; however, location, rate, extent and metabolism should be known and the rate constant(s) should not be too large. It can be assumed that a controlled release product can be developed but its pattern of metabolism must be predictable.

15.2.2.3 Elimination or biological half-life

The rate of drug elimination quantitatively defines its biological half-life. Drugs with short half-life and high dose impose constraints, as the controlled release system of such drugs demands large dose size and those with long half-lives are inherently controlled.

15.2.2.4 Safety considerations and side-effects

For certain drugs, side-effect is supposed as a function of plasma concentration. Through a controlled release system, side-effects for a particular drug can be minimized by controlling its plasma concentration and consuming a lesser dose over the course of therapy. Drugs with narrow therapeutic index (TI) are usually poor candidates for customizing into controlled release products, primarily because of technical limitations of precise control over release rates. A drug with TI value exceeding 10 is considered relatively safe.

15.2.2.5 Protein binding

The characteristics of the protein binding of a drug play a significant role in its therapeutic effect, regardless of the type of dosage form. Drugs displaying extensive plasma protein binding have long elimination half-life and do not require a sustained release dosage form.

15.2.2.6 Disease state

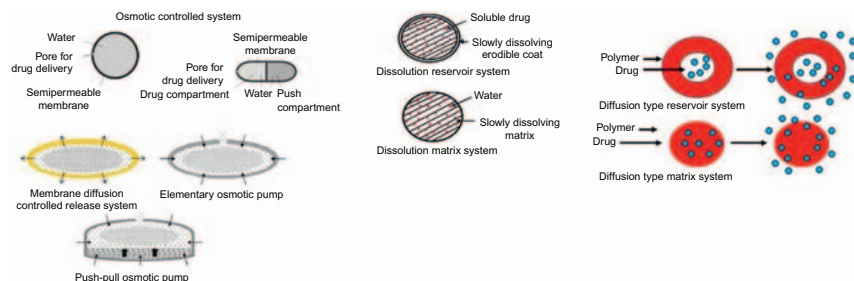
For considering a drug under controlled release system, the disease state is an important factor. The drug formulated as a controlled release system offers improved disease management. For example, in rheumatoid arthritis, sustained release form of aspirin would provide desired drug plasma levels, particularly throughout the night, and accordingly will relieve morning stiffness. Other examples include nitroglycerin in the management of angina pectoris, and belladonna alkaloids and synthetic anticholinergics in the treatment of peptic ulcers.

15.2.2.7 Circadian rhythm

The circadian rhythm affects several biological parameters such as liver enzyme activity, blood pressure, intraocular pressure, and disease states (asthma, acute myocardial insufficiency, and epileptic seizures). Therefore, for certain drugs such as digitalis glycosides, diuretics, amphetamines, barbiturates, carbamazepine, ethyl alcohol, and chlordiazepoxide, the pharmacological response displays a time-dependent nature.

15.3 POLYMERS FOR FABRICATION OF ORAL MODIFIED DRUG DELIVERY SYSTEMS

There are a lot of different synthetic polymers, but not many have been used in the pharmaceutical industry, especially in oral controlled release formulations. The most common synthetic polymers used in oral controlled release formulations are poly(vinyl alcohol), poly(acrylic acid), poly(ethylene oxide), poloxamers, pluronics, and polymethacrylate. Poly(acrylic acid) and its derivatives are often used in enteric coating owing to their insolubility at low pH. Carbopol is a high molecular weight cross-linked poly(acrylic acid) polymer. Polymethacrylate and

**FIGURE 15.2**

Classification of biopolymers used in oral drug delivery systems.

derivatives are the main constituents of Eudragit and frequently used for sustained release coating. Polymers are widely used for developing various modified release pharmaceutical products, as shown in Fig. 15.2. The release rate of a drug from an oral delivery system can be controlled by developing either matrix systems or membrane-controlled release systems. Both systems can be fabricated using polymers. In general, polymers from both natural and synthetic sources are employed for developing oral modified release formulations. Synthetic polymers are generally preferred over natural polymers due to their consistency in quality and other desirable properties. Polymers used in engineering the oral modified release formulations include albumin, sodium alginate, carbomers, carboxy methyl cellulose, chitosan, dextrin, ethyl cellulose (EC), gelatin, guar-gum, hydroxyl ethyl cellulose (HEC), hydroxyl propyl cellulose, hypromellose, maltodextrin, methyl cellulose, microcrystalline cellulose, polycarbophil, polydextrose, polyethylene glycol, povidone, tragacanth, and xanthan gum (Nicolas and Couvreur, 2017; Zołtowska et al., 2017; Sathuvan et al., 2017; Tabasum et al., 2017; Sharma et al., 2017; Debotton and Dahan, 2017; Jain et al., 2013).

15.3.1 POLYMERS FOR ORAL MATRIX SYSTEMS

Hydrophilic matrix systems are popular and broadly used systems to achieve controlled drug release since the 1950s. Polymers for oral hydrophilic matrix systems are generally water soluble, gel-forming, and swellable. Natural hydrophilic swellable polymers include chitosan, pectin, and alginate. The synthetic or semisynthetic hydrophilic polymers include cellulose derivatives and carbomers. Drug release from hydrophilic matrix tablets is controlled by the formation of a hydrated viscous layer around the dosage form, which acts as a barrier to drug release and thereby opposes penetration of water into tablet. This subsequently controls the release of dissolved solutes from the matrix (Jedinger et al., 2014; Ma et al., 2014; Moodley et al., 2012; Aminabhavi et al., 2014).

15.3.1.1 Chitosan

Chitosan, a poly(amino saccharide), has been used as a hydrophilic matrix material due to its swellability and erodibility. Chitosan is produced by partial alkaline deacetylation of chitin. The term chitosan refers to a family of amino polysaccharides, which can be differentiated according to their degree of deacetylation (2%–60%) and molecular weight (50–2000 kDa). Chitosan is used for formulating polymeric beads, tablets, floating microspheres, and colon-specific drug delivery systems (Jyoti et al., 2016; Kaur et al., 2015; Kumar et al., 2013; Kulkarni et al., 2015).

15.3.1.2 Alginate

Alginate is one of the most studied and applied natural polysaccharide polymers in oral controlled delivery systems. It is composed of sequential β -D-mannuronic acid monomers (M-blocks), α -L-guluronic acid (G-blocks), and interconnected M and G units. The ability of sodium alginate to rapidly generate viscous solution upon contact with aqueous medium and to form a gel upon contact with acid or di- or trivalent cationic ions is ideal for its use as a hydrophilic matrix in oral controlled release dosage forms. Matrices in the form of microparticles or tablets incorporating alginate salts or a combination of alginate with other polymers have been successfully employed to prolong the release of many drugs. It is used for the preparation of microcapsules for cells, microparticles of peptides and matrix tablets (Awasthi et al., 2017; Wang et al., 2016; Fan et al., 2016; Pistone et al., 2017).

15.3.1.3 Pectin

Pectin displays excellent swelling and erosion properties and is accordingly useful for colon targeting systems. Pectin is a complex polysaccharide consisting mainly of esterified D-galacturonic acid residues linked by α -(1–4) chain. The acid groups along the chain are largely esterified with methoxy groups in the natural product. Pectin is widely explored for fabricating the colon-biodegradable drug delivery systems. Moreover, pectin can be used as an adsorbent, emulsion stabilizer, bulk-forming agent and coating material for tablets, pellets, microparticles, and beads (Dafe et al., 2017; Izadi et al., 2016; Islan et al., 2015; Keohane et al., 2016; Sawarkar et al., 2015).

15.3.1.4 Hydroxypropyl methylcellulose

Hydroxypropyl methylcellulose (HPMC) or hypromellose refers to soluble methylcellulose ethers. HPMC is used as a thickening agent, binder, film former, and hydrophilic matrix material. HPMC polymers for fabricating hydrophilic matrix systems are available in various viscosity grades ranging from 4000–100,000 mPa s. HPMC is a popular matrix material in oral controlled delivery systems and HPMC matrices show sustained release pattern by two mechanisms, i.e., diffusion and erosion of the gel layer. The viscosity of the polymer affects the diffusion pathway. HPMC can

be employed as a matrix for controlling the release of both hydrophilic and hydrophobic drugs. It is being used in buccal adhesive tablets of pentazocine, gastroretentive systems, and colon delivery systems (Al-Tabakha, 2010; Caraballo, 2010).

15.3.1.5 Other cellulose ethers

Cellulose derivatives, such as hydroxypropyl cellulose (HPC) and HEC, are also used to yield desirable drug release rates in oral controlled delivery systems. The molecular weights depend on the degree of polymerization of the cellulose backbone, which in turn, controls the viscosity of HPC. Higher viscosity grades of HPC and HEC have been used as hydrophilic matrix materials for several purposes such as fabrication of bioadhesive sustained release systems and gastric buoyant sustained release tablets (Moscovici, 2015).

15.3.2 POLYMERS FOR ORAL MEMBRANE CONTROLLED SYSTEMS

An osmotic pump is composed of a core and a semipermeable coating, where the core contains drug and osmotic agents. Polymers used for semipermeable coating are generally insoluble materials bearing adequate film-forming characteristics. Numerous natural, semisynthetic, and synthetic polymers are being used to fabricate osmotic pumps or microosmotic delivery systems.

15.3.2.1 Cellulose acetate

Cellulose acetate is an insoluble cellulose derivative regarded as a nontoxic, non-irritant, and biodegradable material. It is heat-resistant and less hygroscopic. Cellulose acetate is partially acetylated cellulose, in which the acetyl content ranges from 29.0% to 44.8%, corresponding to mono-, di-, and triacetate. Cellulose acetate is used for constructing semipermeable coating on tablets, especially in osmotic pump-type tablets and microparticles for controlled release of drugs. Cellulose acetate films, in conjunction with other materials, offer controlled release without any necessity of drilling a hole in the coating as with typical osmotic pumps (Riaz and Masud, 2013; Arjun et al., 2016).

15.3.2.2 Ethyl cellulose

EC is hydrophobic cellulose ether used as a coating material, tablet binder, and matrix former in microcapsules, microspheres, and controlled release dosage forms. EC membrane is not swellable and is compatible with most of the plasticizers and pore-forming substances. Therefore, it is possible to adjust the release rate of drugs by optimizing the coating solution without laser drilling for osmotic pump delivery systems. Plasticizers or softening agents are added to EC to obtain proper degree of suppleness, lower the softening point, and improve its thermoplasticity. When EC is used as a coating material for pellets, pore-forming agents such as poly(ethylene glycol) are usually included (Murtaza, 2012).

15.3.2.3 Polymethacrylates

Acrylic resins such as polymethacrylates have brilliant film-forming characteristics and are used for coating of tablets, pellets, capsules, and granules. Acrylic resins are pharmacologically inactive and have good compatibility with skin and mucosal membranes. Eudragit is a trade name of copolymers derived from esters of acrylic and methacrylic acids, whose properties are determined by functional groups. Eudragit grades vary in their proportion of neutral, alkaline, or acid groups, resulting in different physicochemical properties. Enteric methacrylate polymers such as Eudragit L/S coatings provide a better barrier against drug release in the stomach and enable controlled release in the intestine. Enteric Eudragit FS is used for controlled release of drugs in the colon (Brunner et al., 2006). Eudragit RS and RL types are widely used in controlled release coating. The amount of quaternary ammonium groups in the RS type is between 4.5% and 6.8%, and in the RL type it is ranged from 8.8% to 12%. Both RS and RL types are insoluble at physiological pH values and capable of swelling. Eudragit RL100 and RS100 are pH-independent cationic polymers having low chemical reactivity (Dillen et al., 2006). Eudragit RL films are more permeable than the Eudragit RS films. These methacrylate polymers are dissolved in organic solvents such as acetone and isopropanol for coating applications. Organic coating dispersions of methacrylates are generally turbid and disturb the coating process. Incorporation of a small amount of water improves the appearance of organic coating dispersions. Aqueous dispersions of polymethacrylates (Eudragit RL 30D and Eudragit RS 30D) are being used for coating applications in recent years (Zimmer et al., 2014).

15.3.3 POLYMERS FOR BIOADHESIVE CONTROLLED RELEASE SYSTEMS

A bioadhesive polymer adheres to a biological surface such as mucus and provides an extended time for drug absorption. Bioadhesive controlled delivery systems are emerging as potential drug delivery systems, due to their applicability for prolonging gastrointestinal residence time of dosage forms by enhancing contact between the drug and its absorbing surface, and hence improving bioavailability. Many hydrophilic polymers, such as carbopol, alginate, polyethylene oxide, and pectin, adhere to mucosal surfaces upon hydration at the mucus gel layer of the epithelial surface. This mechanism of adhesion is known as adhesion by hydration. Bioadhesion of a polymer depends on the porosity, flexibility, and hydration rate. The force of attachment is related to the depth of interpenetration of polymers and mucus. Nonhydrogel forming polymers that contain polar groups or active interaction sites also show intense bioadhesive properties by formation of chemical bonds such as ionic bonds, van der Waals interactions, or hydrogen bonds with the mucus. Many synthetic polymers such as cellulose phthalate, carboxyethyl cellulose, HPC, hydroxyethyl cellulose, HPMC, hydroxyethyl

methacrylate, polyvinylpyrrolidone, polyvinyl alcohol, pectin, polycarbophil, polyacrylic acid, and polymethacrylic acid; and natural polymers such as chitosan and sodium alginate exhibit bioadhesive properties. Recently, thiolated polymers have been investigated as mucoadhesive materials. Polyacrylic acid polymers (carbomers) and polyethylene oxides (Polyox) have been successfully used in bioadhesive drug delivery systems and commercialized (Mizrahi and Domb, 2008).

15.3.3.1 Carbomers

Carbomers are acid-based polymers and demonstrate good mucoadhesion, particularly at low pH value where they present in a protonated state. They are high molecular weight and cross-linked acrylic polymers. Depending upon the degree of cross-linking and manufacturing conditions, various grades of carbomers are available. Each grade has its own significance and applicability in pharmaceutical dosage forms. Carbopol 934 is cross-linked with allyl sucrose and polymerized in benzene. Carbopol 71, 971, and 974 are cross-linked with allylpentaerythritol and polymerized in ethyl acetate. Carbomer polymers contain 56%–68% of carboxylic acid ($-\text{COOH}$) groups and 0.75%–2% of cross-linking agents. Carbomers absorb water readily upon contact with water, become hydrated, and swell. In tablets, carbomers are employed as a binder and control the release of active medicament. It is suitable for both wet and dry granulation processes. Aqueous, hydroalcoholic, anhydrous organic solvent dispersions of carbomers are used in wet granulation processes. Carbomers are also used in the preparation of controlled release matrix beads and microspheres. Moreover, bioadhesive oral formulations containing carbomers increase the gastrointestinal residence time and improve the bioavailability of drugs (LaFontaine et al., 2017; Singla et al., 2000).

15.3.3.2 Polyethylene oxide

Polyethylene oxide is a nonionic homopolymer of ethylene oxide. Polyethylene oxide is an excellent bioadhesive polymer and employed as a binder. Moreover, this polymer controls the release of component in tablet formulations. These polymers show pH-independent swelling, a property that is considered superior in comparison to other bioadhesive and swelling type polymers. Polymeric compacts made of HPMC, carbopol 974, and polyethylene oxide show regional variations in their adhesion with porcine buccal, gastric, and intestinal mucosa. The swelling, bioadhesion, and rate of drug release from polyethylene oxide matrices can be modified by mixing with other hydrophilic polymers such as polyvinylpyrrolidone (Merkle, 2015).

15.3.4 STIMULI-RESPONSIVE POLYMERS

Stimuli-responsive polymers offer a drug delivery platform which delivers drugs at a controlled rate and in a stable and biologically active form. A stimuli-sensitive or smart polymer undergoes an abrupt change in its physical attributes

in response to a small environmental stimulus. These polymers are also named intelligent polymers, as a small trigger turns on the release of active component and polymers regain their shape as the trigger turns off. These reversible transitions comprise of changes in physical state, shape, solubility, solvent interactions, hydrophilic and lipophilic balances, and conductivity. The driving forces behind these transitions include neutralization of charged groups by the addition of oppositely charged polymers or by pH shift and change in the hydrophilic/lipophilic balance, or changes in hydrogen bonding due to increase or decrease in temperature. The benefits of smart polymer-based drug delivery systems include reduced dose-dosing frequency, ease of preparation, maintenance of desired therapeutic concentration with a single dose, prolonged release of incorporated drug, reduced side-effects and improved stability. Responsiveness of a polymeric solution initiated by physical or chemical stimuli is limited to the destruction and formation of various secondary forces such as hydrogen bonding, hydrophobic forces, van der Waals forces, and electrostatic interaction. Chemical events include simple reactions such as oxidation, acid-base reaction, reduction, and hydrolysis of moieties attached to the polymer chain. In a few cases, dramatic conformational change in the polymeric structure occurs, such as degradation of the polymeric structure due to irreversible bond breakage in response to an external stimulus (Jones and Steed, 2016).

15.3.4.1 Temperature-responsive polymers

Thermosensitive polymers undergo unexpected change in their solubility in response to a small change in temperature. An aqueous thermosensitive polymeric solution exhibits temperature-dependent and reversible sol-gel transitions close to body temperature that control the rate of release of incorporated drug along with maintaining physicochemical stability and biological activity. The most commonly used thermosensitive polymers include poly(*N*-isopropylacrylamide), poly(*N,N*-diethylacrylamide), poly(*N*-vinylalkylamide), poly(*N*-vinylcaprolactam), phosphazene derivatives, pluronics, tetronics, polysaccharide derivatives, chitosan and poly(lactide-co-glycolide)-poly (ethylene) glycol-poly (lactide-co-glycolide) triblock copolymers (Thakur et al., 2016; Klouda, 2015).

15.3.4.2 pH-responsive polymers

All pH-sensitive polymers consist of pendant acidic or basic group and release a proton in reaction to alterations in environmental pH. As the environmental pH changes, the pendant acidic group undergoes ionization at specific pH called as pK_a. This rapid change in net charge of the attached group causes modification in the molecular structure of the polymeric chain. This transition to expanded state is mediated by the osmotic pressure exerted by mobile counter ions neutralized by network charges. Poly (*N,N*-dimethylaminoethyl-methacrylate) and poly(*N,N*-diethylaminoethyl-methacrylate) are the most commonly used pH-responsive polymeric bases (Du et al., 2015; Shen et al., 2013).

15.3.4.3 Bioresponsive polymers

Biologically responsive polymer systems are very important in various biomedical applications. The greatest advantage of bioresponsive polymers is that they can respond to the stimuli that are inherently present in the natural system. Bioresponsive polymers can be classified as antigen-responsive polymers, glucose-sensitive polymers, and enzyme-responsive polymers.

15.3.4.3.1 Glucose-responsive polymers

Glucose-responsive polymers have the ability to mimic normal endogenous insulin secretion, which minimizes diabetic complications and can release the bioactive compound in a controlled manner. These are sugar-sensitive and show variability in response to the presence of glucose (Vancoillie and Hoogenboom, 2016; Li et al., 2016).

15.3.4.3.2 Field-responsive polymers

Field-responsive polymers show a response to an application of electric, magnetic, sonic or electromagnetic fields. The additional benefit over traditional stimuli-sensitive polymers is their fast response time, an isotropic deformation due to directional stimuli, and also a controlled drug release rate simply by modulating the point of signal control (Zhou et al., 2016; Sung et al., 2016).

15.3.4.3.3 Light-sensitive polymers

A light-sensitive polymer undergoes a phase transition in response to exposure to light. On the basis of the wavelength of light that triggers the phase transition, these polymers can be classified into UV-sensitive and visible-sensitive systems. Naturally occurring polymers such as chitosan, alginate, and hyaluronic acid are commonly employed to prepare light-responsive materials. The most important synthetic polymers that have been used include allylamine, vinyl alcohol, acrylonitrile, methacrylic acid and vinylacrylic acid (Chiappetta et al., 2010; Morales et al., 2015; Ahmed et al., 2016).

15.3.4.3.4 Hydrogels

Hydrogels are three-dimensional high-molecular weight networks comprising of a polymer backbone, water, and a cross-linking agent. Moreover, hydrogels are polymeric materials that do not dissolve in water at physiological temperature and pH. They swell significantly in aqueous medium and demonstrate extraordinary capacity (> 20%) for imbibing water into the network structure. Gels exhibiting a phase transition in response to change in external conditions, such as pH, ionic strength, temperature and electric currents, are known as stimuli-responsive or “smart” gels. Hydrogels have been developed as stimuli-responsive materials, which undergo unexpected volume change in response to small changes in environmental parameters, such as temperature, pH, ionic strength, etc. (Hamlet et al., 2017; Manga and Jha, 2017; Huang et al., 2017).

15.4 ORAL CONTROLLED RELEASE DOSAGE FORMS

15.4.1 DESIGN AND CONCEPTS IN ORAL CONTROLLED RELEASE DOSAGE FORMS

Oral controlled release formulations are mainly designed on the basis of physical mechanisms rather than chemical degradation, enzymatic degradation, or prodrug approach. Therefore, controlled release formulations can be designed on the basis of a single or couple of mechanisms (Ummadi et al., 2013).

15.4.1.1 *Diffusion-controlled products*

In diffusion-controlled formulations, drug molecules diffuse through a polymeric membrane (Lachman and Lieberman, 2002). Diffusion occurs through pores in the polymer matrix or passes through the polymer chains. Diffusion-controlled formulations can be divided into reservoir and monolithic systems, subject to whether a drug is surrounded by a polymer membrane or distributed through the polymer matrix. Water-insoluble polymeric material in reservoir system encloses a core of drug. The rate-limiting membrane controls the diffusion of active ingredient into the surrounding environment. The drug delivery rate remains moderately constant in reservoir systems. In nonporous reservoir systems, drug molecules must diffuse through the polymer membrane, but in microporous reservoir systems, drug molecules are released by diffusion through micropores that are generally filled with either water or oil. In addition to nonporous and microporous systems, diffusion-controlled monolithic systems can be further classified based on the concentration of loaded drug. The monolithic system is called a monolithic solution if a drug is loaded by soaking a polymer matrix with a drug solution, in which drug concentration inside the matrix cannot be higher than its solubility, if the partition coefficient of a drug is 1.

The matrix system in the matrix devices contains drug or active component that is dispersed in the polymer matrix to form an homogeneous system (Kar et al., 2009). Diffusion occurs when the drug passes from the polymer matrix into the external environment. In this type of system, the release rate normally decreases as a function of time, since the active agent travelling a longer distance requires a longer diffusion time to release (Salsa et al., 1997).

15.4.1.2 *Dissolution-controlled products*

In dissolution-controlled products, the drug dissolution rate is controlled by means of slowly soluble polymers or by microencapsulation. By means of slow dissolving polymer, coating of individual particles or granules of drug can be accomplished in the encapsulated dissolution system (reservoir system). The drug release is governed by the thickness and dissolution rate of the polymer membrane surrounding the drug core. Once the coated polymer membrane dissolves, the drug release takes place, such as an immediate release formulation. The coated particles can be compressed directly into tablets or filled in capsules.

Microencapsulation accordingly controls the rate of drug dissolution (and so the availability for absorption). These products are dented if chewed, as the coating may become damaged. As an advantage of encapsulated pellet products the onset of absorption is less sensitive to stomach emptying. Few preparations containing a portion of the total dose as an immediate release component provide a pulse dose soon after the administration (Jain, 1997). In the matrix dissolution system, the drug is homogeneously distributed throughout the polymer matrix. The drug is compressed with a slow dissolving carrier. As the polymer matrix dissolves, drug molecules are released and this phenomenon is called erosion controlled release. Here, factors such as penetration rate of the dissolution medium into the matrix, degree of porosity, presence of hydrophobic additives, wettability of system, and surface topography of particles control the rate of drug release (Mamidala et al., 2009).

15.4.1.3 Osmotic pump systems

Osmosis, the natural movement of water into a solution through a semipermeable membrane, has been employed in the development of zero-order release drug delivery systems (Fig. 15.3). Only water, not solutes, can diffuse through the semipermeable membrane (Herbig et al., 1995). For different polymeric membranes, their water vapor transmission value is distinguishable, and the selection of a semipermeable membrane depends on the nature of application. The rate of release of drug from these products is determined by the constant flow of water across a semipermeable membrane into a reservoir that contains an osmotic agent called as osmogen. The rate of release is constant and can be controlled within fixed limits, yielding relatively constant drug concentration. The osmotic pump is similar to a reservoir device but it contains an osmogen. Osmogen is the active agent in salt form, which acts to imbibe water from the surrounding medium through a semipermeable membrane (Gupta et al., 2011). Pressure is developed within the reservoir which forces the active agent out of the reservoir device through an orifice. The rate of release can be adjusted by changing the osmogen and size of the orifice. The constant release is unaffected by the environment of the gastrointestinal tract and relying simply on the passage of water into the dosage form, acts as an advantage for this type of system (Lachman et al., 1991).

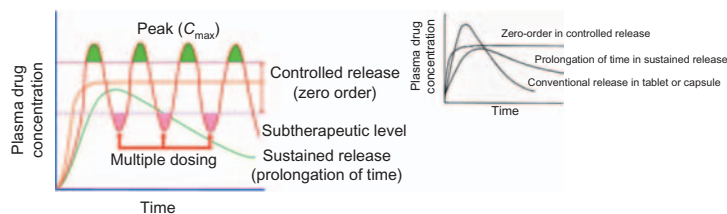


FIGURE 15.3

Schematic representation of different oral controlled release dosage forms.

Consequently, osmotically controlled systems can be classified in to single chamber osmotic pump (elementary osmotic pump) and multichamber osmotic pump (push-pull osmotic pump, osmotic pump with nonexpanding chamber) (Fig. 15.3). Specific types: are controlled porosity osmotic pump, monolithic bursting osmotic pump, osmotic bursting osmotic pump, osmotic release oral system-colon targeted (OROS-CT), multiparticulate delayed release systems, and liquid oral osmotic system (Vyas and Khar, 2000; Bechgaard and Nielson, 1978).

15.4.1.4 Ion exchange resins

Ion exchange-controlled release systems use ion-exchange resins that are water-insoluble polymeric materials having ionic groups (Guo et al., 2009). Drug molecules can be attached onto the ionic groups having opposite charge through electrostatic interaction. Accordingly, the drug molecules can be replaced with other ions having the same charge and released from the ion-exchange resin. The drug release from ion-exchange systems depends on replacement of drug molecules by other electrolytes (Conaghey et al., 1998). For more predictable drug release, the ion-exchange resins can be coated with water-insoluble polymers such as EC to provide diffusion-controlled drug release. The rate of drug release depends on the area of diffusion (surface area of resin particles), cross-linking density, ionic strength (concentration of replacing ions such as Na^+ or K^+ for cationic drugs and Cl^- for anionic drugs), and coating of the drug resin complex. Drug-resin complexes (“resonates”) are well established for extended release dosage forms and used successfully for commercial purposes. Here, a drug candidate bounds to the resin and releases by exchanging process with appropriately charged ions. This technique can be applied to certain drugs that possess specific features in terms of their relative affinity for the polymers being used.

15.4.1.5 Swelling controlled drug release system

This system is convenient for prolonging the release of highly soluble drugs. The materials for such matrices are hydrophilic gums of natural origin (guar-gum and tragacanth gum), semisynthetic (HPMC, CMC, Xanthan gum) or synthetic (polyacrylamides). The drug and gum are granulated together with a solvent such as alcohol and compressed into tablets. The release of drug from initially dehydrated hydrogels encompasses adsorption of water and desorption of drug from a swelling controlled diffusion system. The drug diffuses out from the gum upon swelling.

15.4.1.6 Hydrodynamic pressure controlled release system

A hydrodynamic pressure-activated drug delivery system can be fabricated by enclosing a collapsible, impermeable container, which restrains a liquid drug formulation to form a drug reservoir compartment, inside the rigid shape retaining casing. A composite laminate of an absorbent layer and swellable hydrophilic polymer layer is sandwiched between the drug reservoir compartment and the casing. In the GI tract, the laminate absorbs the gastrointestinal fluid through the

annular opening at the lower end of the housing and becomes swollen, which generates hydrodynamic pressure in the system. The hydrodynamic pressure forces the drug reservoir compartment to reduce in volume and causes the liquid drug formulation to release through the delivery orifice at the specific rate (Robinson and Lee, 1987). Such systems are also called push-pull osmotic pumps.

15.4.1.7 pH-independent formulation

pH-independent systems are designed to eliminate the impact of gastrointestinal pH on dissolution and absorption of drugs by formulating them with sufficient amounts of buffering agents (salts of phosphoric, citric, or tartaric acids) that adjust the pH to the required value as the dosage form passes along the GIT and permits drug dissolution and drug release at a constant rate independent of gastrointestinal pH. The dosage form containing drug and buffer is coated with a permeable substance that allows entry of aqueous medium but prevents dispersion of tablets (Allen et al., 2004).

15.4.1.8 Altered density system

Generally, less than 24 h is the transit time of GI contents. This is the major limiting factor in the design of oral controlled release formulations, which can reduce the frequency of dosing to a time period little more than the residence time of drug. If the residence time of a drug in the stomach or intestine is prolonged in some, then the frequency of dosing can be reduced. There are two ways by which this goal can be achieved: altering the density of drug particles by using the mucoadhesive polymer, and manipulating the size of the dosage form (Lapidus and Lordi, 1968). The density of the pellets should exceed the normal stomach contents and must be at least 1–4 g/cc.

15.4.1.9 Mucoadhesive system

A bioadhesive polymer, such as cross-linked polyacrylic acid in a tablet, allows it to adhere to the gastric mucosa. Such a system constantly releases a fraction of the drug into the intestine over an extended period of time.

15.4.2 BIOPHARMACEUTICS AND PREFORMULATION CONSIDERATIONS IN ORAL CONTROLLED RELEASE DOSAGE FORMS

Many parameters of a drug affect the design of controlled release formulations and absorption of drug from the formulation. Many properties of drug substances, such as pH, solubility, pKa, permeability, particle size distribution, thermal properties, hygroscopicity, compactibility, and excipient compatibilities, affect oral controlled release formulation design, processing, storage, and drug absorption. A successful formulation of a controlled release dosage form begins from the

understanding of the physical, chemical, and biopharmaceutical properties of the drug substance.

15.4.2.1 Biopharmaceutical factors

The major biopharmaceutical factors include PD factors (therapeutic objectives, toxic effects and adverse reactions), drug properties (chemical and physical properties of drug), drug product factors (PKs and bioavailability of drug, route of administration, desired drug dosage form, and desired dose of drug), patient factors (compliance and acceptability of drug product), and manufacturing factors (cost, availability of raw materials, stability and quality control). For a drug to be absorbed from an oral delivery system, the drug must first release from the formulation, dissolve in the intestinal fluid, and then pass through the GI membrane. Unlike an immediate release formulation where the drug is released and absorbed in the upper GI tract, the drug in the oral controlled release formulation is targeted to release and be absorbed continuously throughout the GI tract or targeted to a specific region. It is well known that the GI tract is physiologically complex and varies from stomach to colon in terms of composition, structure, and functions. For example, pH, surface area, motility, and the variety of dosing conditions (fed or fasting state) factors have a major impact on the rate and extent of absorption (Macheras and Argyrakis, 1997; Kimura and Higaki, 2002; Dressman et al., 1990).

15.4.2.1.1 Solubility

The aqueous solubility of a drug depends on its chemical structure, physicochemical properties of the functional groups, any variation in its stereochemical configuration, and its solid-state properties (Wassvik et al., 2008). A drug with high solubility displays faster release, while drugs with poor aqueous solubility often show incomplete release because of the impact of solubility on dissolution rate from a formulation matrix. Depending on the drug's chemical structure and properties, it demonstrates a pH-dependent solubility profile based on its ionizable group. For example, a weakly acidic drug shows lower solubility at acidic gastric pH, whereas at a pH greater than its pKa, it shows higher solubility. The drug exhibiting pH-dependent solubility, particularly in the GI pH range, is generally a poor candidate for a matrix-based oral controlled release delivery system (Varma et al., 2004). Dose/solubility ratio defines a volume that is needed to dissolve the drug and creates a drug solution for absorption (Rinaki et al., 2003). According to Thombre (2005), among single unit and multiparticulate unit (tablets/capsules), multiparticulate units offer more flexibility than single unit matrix tablets and are considered a better formulation platform for a drug that poses a dose/solubility challenge.

15.4.2.1.2 Permeability

In the oral absorption process, a drug must be permeable after dissolving in the GI lumen to be absorbed into the blood stream. Drugs administered into the

gastric lumen cross the mucosal epithelium by the paracellular transport route (the main route of permeation for hydrophilic compounds) or the transcellular transport route (the main route of permeation for hydrophobic compounds). Generally, the transport pathways can be divided into passive paracellular transport, passive transcellular transport, carrier-mediated transcellular transport, and vesicular transport (Smith et al., 1992). The intestinal mucosa serves as a physiologically selective permeability barrier to control a drug's permeation. Properties such as molecular size and hydrophobicity influence the drug's transport across the intestinal mucosa. Site-dependent differences in drug permeability would not only lead to differences in absorption profiles from the upper small intestine to the colon in a single subject, but also cause a high degree of variability in the absorption data due to transit time variations between subjects. For controlled release formulations, drugs not only need to release throughout the GI tract but also need to be absorbed throughout the GI tract. Consequently, for controlled release formulation whose oral absorption rate is supposed to be controlled by dissolution or dosage release, the drug must have high permeability. According to BCS and controlled release approach requirement, BCS Class III and IV drugs that have low and probable region-dependent permeability might not be the suitable candidates for controlled release formulation development.

15.4.2.1.3 Colonic stability

Another vital factor to colonic drug absorption and controlled release formulation development is colonic stability. A unique feature of the human colon is that it contains a remarkably higher amount of complex microorganisms than the upper part of the GI tract. Unlike the aerobic nature of the bacteria in the upper GI tract, colonic bacteria are predominantly anaerobic in nature. These anaerobes serve to control colonic pH, aid digestion, stimulate the immune system, and inhibit the growth of other food-poisoning or disease-causing bacteria. The colonic microfloras are responsible for numerous metabolic reactions having a wide spectrum of enzymes and potential to metabolize drugs (Shamat, 1997).

15.4.2.1.4 Gastrointestinal metabolism and elimination

The metabolism of a drug via presystemic extraction by intestinal and/or hepatic enzymes influences the bioavailability and hence the feasibility of the controlled release approaches. Intestinal CYP3A4 and P-gp (pumping glycoprotein) provide a couple of pathways for intestinal elimination of orally administered drugs that affect their bioavailability (Zhang et al., 1998). Controlled release delivery reduces CYP3A4-related drug-drug interactions by delivering the drug mainly to the colon. pumping glycoprotein synthesis has been reported to increase from the proximal to distal regions of the small intestine (Mouly and Paine, 2003), resulting in a decrease in the absorption of drugs that are P-gp substrates as they transit through the small intestine.

15.4.2.2 Preformulation parameters

There are four main categories of preformulation parameters: polymorphism/crystallinity, bulk characterization, solubility/permeability, and stability/compatibility. Properties that impact the controlled release formulations are stability, solubility, excipient compatibility, and bulk powder mechanical properties.

15.4.2.2.1 Particulate and mechanical properties of drug substances

The physical properties of drug substances have a great impact on the selection of controlled release formulation and manufacturing process. For low-dose drugs, content uniformity is a critical quality factor from both patient safety and efficacy perspective. To improve content uniformity, the drug particles must be small enough to distribute evenly from one tablet to another tablet, and mix well with the added excipients. The flowability and compressibility of the drug substance are vital for stability of the final product. Large crystal size and spherical shape improve the flowability. Particle size reduction often leads to poor flow properties except when the crystal shape is modified from needle or plate to a more spherical geometry.

Particle size analysis Large drug particles generally pose the issue of over-potent dosage units with low potency for the batch because a few large particles are usually present in every dose unit (Zhang and Johnson, 1997; Rohrs et al., 2006). Consequently, micronization is an alternative to reduce the particle size and therefore to improve drug content uniformity in routine practices. Methods for particle size analysis include examination of microscopic image, laser light scattering method and sieve analysis.

Bulk powder properties The surface characteristics of a particle depend on physiochemical traits, spatial arrangement, and orientation of the molecules. Several tests used to evaluate the bulk powder properties are powder density (Carr's index), angle of repose, flowability, and compression properties.

15.4.2.2.2 Stability and compatibility

A drug substance is usually more stable by itself than in a formulation with excipients, and as the drug concentration decreases, the stability decreases in a corresponding manner. Demonstration of drug substance stability and excipient compatibility under stressed conditions lends credence to long-term storage stability of the drug product. Several analytical methodologies have been employed for preformulation stability testing including spectroscopy, chromatography, and thermal analysis. Drugs that are denatured in the GI tract may undergo excessive degradation when slowly released in stomach from the controlled release formulations. Consequently, stability studies should be carried out under the parameters such as solution-state forced degradation, solid-state forced degradation, pH-stability profile, and stabilization by microenvironmental pH modulation.

15.4.2.2.3 Active ingredient solubility

Solubility is the most critical drug substance property for generating controlled release dosage forms. The intent of controlled release dosage forms is to alter the release mechanism from the formulation. Consequently, a freely soluble drug is generally considered for translating in to controlled release dosage form.

15.4.2.2.4 Partition coefficient

Biological membranes are predominantly lipophilic in nature and, therefore, unionized drugs penetrate these barrier membranes through passive diffusion. The partition coefficient can be defined as the partition of a neutral noncharged species into a lipid phase, a process that is not pH-dependent (Ku et al., 1985). In contrast, for ionic compounds, the distribution constant, which accounts for partitioning of both charged and noncharged species, is pH-dependent and can be measured at various pH values. For controlled release, pH partition over pH 1–8 is even more pertinent, since a wide absorption window utilizing the entire GI tract is needed.

15.4.3 COATING SYSTEMS FOR ORAL CONTROLLED RELEASE SYSTEMS

Pharmaceutical solids can be coated with polymeric materials for functional, protective, and decorative purposes. Polymeric material coating helps to improve the aesthetic appearance of a drug product. These coatings led to unique appearance of drug product which aid easy identification and also reduce the potential for counterfeiting. Coatings can be used to enhance the chemical stability of a drug by providing a physical barrier to environmental storage conditions such as light, oxygen, or water vapor (Berchard et al., 1992; Felton and Timmins, 2006; Zheng et al., 2005; Mwesiwa et al., 2005). The physical strength and fracture resistance of the dosage form can be increased by the application of such coatings. The drug can be coated to prevent its reaction with a secondary chemical moiety within a single multicomponent product. The taste and odor of drugs may be masked by using coatings (Cerea et al., 2004). Moreover, a number of modified release mechanisms can be achieved in desired drug product using coatings. A sustained release effect can be achieved by a simple diffusional reservoir system (Fig. 15.3), where drug inside the tablet core diffuses out through a semipermeable polymeric membrane.

15.4.3.1 Materials used in coating processes

Sugar coating led to the beginning of coating process in the pharmaceutical industry. Since sugar is a water soluble material, these types of coatings cannot be used to modify drug release characteristics. Other commonly used water soluble materials include HPMC, HPC, polyvinyl pyrrolidone-vinyl acetate copolymer, and polyvinyl alcohol-polyethylene glycol copolymer. The polymeric film of

these sustained release systems controls the rate at which fluid from the gastrointestinal tract diffuses into the core substrate. This gastrointestinal fluid dissolves the drug and the drug then diffuses out of the dosage form owing to the concentration gradient. Spray atomization technique generally helps to coat solid substrates. A coating film between 50 and 150 nm thick offers a longer, tortuous diffusional pathway. Polymers used to achieve such sustained drug release include ethylcellulose, polymethacrylates with quaternary ammonia groups, and polyvinyl acetate. Polymer may also be blended to adjust film permeability or mechanical properties (Zheng et al., 2005; Zheng and McGinity, 2003). In addition, hydrophilic materials may be included in the coating process to create pores and facilitate drug release, an approach that is especially useful for drugs with limited water solubility.

15.4.3.2 Excipients used in coating

Excipients are required in the coating formulation to facilitate film formation, modify the permeability or mechanical properties of the polymer, or aid in processing.

15.4.3.2.1 Solvents

Most polymeric films are formed upon solvent evaporation. There are a number of solvents used in film-coating processes. Film-coating technology is mainly aqueous-based due to environmental, economic, and toxicological reasons. With water-based systems, the risk of explosion may be diminished and the costs related to the purchase and disposal of organic solvents can be reduced. In addition, potential toxicity issues due to residual solvents can be eliminated.

15.4.3.2.2 Plasticizers

Many of the polymers used in pharmaceutical film-coating processes are brittle and require the addition of a plasticizing agent. Plasticizers function by weakening the intermolecular attractions between the polymer chains. The addition of a plasticizer to a coating formulation generally increases the flexibility and decreases the tensile strength of the film. Plasticizer type and concentration have been shown to remarkably influence drug release from coated solids (Amighi and Moes, 1996; Bodmeier and Paeratakul, 1991). Consequently, mechanical (Wu et al., 2000) and adhesive properties of the film (Felton and McGinity, 1997) are the critical parameters in coating performance. Phthalate esters, sebacate esters, and citrate esters are commonly used as plasticizing agents. Various glycol derivatives, such as propylene glycol and polyethylene glycol, have also been used as plasticizers. In addition, surfactants, preservatives, and other compounds also plasticize the cellulosic and acrylic polymers (Wu and McGinity, 1999; Lindholm et al., 1986). The effectiveness of a plasticizing agent is dependent on the concentration of plasticizer added to the coating formulation and the extent of interaction with the polymer.

15.4.3.2.3 Antiadherents

Antiadherents are added to film coating formulations to prevent agglomeration of the substrate. The degree of tackiness correlates with the minimum film-forming temperature of the polymer or the minimum temperature necessary for coalescence to occur. This effect has been reported to increase with increasing plasticizer concentration (Wesseling et al., 1999). One of the most common antiadherents for pharmaceutical coating formulations is talc. Talc has been demonstrated to decrease water vapor permeability and the dissolution rate of drugs (Fassihi et al., 1994; Maejima and McGinity, 2001), presumably due to its hydrophobic nature. Glyceryl monostearate at a concentration of 2%–10% (based on dry polymer weight) has been suggested as an alternative to talc (Nimkulrat et al., 2004).

15.4.3.2.4 Pigments

Pigments are added to film coating formulations to produce a more pharmaceutically elegant dosage form and to provide easy product identification for both healthcare workers and patients. Opacifying agents such as titanium dioxide (TiO_2) have been incorporated into coatings to improve the stability of photolabile or light-sensitive drugs (Berchard et al., 1992; Rowe et al., 2006). The most common pigments used in the pharmaceutical industry are the water-insoluble lakes and the iron oxides. Color migration and stability issues have diminished the use of water soluble dyes in film coating formulations (Felton and McGinity, 1999, 2002). Surface polarity of pigments also influences the drug release (Maul and Schmidt, 1997). Some commonly used pigments are black iron oxide, red iron oxide, yellow iron oxide, and TiO_2 .

15.4.3.2.5 Surfactants

In film coating formulations, surfactants such as polysorbate 80, sorbitan monooleate, and sodium dodecyl sulfate may be incorporated to emulsify water-insoluble plasticizers. In addition, these surfactants also increase the substrate wettability and enable spreading of the polymer-containing droplets on the substrate surface. Moreover, these surfactants also help to stabilize the suspensions. The concentration of surfactants in such pharmaceutical coatings is typically very low, generally in the range of 0.25%–1% (Bodmeier and Paeratakul, 1991).

15.4.4 CHALLENGES AND NEW TECHNOLOGIES IN ORAL CONTROLLED RELEASE DRUG DELIVERY

15.4.4.1 Limiting factors for oral controlled release formulations

There are various challenges in the development of controlled release formulations for drugs with poor aqueous solubility. Consequently, poorly soluble drugs necessitate both solubilization and engineering of release profile. Furthermore, large number of new therapeutics such as peptides, proteins, oligonucleotides, and

vaccines are under development. However, physical, chemical, and biopharmaceutical characteristics of such high molecular weight components unlike small molecule drugs demand novel controlled release technologies to diminish obstacles for oral delivery, such as instability in GI tract and poor absorption. Accordingly, there are several opportunities for research, development, and innovation in the field of peptide and protein oral delivery. The main limitation to present controlled release principles is their suitability for water soluble drugs only. Drugs which are absorbed in limited areas of the gastrointestinal tract present other hurdles. Therefore, new techniques are required to address the issues of drug release mechanisms, drug absorption processes and gastrointestinal transport.

15.4.4.1.1 Dose dumping

Dose dumping is a phenomenon in which relatively large amount of drug in a controlled release formulation is quickly released and a potentially toxic quantity of the drug is introduced into systemic circulation. Additionally, dose dumping proves to be fatal in the case of potent drugs, which have a narrow TI.

15.4.4.1.2 Less flexibility in accurate dose adjustment

In conventional dosage forms, dose adjustment is simple, as a tablet can be divided into two fractions. However, in the case of controlled release formulations, it is more complicated. Controlled release property may be lost, if the dosage form is fractured.

15.4.4.1.3 Poor in vitro in vivo correlation

In controlled release dosage form, the rate at which a drug is released is purposely reduced to achieve drug release probably over a large region of gastrointestinal tract. Here the so-called absorption window becomes important and may give rise to inadequate drug absorption in vivo despite excellent in vitro release characteristics.

15.4.4.1.4 Increased potential for first pass clearance

Hepatic clearance is a saturated process. After oral dosing, the drug reaches the liver by travelling through portal vein. The concentration of drug that reaches the liver determines the amount metabolized. Higher the drug concentration, greater is the amount required for saturating an enzyme surface in the liver. Conversely, the smaller the concentration bound to controlled release and a sustained release dosage form, lesser the chance of saturating the enzyme surface. The probability of reduced drug availability due to first pass metabolism is therefore more with controlled release and sustained released formulation, compared to conventional dosage form.

15.4.4.1.5 Patient variation

The mandatory time period for absorption of drug released from the dosage form may fluctuate among individuals. Coadministration of other drugs, presence or

absence of food, and residence time in gastrointestinal tract differs among patients. This also yields variation in clinical outcome among the patients.

15.4.4.1.6 Less gastric retention time

Relatively short gastric emptying, intestinal transit time and varying pH values are considerable factors. Consequently, most of the oral controlled release formulations are designed to release the entire amount of drug within 12–18 h. The presence of food in stomach tends to delay the gastric emptying. Among different foods, carbohydrates and proteins tend to be emptied from the stomach in less than 1 h, while lipids stay in the stomach for more than 1 h (Yu and Amidon, 1998; Yu et al., 1996). The small intestinal transit time is more reproducible and is typically about 3–4 h (Yu and Amidon, 1998; Yu et al., 1996). Accordingly, the transit time from mouth to cecum (the first part of large intestine) ranges from 3 to 7 h. Colonic transit is highly variable and is typically 10–20 h (Wagener et al., 2004). Since most drugs are absorbed from the small intestine, the time interval from mouth to cecum for oral controlled release dosage forms is too short, unless the drug can be equally well absorbed from the large intestine. Accordingly, the release profiles of most oral controlled release dosage forms are effective for only about 8 h. If the drug absorbs from large intestine, then the time interval for drug absorption can be increased to 1 day. Accordingly, certain drugs can be delivered for 24 h by a single administration of an oral controlled release dosage form. However, many drugs require more than one administration if they have the upper GI tract absorption window and short half-life, unless the release of those drugs can be controlled at the upper GI tract with special design. The study of GI transit time of once-a-day OROS tablets of both oxprenolol and metoprolol exhibited that the median total transit time was 27.4 h with a range of 5.1–58.3 h (Grundty and Foster, 1996; John et al., 1985).

15.4.4.1.7 Absorption window

Absorption in different segments of the GI tract is not uniform, and in general, it tends to decrease as the drug moves along the GI tract. If a drug is absorbed only from the upper segment of the GI tract, it is known to have a “window for absorption” (Alvisi et al., 1996). For the drugs with a window for absorption, adjusting drug release rate on different segments of the GI tract may be needed to compensate decreased absorption, in order to maintain relatively constant blood concentration. For example, to achieve a plateau-shaped profile of plasma concentrations at steady state throughout the 24 h dosing interval, nisoldipine controlled release formulation releases the drug slowly in the upper GI tract that has fast absorption and quickly in the colon that has decreased absorption rate (Heinig et al., 1997). Besides adjusting drug release rate, increasing the residence of drug formulations at or above the absorption window also enhance the absorption of such drugs. Currently, two main approaches have been widely explored, including bioadhesive microspheres that have a slow intestinal transit and the gastroretentive dosage system (Davis, 2005). Presystemic clearance may occur at some sites of the GI

tract and affect drug absorption. Degradation of orally administered drugs occurs by hydrolysis in the stomach, enzymatic digestion in the gastric and small intestinal fluids, metabolism in the brush border of the gut wall, metabolism by microorganisms in the colon, and/or metabolism in the liver prior to entering the systemic circulation (i.e., first pass effect). Such degradations may lead to highly variable or poor drug absorption into the systemic circulation. For example, digoxin undergoes microbial metabolism before absorption (Lindenbaum et al., 1981; Saha et al., 1983). For this type of drugs, for which presystemic clearance is determined by the site of absorption, drug bioavailability can be enhanced by restricting drug delivery to the upper segment of the gut, or to the stomach. For example, among metoprolol intragastric infusion (administered for 13.5 h continuously at predetermined rate) and OROS tablet of metoprolol at identical doses, the intragastric infusion had higher plasma concentration than metoprolol OROS tablet (Warrington et al., 1985).

15.4.4.2 Oral controlled delivery for water-insoluble drugs

Drug dosage forms must be dissolved in GI fluids for effective absorption. For a water-insoluble drug, the absorption and bioavailability are limited, owing to poor dissolution rate and solubility in the GI tract. There are many established approaches for translating water-insoluble drugs in to oral dosage forms. Strategies such as salt formation, microenvironmental pH control, inclusion complexation, surfactants mediated solubilization, solid-dispersions, biodegradable lipid-based formulations, and nanoparticles have been widely employed. It is very challenging to develop a controlled release formulation for a water-insoluble drug. A controlled oral delivery may be required to achieve prolonged exposure or time-based release for a water-insoluble drug under certain circumstances. A combination of solubilization and modulating the release is desired to achieve controlled release for a water-insoluble drug. If a drug could be solubilized by a surfactant or a complexing agent, inclusion of a solubilizing agent in polymer-based matrix tablets may solve the issue. Many drugs need more complicated formulation approaches to enhance the dissolution, such as amorphous solid dispersion, emulsion, microemulsion, self-emulsifying, and nanoparticles. Among them, amorphous solid dispersion is the most popular approach for enhancing solubility and dissolution properties. The physical stability of solid dispersions must be monitored for polymer-based matrix systems, membrane coatings, or osmotic systems during extended release. All three approaches need water to diffuse inside the formulation to solubilize drugs and advance the release. Drugs may crystallize out during the prolonged exposure to water, owing to supersaturation inside the dosage form or change of glass transition temperature because of interaction with water. As a substitute to dissolution/partition/diffusion-based devices, osmotic pumps have been produced to provide zero-order release profile. An elementary osmotic pump is a tablet or a capsule comprising of a core of drug enclosed by a membrane that is permeable only to water and not to the drug. A small orifice is drilled into the membrane. When ingested, water is osmotically imbibed into the

core through the semipermeable membrane; accordingly, constant osmotic pressure gradient is established between the core and external dissolution medium. This phenomenon establishes the stage for water influx that displaces drug through the orifice at a constant rate. Nanoparticles can be used to improve the bioavailability of poorly soluble drugs. Consequently, the drug dissolution rate is ultimately augmented due to the increase of surface area.

15.4.4.3 New formulation designs for achieving desired release profiles

Many formulation designs have been followed to obtain controlled release and diminished the effect of the GI environment. Osmotic pump drug delivery system is one of the promising approaches for achieving the controlled release. Disintegration controlled matrix tablet and erodible molded multilayer tablet are based on the principle of erosion and showed some promises. Conversely, bioadhesive polymers have been extensively investigated for improving gastroretentive delivery and to increase the localized action in GI tract. Moreover, significant progress has also been made in computer modeling to design controlled release formulations.

15.4.4.3.1 Disintegration-controlled matrix tablet

A disintegration-controlled matrix tablet is an erosion-based controlled release platform. It was developed for the sustained release from solid dosage form. Disintegration-controlled matrix tablets contain hydrogenated soybean oil as the wax matrix with uniformly distributed solid dispersion granules. The solid dispersion granules are prepared with low-substituted HPC as a disintegrant. The process of tablet erosion controls the drug release. The wax allows the penetration of water into the surface layer of tablet, and in this way, water leads to the swelling of the disintegrant on the surface. As a consequence, erosion in tablet causes the separation of solid dispersion granules from the tablet. A constant rate of tablet disintegration/erosion can be achieved by repeating the water penetration and swelling/separating of solid dispersion granules.

15.4.4.3.2 Erodeable molded multilayer tablet

Similar to disintegration-controlled matrix tablet, Erodeable molded tablet is an erosion-based platform. It offers the advantage such as zero-order drug delivery or delayed release of drug without any interference by the gastrointestinal conditions. Erodeable is a more sophisticated engineered delivery system where erosion is one-dimensional; however, in disintegration-controlled matrix tablet, erosion occurs in three dimensions. Accordingly, Erodeable achieves a better zero-order release. Drug is dispersed in the matrix and the release is controlled by the erosion rate in the two ends of tablets. The surface area from where erosion takes place is constant. Erodeable tablets are prepared by injection molding (IM) (Pederson, 2006). Release of drug is controlled by the gradual erosion of the matrix part. The mode and rate of release can be regulated by altering the matrix, coat, and geometry to achieve either a zero-order release or a delayed release. A drug is distributed in the matrix for a zero-order release. Biodegradable coat has poor water permeability to prevent its

penetration. The matrix tends to erode when it comes in contact with available water and the erosion of the matrix is caused by GI fluids and promoted by gut movements in the GI tract. The drug release is mediated completely by erosion because the dosage form is designed to slow down the water diffusion into the matrix. The Egalet delivery system can be easily fabricated for delayed release, which can be delayed for specified period of time. This approach is applicable in achieving sustained release in colon delivery of therapeutic moieties. In addition, delayed release of the drug can be regulated to synchronize the natural rhythms of symptoms of disease such as morning stiffness and pain experienced by patients having arthritis on waking. A delayed release can be accomplished through three compartments of a tablet, such as coat, drug release matrix, and lag component. The lag component tends to provide a predetermined delay for the drug release. After the lag component is eroded, the drug release occurs in a designed mode. Egalet delivery technology was developed on the basis of standard plastic IM to ensure accuracy, reproducibility, and low production cost. It is actively using by several companies for the development of numerous controlled release formulations.

15.4.4.3.3 Bioadhesive oral delivery

Bioadhesive delivery could be applied for the development of oral controlled release formulations. Bioadhesive polymers tend to adhere to the mucin and are accordingly used for ocular, buccal, vaginal, and nasal drug delivery. Moreover, bioadhesive polymers also help increase the residential time of solid dosage forms in the GI tract to improve gastroretentive delivery. Conversely, bioadhesive polymers allow oral dosage forms to stay close to epithelial layer and allow the quick flux of drugs after dissolution. Bioadhesive systems enhance oral absorption or enhance localized action if disease occurs in the GI tract. The GI tract is covered by a layer of mucus. Polymers having the ability to form hydrogen bonds tend to bind to the mucus layer. However, the mechanism of mucoadhesion is not completely implicit. It is anticipated that the attraction forces, such as hydrogen bonding, van der Waals forces, and charge, make polymers have good contact with the mucus. This close contact further promotes the penetration of polymers and formation of entanglements with the mucin ([Andrews et al., 2009](#)). Polymers such as carbomers, chitosan, starch, polymethacrylic acid, HPC, HPMC, and sodium carboxymethyl cellulose have been widely investigated for developing oral bioadhesive drug delivery systems. Bioadhesive polymers can be used to fabricate drug delivery systems in monolithic or multiparticulate forms to achieve controlled release. Bioadhesive delivery allows the controlled release of drugs with narrow absorption window. Several drugs have a narrow absorption window from the proximal part of the GI tract due to transporter-mediated absorption. Increasing the residence time in upper GI tract could extend and enhance the absorption phenomena. Bioadhesive delivery is advantageous in providing sustained release for localized therapy. The microspheres prepared from pectin and chitosan have displayed desirable mucoadhesive properties that further prolonged the drug release in physiological dissolution medium.

15.4.4.3.4 Computer modeling

The major challenge for developing controlled release products is its in vitro-in vivo correlation (IVIVC). Fabricating a drug formulation with a zero-order release in vitro is less challenging than predicting the in vivo performance. The GI tract is more complex than a dissolution unit. Effects of pH, food, GI mobility, regional absorption, bile salts, and gut metabolism are too complicated to formulate as simple mathematical equations. Computer simulation software is a useful tool for formulators to study the drug molecules and recommend target release profiles. Moreover, software also helps in the deconvolution of animal and human PK data and provides feedback for further optimization of the formulation. Advanced software packages for modeling oral absorption are physiology-based simulation software such as iDEA, GastroPlus, and SimCYP. TheiDEA software for predicting oral drug absorption, has been developed according to a physiological multiple-compartment model invented by Grass ([Grass, 1997](#)). The Grass model divides the GI tract into five compartments including stomach, duodenum, jejunum, ileum, and colon. Oral absorption of a drug can be simulated on the basis of three parameters such as solubility, permeability, and tissue surface area. TheiDEA uses permeability data of Caco-2 cell model and has the capability to calculate solubility and permeability from a simple structure input. The iDEA is convenient to use for predicting oral absorption of new chemical entities in early discovery. GastroPlus is one of the widely used software packages for modeling oral absorption of solid dosage forms. GastroPlus is advanced software to simulate oral absorption, PKs, and PDs in animals and humans based on molecular, physical, biopharmaceutical, and pharmacological attributes of a drug. The oral absorption module is based on an absorption model called advanced compartmental and transit model ([Agoram et al., 2001](#)). In GastroPlus, there are nine physiological compartments for human in line with different segments of the GI tract ([Lukacova et al., 2009](#)). GastroPlus is very useful in modeling the effect of solubility, particle size, and dose on the extent of oral absorption for a specific drug. Moreover, GastroPlus also contains sophisticated PK simulations. In addition, GastroPlus has been used as a tool in preclinical compound selection to assess the absorption liability, establish IVIVC to justify biowaiver, and to study the effect of food on PKs. A study by Lukacova et al. demonstrated that GastroPlus is very useful in modified release formulation development. GastroPlus has been applied to obtain simulated models for adinazolam and metoprolol to assess the absorption, PK, and PDs in oral administration ([Lukacova et al., 2009](#)). Similar to GastroPlus, SimCYP ADME simulator has seven compartments for small intestine. This software displays physiological variables such as gastric emptying time, small intestinal transit time, the radius, and length of the small intestine. Moreover, it considers the dynamics of drug dissolution, precipitation, degradation, intestinal metabolism, and active transport. It calculates the effect of bile salts on dissolution and the effects of pH and transporter on permeability.

15.4.5 QUALITY BY DESIGN APPROACH IN ORAL CONTROLLED RELEASE DRUG DELIVERY

Quality by design (QbD) is a new concept for the pharmaceutical industry that has been extensively applied in the automotive, semiconductor and petrochemical industries. Deming and Juran documented the concept of building quality into products. “Planning for quality” is a common theme of the various initiatives, that is, building quality into the products in comparison to traditional paradigm of testing the product to ensure quality. The Juran trilogy concept identifies quality planning, quality control, and quality improvement as three essential aspects of quality planning (Juran, 1992). Quality planning is the practice of identifying the needs of the customer and designing the product and the process to meet the needs of the customer. In the QbD paradigm, it is possible to use knowledge and data from product development studies to create a design space where continuous improvement can be implemented, but the management for such change control lies with the industry without the need of regulatory approval. Prior knowledge and risk assessment are central to the understanding of the product and process. In this paradigm, the processes can be adjusted to accommodate the variations in the input material, thereby resulting in a product that will be of consistent quality. This can be accomplished only with enhanced process/product understanding. Tools such as process analytical technology and design of experiments (DoE), in combination with enablers such as quality risk management (QRM), knowledge management, and so on, help to understand the process or product. Alignment of quality systems to manage the concept of QbD will enable the implementation of QbD at the manufacturing sites and thereby facilitate regulatory flexibility, an outcome of the process/product understanding. The principle of QbD can be applied to all types of drugs and drug delivery systems. However, complexity of the drug or delivery platform and model influences the application of QbD. From a development perspective, oral controlled release products are not different from the traditional formulations and/or the immediate release products; however, more attention is required on release mechanism and associated complexities in the technology used therein. The choice of excipients, type of delivery system, active pharmaceutical ingredient properties, and manufacturing platform influence the quality attributes that are critical to safety, efficacy, and manufacturability (elements of the target product quality profile).

15.4.5.1 Enablers of quality by design

Pharmaceutical Development Annex (ICH Q8 (R2)) discusses the various elements of QbD. These in combination with enablers form the fundamental basis for QbD approach to development.

15.4.5.1.1 Quality risk management

QRM is a key enabler for the development and application of QbD. It is one of the tools that provides a proactive approach for identification, scientific

evaluation, and controlling potential risks to quality. It also facilitates persistent improvement in product and process performance throughout the product life cycle. Quality risk assessment in combination with the prior scientific and engineering knowledge helps to identify the material attributes and process parameters that may have a potential effect on the product critical quality attributes (CQAs). The various phases of development and gains in process/product knowledge and risk assessment enable to differentiate between true versus perceived risks. This further proves that attention should be given to high-risk areas and strategies should be developed to mitigate them.

15.4.5.1.2 Knowledge management

Product and process knowledge management is an important component of QbD and is managed through the commercial life of the product including discontinuation. It is an organized approach for acquiring, analyzing, storing, and disseminating information related to products, processes, and components. This also emphasizes the transfer of information from development to commercial and vice-versa. Prior knowledge comprises previous experience and understanding of success and failure, and recognition of issues, problems, or risks that may occur and need to be addressed. Prior knowledge from the literature, and experience with prior compounds/processes provide the basis for initial risk assessments and influence a number of decisions. Therefore, a good understanding of the documentation relating to prior knowledge referred to in risk assessments and DoEs are necessary for the success of QbD.

15.4.5.2 Elements of quality by design

Various elements in combination with the enablers form the fundamental basis from the QbD approach to development.

15.4.5.2.1 Identifying a quality target product profile

The quality target product profile (QTPP), as defined in ICH Q8(R1), is a summary of the quality characteristics of a drug product that ideally will be achieved and thereby ensures the safety and efficacy of a drug product. The QTPP may be updated at various stages of development as new information is obtained during the development process. According to FDA guidelines, target product profile (TPP) focuses on the consumer and the desired product label. The subset of TPP is QTPP which is more oriented towards the chemistry, manufacturing, and controls (CMC) aspects of development. QTPP is a quantitative surrogate for clinical safety and efficacy and QTPP can be employed to design and optimize both the formulation and the process ([Lionberger et al., 2008](#)). A typical QTPP includes quantitative targets such as dissolution, potency, impurities, stability, release, and other product-specific requirements. Depending on the type of controlled release, certain functionality related test(s) could be incorporated if they are important, for example, polymer viscosity, tortuosity, and glass transition of composite. For a

generic controlled release product, bioequivalence is a quality attribute that has extra significance.

15.4.5.2.2 Identification of critical quality attributes

Critical quality attribute According to ICH Q8(R2), a CQA can be defined as a physical, chemical, biological, or microbiological property or characteristic that should be within suitable limit or range to guarantee the required product quality. In general, CQAs are associated with raw materials, such as drug substance and excipients; intermediates, such as in-process materials; and drug product. Moreover, CQAs comprise of properties of a raw material that may affect the drug product performance or manufacturability.

Quality attributes important for the performance of the drug product From a clinical perspective, the first and foremost importance for a drug product is its safety and efficacy. Accordingly, for an oral controlled release product, it is important to consider characteristics that are essential for performance. This may be drug dissolution/release, potency, polymer concentration, polymer viscosity, and glass transition temperature (T_g) of composite. There are several issues and challenges related to availability and characteristics of controlled release excipients. Most of the oral dosage form designs depend on the material attributes of the active and the excipient components. These may be linked to the drug product CQAs or specifications such as purity, uniformity, dissolution, and appearance.

Quality attributes impact the manufacturability of the drug product Physical and mechanical properties of either the drug substance or the excipient influence the CQAs of drug product (hardness, appearance, moisture, friability, etc.) and its manufacturability (Amidon, 1995). The knowledge of mechanical properties of the drug substance and excipients plays a critical role in dosage form development and processing (Dave et al., 2015). A good understanding of the drug substance and excipient properties can help in rational selection of excipients. This enables the development of a robust process which helps to address the challenges during scale-up or manufacturing.

15.4.6 ADVANTAGES AND DISADVANTAGES OF CONTROLLED RELEASE DRUG DELIVERY SYSTEMS

The main benefits of controlled release drug delivery systems include maintenance of optimum drug concentration and augmented duration of therapeutic effect, improved efficiency of treatment with lesser amount of drug, reduced side-effects, less frequent administration, increased patient convenience and compliance, reduced drug plasma level fluctuation, and maintenance of a steady-state plasma level of the drug over a prolonged period of time. Drug plasma levels are kept within a narrow window with no sharp peaks. Oral drug delivery is the most used and convenient route for patients and a reduction in dosing frequency enhances the compliance. The total cost of treatment with the controlled release

product is comparable or lower than the immediate release product with reduction in side-effects. The overall expenses in disease management also become reduced. It is also recommended for patients to avoid the dosing at night time (Vyas and Khar, 2000; Shargel and Yu, 1999). The major disadvantage of controlled release formulation is dose dumping. In controlled release formulation, dose adjustment is more difficult. Controlled release property may be lost, if the dosage form is broken. Moreover, in controlled release dosage form, the rate of drug release is intentionally reduced to achieve drug release possibly over a large region of gastrointestinal tract. Here, the so-called “absorption window” becomes important and may give rise to inadequate drug absorption in vivo, despite excellent in vitro release characteristics. The possibility of reduced drug availability due to first pass metabolism is greater with controlled release and sustained release formulation compared to conventional dosage form. Probabilities for difference in clinical response among the patients are associated with controlled release products because the time period required for absorption of drug released from the dosage form may differ among individuals. There is a risk of an ineffective action or even absence of it if the therapeutic ingredient is poorly absorbed from GIT. Drugs for which a single dose exceeds 1 gm, the technical process requirements may make the product very difficult to scale-up. Moreover, therapeutic agents absorbed through active transport mechanism are not good candidates for controlled release dosage form. One such example is riboflavin. Processes and equipment which are used in manufacturing of many controlled release dosage forms are costly or expensive (Welling and Dobrinska, 1978).

15.5 ORAL SUSTAINED RELEASE DOSAGE FORMS

Sustained release dosage form can be defined as any dosage form that provides the drug over an extended period of time or the system is able to provide some definite therapeutic control either of a temporal nature, spatial nature or both. Sustained release systems generally do not attain zero-order type release and usually try to mimic zero-order release by providing drug in a slow first-order. A sustained release dosage form provides a therapeutic concentration of the medication in the blood that is maintained throughout the dosing interval with reduction in peak concentration ratio (Prajapati et al., 2008; Jantzen and Robinson, 1995).

15.5.1 DESIGN AND FABRICATION OF ORAL SUSTAINED RELEASE SYSTEMS

The majority of oral sustained release drug delivery systems rely on dissolution and diffusion or a combination of both mechanisms to create slow release of drugs in gastrointestinal milieu. The following techniques are generally used in the design and fabrication of oral sustained release dosage forms.

15.5.1.1 Dissolution controlled system

Slowing the dissolution rate of a drug in GI medium, incorporation of drug in an insoluble polymer, and coating of drug particles or granules with polymeric materials of varying thickness are the parameters required to obtain the dissolution-controlled release system. The rate-limiting step for dissolution of a drug is the diffusion across the aqueous boundary layer (Mandal et al., 2010). The solubility of the drug provides the source of energy for drug release, which is opposed by the stagnant-fluid diffusional boundary layer. It is possible to prepare sustained release product by reducing the dissolution rate of drug which are highly water soluble. This can be done by preparing suitable salt, coating the drug with slowly dissolving materials, or incorporating the drug into the tablet having a slowly dissolving carrier. The drugs with poor water solubility (BCS Class II and IV) are inherently sustained release forms. Conversely, for water soluble drugs, it is possible to incorporate a water-insoluble carrier to reduce dissolution of the drug. Poly(ethylene glycol) is usually added to reduce the dissolution rate of highly aqueous soluble drug. Most of the formulations relying on dissolution to release the drug can be categorized as follows:

15.5.1.1.1 Encapsulated dissolution systems (soluble reservoir system)

Encapsulated dissolution systems can be prepared either by coating granules of drug with varying thickness of slowly soluble polymers or by microencapsulation. In this system, drug is coated with a slowly soluble polymer, which slowly dissolves in the gastrointestinal fluid and modifies the drug release using rate controlling coats. These coated drug particles can be compressed into tablets called Spacetabs or placed in capsules such as Spansules.

15.5.1.1.2 Matrix dissolution systems (soluble matrix system)

A matrix dissolution system can be either a drug-filled sphere or a drug-filled tablet, which is subjected to slow erosion. Matrix dissolution devices are prepared by dispersing a proper dose of micronized drug particles homogeneously throughout a gradually dissolving polymer matrix by congealing or by aqueous dispersion methods and compressing into a tablet.

15.5.1.1.3 Multilayer matrix tablet

Multilayer matrix tablet offers much diversity in achieving preferred release profile by combining various types of subunits in tablets. Usually, an immediate release and sustained release layer are added to the bilayer tablet for maintaining plasma drug level.

15.5.1.2 Diffusion systems

Diffusion is a process in which movement of drug molecules occurs from high concentration in the tablet to lower concentration in GI fluids. This movement

depends on surface area exposed to gastric fluid, diffusion pathway, drug concentration gradient, and diffusion coefficient of the system. In diffusion systems, the release rate of drug is determined by its diffusion across a water-insoluble polymer. Here, the diffusion of drug through a polymeric barrier is a rate-limiting step. The drug release rate is never zero-order, since the diffusional path length increases with time as the insoluble matrix is progressively depleted of drug. Diffusion of a drug molecule through a polymeric membrane forms the basis of these controlled drug delivery systems. The diffusion-controlled devices are manufactured either by encapsulating the drug in a polymeric membrane or by dispersing the drug in a polymeric matrix. In contrast, drug in dissolution-controlled systems is available as a result of partitioning through the polymer (Mandal et al., 2010; Thakore et al., 2010; Dusane et al., 2011). In practice, one can follow either of the two methods. In the first method, drug particles are coated with polymer of defined thickness so the portion of drug should be slowly diffuse through the polymer to maintain constant plasma drug level. Conversely, in the second method, the drug is formulated in an insoluble matrix; the gastric fluid penetrates the dosage form and dissolves the medicament and releases the drug through diffusion.

15.5.1.2.1 Diffusion reservoir devices

In diffusion reservoir devices, a water-insoluble polymer covers a core of drug. Drug is partitioned into the membrane and is exchanged with the fluid surrounding the particle or tablet. The release of drug in diffusion reservoir devices is governed by Fick's first law of diffusion.

$$J = - \frac{D dC_m}{dx}$$

where J is the flux of drug in the direction of decreasing concentration across a membrane, D is the diffusion coefficient of the drug in the membrane, dC_m/dx is the change in the concentration of the drug in the membrane over a distance x . Reservoir type devices contain microencapsulation and coating in the tablet.

15.5.1.2.2 Diffusion matrix devices

A diffusion matrix device can be defined as a well-mixed complex of one or more drugs with a gelling agent such as hydrophilic polymers. It is the system which extends and controls the release of drug that is dissolved or dispersed homogeneously in an inert polymeric matrix. The rate of release of drug can be described by Higuchi equation: the change in amount of drug released per unit area (dm), with change in the depleted zone thickness, dh

$$dm = C_0 dh - (C_s/2) dh$$

where C_0 is total amount of drug present per unit volume in the matrix, C_s is the saturated solubility of drug per unit volume in the matrix.

The most common method for the preparation of a matrix device is to mix the drug with the polymeric matrix material and compress the mixture into tablets or multilayered tablets, as per the required release rate; the drug is dispersed in molten wax or rate-controlling material, which is then congealed and compressed to form the tablets. Matrix diffusion-control devices contain a solid drug dispersed in an insoluble matrix. Matrix systems are simpler to develop than reservoir or encapsulated devices, having versatility, effectiveness, and low cost. Furthermore, it is possible to formulate high molecular weight drugs. The stability of the drug can be increased by protecting it from hydrolysis or other probable changes in gastrointestinal tract. There are certain limitations, such as the host matrix needs to be removed after the drug has been released. The release rates are affected by several factors such as food and the rate of transit across the gut. Consequently, it does not provide pure zero-order release.

15.5.1.3 Ion-exchange resin-drug complexes

Ion-exchangers are water-insoluble resinous materials comprising salt-forming anionic or cationic groups. During manufacturing, the drug solution is mixed with resin and dried to form beads that can be compressed to formulate the tablets. The drug from this complex is exchanged with Na^+ and Cl^- ions present in GI tract. Generally, this system employs a resin compound of insoluble cross-linked polymer. It encompasses a salt forming functional group at a polymer chain in repeating position (Patel et al., 2012). The drug release depends on the presence of high concentration of ions in GI tract, where the drug molecules are exchanged and diffused out of the resin into the surrounding fluid. This mechanism is based on the ionic environment of resin and not the pH or enzyme at the absorption site.

15.5.1.4 pH-independent formulation

Generally, the release of drug from sustained release formulation is pH-dependent. Consequently, to maintain constant pH value, a buffer such as salt of citric acid, amino acid, and tartaric acid can be incorporated to the formulation. The scale-up of a buffer sustained release formulation requires the mixing of a basic or acidic drug, one or more buffering agents, granulation with appropriate excipients, and coating with gastrointestinal fluid permeable film forming polymer. When gastrointestinal fluid permeates through the membrane, the buffering agent adjusts the fluid pH at a suitable constant pH and accordingly offers a steady rate of drug release (Gupta et al., 2002).

15.5.1.5 Osmotic pressure controlled systems

In osmotic pressure controlled systems, a semipermeable membrane is placed around the tablet, particle or drug solution that allows transport of water into the tablet with subsequent pumping of drug solution out of the tablet through the small delivery aperture in tablet core. Two types of osmotic pressure controlled systems are reported in literature. Type 1 contains an osmotic core with drug,

whereas, type 2 holds the drug in flexible bag that is surrounded with osmotic core. By optimizing the formulation and processing factors, it is possible to develop osmotic system to deliver the drug of diverse nature at predetermined rate (Dusane et al., 2011). Osmosis is a phenomenon in which fluid flow occurs from lower concentration to higher concentration through a semipermeable membrane which allows transfer of liquid only. The drug is coated with a semipermeable membrane with a hole at one end of the tablet made by a laser beam. The gastric fluid permeates through the membrane, solubilizes the drug, and increases the internal pressure, which pumps the drug solution out of the aperture and releases the drug into the gastric environment. The delivery rate remains constant if an excess amount of drug presents inside the tablet. However, it declines to zero once the concentration drops below the saturation point.

15.5.1.6 Altered density systems

Altered density systems are intended to prolong their residence time in the GI tract where these systems release the active medicament. Generally, these dosage forms are fabricated to retain in the stomach and hence the drug present inside should be stable at gastric pH value. Systems included in this category are mucoadhesive systems and size-based systems.

15.5.1.7 Sustained release matrix tablets

A method to develop the sustained release dosage form is the direct compression of blend of drug, retardant materials, and an additive in which therapeutic agent is embedded in a matrix core of the retardant. Conversely, a retardant drug blend may be granulated before compression. Matrix tablets can be considered as the commercially sustained action dosage form that involves minimum processing variables, employs conventional facilities, and accommodates drugs in large doses. A major drawback of matrix tablets or devices is the rate of drug release continuously decreases with time, which is the result of increased diffusional distance and reduced surface area at the penetrating solvent front. Additives such as poly(vinyl pyrrolidone) or hydrophilic polymers may control the drug release from materials and in this way, zero-order release can be achieved. Moreover, water-insoluble materials that are easily erodible and polymers that form hydrophilic matrices can be used.

15.5.2 POTENTIAL ADVANTAGES AND LIMITATIONS OF ORAL SUSTAINED RELEASE DOSAGE FORMS

15.5.2.1 Advantages

The main advantages include decreased local, systemic, and gastrointestinal side-effects. Moreover, minimum drug accumulation occurs upon chronic dosing. Bioavailability of drug is improved. Superior efficiency in the treatment appears owing to uniform and therapeutic plasma drug concentration level, reduced

toxicity owing to slow drug absorption, and reduction in fluctuation in plasma drug level. Oral sustained release dosage forms enhance the stability by protecting the drug from hydrolysis or degradation in GI tract (Patel et al., 2012). Improved patient compliance, owing to reduced dose-dosage frequency, is another advantage of oral sustained release dosage form. Although the primary unit cost of sustained release products is usually greater than that of the conventional dosage form because of the distinct nature of these products, the average cost of treatment over a prolonged time period is less (Chugh et al., 2012; Mandal et al., 2010).

15.5.2.2 Disadvantages

Extended release formulation contains a higher amount of drug and accordingly any kind of alteration in the release characteristics of the dosage form may cause toxicity. Furthermore, the larger size of extended release formulations may cause difficulty in ingestion or passage through the gut. The release rates are affected by several factors, such as food and the rate of transit across the gut. Cost of preparation is high. Sometimes, the target tissue is exposed to a constant quantity of drug over prolonged period of time that may cause drug tolerance. Toxicity due to dose dumping, unpredictable and poor IVIVC, risk of side-effects or toxicity owing to immediate release of contained drug (mechanical failure, chewing or masticating, alcohol intake) and increased possibility for first-pass clearance are several other disadvantages associated with oral sustained release dosage forms. Moreover, additional patient education and counseling is also required for proper handling of the oral sustained release dosage forms (Hoffman, 1998; Munday and Cox, 2000).

15.5.3 KEY FORMULATION CONSIDERATIONS IN ORAL SUSTAINED RELEASE DOSAGE FORMS

15.5.3.1 Biological factors

15.5.3.1.1 Biological half-life

The main purpose of the oral sustained release dosage form is to maintain therapeutic drug plasma level over a prolonged period of time. To accomplish this purpose, the drug should enter the circulation at an equivalent rate at which it is eliminated. The elimination of the drug depends on the half-life ($t_{1/2}$) of therapeutic moiety. Short half-life compounds are usually an excellent candidate for sustained release formulation because of reduced dosing frequency. Generally, drugs with shorter half-lives (less than 2 h), such as furosemide or levodopa, act as poor candidates for sustained release formulation. Furthermore, drugs with long half-lives (more than 8 h), such as digoxin and phenytoin sodium, are not generally used in sustained form, as their effect is already sustained (Chugh et al., 2012; Robinson and Lee, 1987).

15.5.3.1.2 Absorption

Generally, the aim of generating a sustained release product is to slow down the rate of drug release than the rate of drug absorption. For most of the drugs, there is about 8–12 h of transit time in the absorptive regions of the GI tract and 3–4 h is maximum half-life that is required for absorption; otherwise, the device will pass out from the potential absorptive regions before completion of the drug release. This can be correlated with minimum apparent absorption rate constant of $0.17\text{--}0.23\text{ h}^{-1}$. Consequently, absorption of the drug must be at a comparatively uniform rate over the complete length of small intestine. However, this is not true for many therapeutic moieties. If a drug is absorbed by active transport mechanism or transport is limited to a particular area of intestine, then sustained release formulation would not be advantageous (Chugh et al., 2012; Shirwaikar et al., 2005).

15.5.3.1.3 Metabolism

A few drugs undergo metabolism before absorption, either in the lumen or tissue of the intestine, and exhibit decreased bioavailability from sustained release dosage forms. Therefore, poorly-water soluble drugs cannot be formulated as sustained release dosage form. A suitable system is required to augment the solubility of drug which can be then formulated as sustained release dosage form. However, a drug may encounter crystallization during its entry in to systemic circulation. This is why utmost care should be taken to prevent the crystallization of drug (Chugh et al., 2012; Popli and Sharma, 1989).

15.5.3.2 Physicochemical factors

15.5.3.2.1 Partition coefficient

When a drug is administered to the GI tract, it crosses different biological membranes to show a therapeutic action in particular tissues of the body. Physiologically, these membranes are composed of lipids; accordingly, the partition coefficient of oil-soluble drugs is crucial to determine the membrane penetration efficiency of drug. Lipophilic compounds have high partition coefficient, poor aqueous solubility, and are retained in tissues for the extended period of time. In the case of compounds having low partition coefficient, it becomes difficult for them to penetrate the membrane, which results in poor bioavailability. Conversely, partitioning effect can be applied equally to diffusion through polymeric membranes. Generally, diffusion-limiting membranes depend on the partitioning features of the drug (Chugh et al., 2012; Popli and Sharma, 1989).

15.5.3.2.2 Dose size

For systems which are orally administered, there is an upper limit to the size of the dose to be administered. Generally, a single dose of 0.5–1.0 g is considered highest for a conventional dosage form, which is also valid for sustained release dosage form. Drugs which are required in large doses can sometimes be given in

multiple quantities or formulated into liquid dosage forms. Administration of higher amount of a drug with a narrow therapeutic window is another aspect that should be determined before considering it for sustained release dosage form (Takayama, 2015).

15.5.3.2.3 Stability

Orally administered drugs experience both acid-base hydrolysis and enzymatic degradation. The rate of degradation is generally slow for solid state drugs, therefore, it is an ideal composition for delivering a drug in sustained manner. An unstable dosage form in stomach prolongs the delivery of drug over whole course of passage in GI tract. Consequently, it is beneficial for sustained release system until the dosage form reaches the small intestine. Drugs that are unstable in the small intestine may exhibit decreased bioavailability when administered from a sustained release dosage form. Ultimately, this effect causes degradation of drug and consequently requires a large dose of the drug. Propentheline and probanthine are representative examples of such category (Shabaraya and Narayanacharyulu, 2000; Roldy et al., 2003).

15.5.3.2.4 Ionization, pKa, and aqueous solubility

Most drugs are weak acids or bases. As the unchanged form of a drug satisfactorily permeates across lipid membranes, it is important to consider the link between pKa of the compound and absorptive environment. Providing the drug in an unchanged form is beneficial for drug permeation. However, the unionized form of a drug is inversely related to its aqueous solubility. Delivery systems that are dependent on diffusion or dissolution are also dependent on solubility of the drug. Consequently, such dosage forms should work in an environment of changing pH (acidic pH of stomach to nearby neutral pH of small intestine). Compounds having low solubility (<0.01 mg/mL) are mainly sustained release, since their release over the long time period in the GI tract will be limited by dissolution of the drug (Robinson and Lee, 1987; Roldy et al., 2003).

15.5.4 COATING SYSTEMS FOR ORAL SUSTAINED RELEASE DOSAGE FORMS

A sustained release effect can be achieved by a simple diffusional reservoir system, where drug inside the tablet core diffuses out through a semipermeable polymeric membrane. Before spraying, the coating material is either dissolved or dispersed in an aqueous or an organic solvent. The substrates are usually preheated in the coating equipment prior to the initiation of coating process. The coating solution or dispersion is atomized with air into tiny droplets that are later transported to substrate surface. Upon atomization, the droplets spread over the surface and coating occurs as the solvent evaporates. For aqueous-based dispersions, the polymer particles closely pack on the surface of the solid and, upon

further evaporation, the particles flow together due to cohesive forces between the polymer spheres and ultimately coalesce to form the film. Polymethacrylate and derivatives, the main compositions of Eudragit, are frequently used for sustained release coatings.

15.5.4.1 *Materials*

Water-insoluble polymers are used to modify the drug release in sustained release dosage forms. These polymers are typically used to achieve a sustained release of drug over an extended time period. Moreover, reduction in the dosing frequency for improving patient compliance is also achieved. Sustained release products reduce blood level fluctuations of the active ingredient, accordingly eliminating high peak concentrations usually associated with side-effects. The polymeric film of these sustained release systems controls the rate at which fluid from the gastrointestinal tract diffuses into the core substrate. This gastrointestinal fluid dissolves the drug and the drug then diffuses out from the dosage form owing to concentration gradient. Films between 50 and 150 μm thick create a longer and tortuous diffusional pathway (Rajabi-Siahboomi and Farrell, 2008; Rohera and Parikh, 2002) for drug release. Examples of polymers used to achieve sustained drug release include EC, polymethacrylates with quaternary ammonia groups, and polyvinyl acetate. A polymer may also be blended to adjust film permeability or mechanical properties (Zheng et al., 2005). Moreover, hydrophilic materials may be included in the coating solutions to create pores and facilitate drug release, an approach that is chiefly useful for drugs with limited water solubility (Sadhegi et al., 2001). An osmotic pump system is one of the subcategories of sustained release dosage forms. These delivery systems contain osmotic agent such as sodium chloride within the core. Subsequently, core is coated with a water insoluble polymer that later drilled with laser light to make an orifice in the coating film. Water diffuses into the core and dissolves the osmotic agent that increases the osmotic pressure. The drug is then released through the orifice. The coating used for these systems must be strong enough to withstand the hydrostatic forces reached during gastrointestinal transit (Verma et al., 2000). One of the most commonly used polymeric materials in osmotic pump delivery systems is cellulose acetate (Sastry and Khan, 1998). In addition, EC has also been used. Several variations in osmotic pump delivery systems are reported in the literature. For example, a bilayer tablet design, referred to as a push-pull osmotic pump, utilizes a swellable layer to maintain the hydrostatic pressure within the device for a more comprehensive release of the drug payload (Sastry and Khan, 1998). In another type of system, additives in the coating dissolve after ingestion to create pores in the film and this circumvents the need for a delivery orifice (Verma et al., 2003). Various excipients used in coating are solvents, plasticizers (triethyl citrate, acetyl triethyl citrate, tributyl citrate, acetyl tributyl citrate, triacetin), antiadherents (talc and glyceryl monostearate), pigments (red iron oxide, yellow iron oxide, titanium dioxide, aluminum lake yellow number 6), and surfactants (polysorbate 20).

Table 15.1 Currently Reported Preclinical Analysis of Oral Controlled or Sustained Release Dosage Forms in Experimental Animals

Category	Drug	Dosage Form	Remarks	Reference
Antihypertensive	Carvedilol	Osmotic pump capsule of carvedilol nano suspension	In vivo test in beagle dogs demonstrated relative bioavailability of novel system 203.5% in comparison to marketed preparation	Liu et al. (2014)
Antihypertensive	Femotidine	Chitosan sulfobutyl ether β -cyclodextrin tablets of famotidine	Improved absorption of femotidine after oral administration to rats	Anraku et al. (2015)
Antidiabetic	Repaglinide	Controlled release matrix pellets of repaglinide	Repaglinide sustained release matrix pellets consisting of avicel, lactose and other polymers released 94% of drug content after 12 h. Blood glucose levels decreased in rats (normal and diabetic both) in 8–12 h.	Tavakoli et al. (2014)
Antifibrinolytic drug	Tranexamic acid	Tranexamic acid loaded gellan gums based polymeric microbeads	Microbeads provided intestinal specific controlled release of tranexamic acid	Bhattachary et al. (2013)
Antihyperlipidemic	Lovastatin	Novel mesoporous nanomatrix-supported lipid bilayers for oral sustained delivery of the water-insoluble drug, lovastatin	Nanocomposite, mesoporous nanomatrix-supported lipid bilayer (MN-SLB) in beagles dogs controlled the dissolution rate and improved the oral absorption	Zhang et al. (2015)
β -Blocker	Propanolol	Oral sustained-release suspension based on a novel taste-masked and mucoadhesive carrier-ion-exchange fiber	Oral taste-masked mucoadhesive sustained-release suspensions of propranolol hydrochloride improved the plasma drug level in rats	Yuan et al. (2014)
Antimalarial	Artesunate	Sustained release artesunate-loaded solid lipid micro particles based on phytolipids	Artesunate-loaded solid lipid microparticles could be used once daily in the treatment of malaria	Chinaeke et al. (2014)

(Continued)

Table 15.1 Currently Reported Preclinical Analysis of Oral Controlled or Sustained Release Dosage Forms in Experimental Animals *Continued*

Category	Drug	Dosage Form	Remarks	Reference
Antiviral	Acyclovir	Gastro retentive sustained-release formulation of acyclovir	Acyclovir has PK limitations, including poor oral bioavailability of 15%–30%, high variability, and short elimination half-life of 2.3 h. A gastro retentive sustained-release formulation of acyclovir, based on a combination of swelling and mucoadhesive mechanisms, has been developed. The developed formulation prolonged retention in the upper gastrointestinal tract, sustained in vitro drug release, prolonged in vivo absorption, and better bioavailability than the immediate release formulation when tested in albino rabbits. It would also improve patient compliance and increase the efficacy of therapy	Sankar and Jain (2013)
Treatment for hypertension, pneumosilicosis, and lung cancer	Tetrandrine	Tetrandrine -phospholipid complex loaded lipid nanocapsules	Tetrandrine-phospholipid complex loaded lipid nanocapsules system developed in this study is a promising carrier that improves the oral bioavailability of tetrandrine in rats	Zhao et al. (2013)
	Cephalexin	Gastro-floating tablets of cephalexin	Gastro-floating tablets of cephalexin were prepared and optimized using HPMC K100M as matrix and sodium bicarbonate as a gas-forming agent. Study in fed and fasted beagle dogs was performed. It was concluded that the gastro-floating tablets had a sustained-release effect in vitro and in vivo, and desired PK properties in both fed and fasted conditions	Yin et al. (2013)

Treatment of bowel conditions	Budesonide	Budesonide loaded core/shell nanofibers	Budesonide loaded ethylcellulose-core/Eudragit S100-shell nanofibers improved colon distribution in rats after oral administration	Xu et al. (2013)
Nonsteroidal antiinflammatory drug	Aceclofenac	Aceclofenac-loaded unsaturated esterified alginate/gellan gum microspheres	Aceclofenac-loaded alginate/gellan gum microspheres showed sustained systemic absorption of aceclofenac in rabbits and excellent antiinflammatory activity in carrageenan-induced rats after oral administration over prolonged period	Jana et al. (2013)
Anticancer	Protopanaxadiol	Novel drug-phospholipid complex enriched with micelles	Mixed micelles consisting of protopanaxadiol (PPD)—phospholipid complexes improved antitumor therapies	Xia et al. (2013)
Antidiabetic	Pioglitazone	Pioglitazone-loaded jackfruit seed starch-alginate beads	Pioglitazone-loaded jackfruit seed starch-alginate beads showed significant hypoglycemic effect in alloxan-induced diabetic rats over prolonged period after oral administration	Nayak et al. (2013)
Treatment of liver diseases	Silymarin	Silymarin loaded porous silica nanoparticles	Silymarin-loaded porous silica nanoparticles gave a significantly higher plasma concentration and larger area under the concentration-time curves than commercial tablets when orally administered to beagle dogs	Cao et al. (2013)
Vaccine	Ulex europaeus agglutinin 1	Ulex europaeus agglutinin I (UEAI)-conjugated liposomes	UEAI-modified bovine serum albumin-encapsulating liposomes (UEAI-LIP) as oral vaccine carriers and examined the feasibility of inducing systemic and mucosal immune responses by oral administration of UEAILIP. In vivo immune-stimulating results were observed in KM mice. The study demonstrated the high potential of lectin-modified liposomes containing the antigen as carriers for oral vaccine.	Li et al. (2011)

(Continued)

Table 15.1 Currently Reported Preclinical Analysis of Oral Controlled or Sustained Release Dosage Forms in Experimental Animals *Continued*

Category	Drug	Dosage Form	Remarks	Reference
Antiplatelet drug	Dipyridamole	Dipyridamole sustained-release pellets	Sustained-release pellets loaded with dipyridamole were tested in fasted beagle dogs after oral administration. Owing to incorporation of a pH-modifier into the core of pellets to modify the inner microenvironment and employing mixtures of enteric soluble and insoluble polymers as a retarding layer displayed no pH-dependence in drug release and enhanced bioavailability	Xu et al. (2012)
Antihypertensive	Captopril	Captopril-cyclodextrin nanoparticles	Captopril–cyclodextrin complex in conscious Wistar rats reduces its oral dose to once per day	De azevedo Mde et al. (2011)
Antidiabetic	Insulin	Insulin chitosan capsules cross-linked with phytic acid	Phytic acid was used as a cross-linking agent for encapsulation of insulin in a chitosan matrix for oral delivery of insulin and the system was tested in vivo by oral administration in diabetic mice. Phytic acid-chitosan capsules reduced blood glucose level	Lee et al. (2011)
β -Lactum antibiotic	Amoxicillin	Gastro retentive controlled release drug delivery system for amoxicillin	Single-dose administration of gastroretentive controlled release system of amoxicillin offered effective antimicrobial course, improved PK-PD profiles, and minimized adverse effects	Horwitz et al. (2011)
α -Glucosidase inhibitor (antidiabetic)	Miglitol	Oral bioadhesive controlled release formulation of miglitol	Controlled release formulation of miglitol containing multilayered pellets were designed evaluated in dogs, displaying higher and prolonged levels of GLP-1 in comparison to placebo or immediate release	Deshpande et al. (2009)

Table 15.2 Clinical Trials of Recently Developed Oral Controlled or Sustained Release Dosage Forms

Category	Drug	Dosage Form	Remarks	Reference
Antihypertensive	Metoprolol	Intellicap controlled release capsules	Intellicap controlled release capsules exhibited extended release of metoprolol	Soderlind et al. (2015)
Antioxidant	Seasamol	Seasamol floating beads	Seasamol floating beads significantly decreased release rate, increased half-life by 50% and reduced in vivo clearance and showed to be better/equivalent to methotrexate	Geetha et al. (2015)
Opioid analgesic	Oxycodone	Controlled release tablet	Oxycodone once a-day tablet comprising of two layers, an inert HPMC layer and a controlled release layer improved patient compliance, safety, and efficacy, which could help researchers to develop new controlled release dosage forms of oxycodone.	Kim et al. (2015)
Opioid analgesic	Tramadol	Tramadol hydrochloride controlled release capsule filled with coated pellets	In vivo evaluation of tramadol controlled release capsules filled with pellets compared to sustained release tablets	Szkutnik-fiedler et al. (2014)
Antianginal	Trimetazidine	Mucoadhesive elementary osmotic pump tablets of trimetazidine	Studies showed bioavailability enhancement and drug release regulated by osmosis and diffusion in humans	Alam et al. (2015)

(Continued)

Table 15.2 Clinical Trials of Recently Developed Oral Controlled or Sustained Release Dosage Forms *Continued*

Category	Drug	Dosage Form	Remarks	Reference
Calcium channel blocker	Isradipine	Isradipine controlled release matrix tablet containing self emulsifying solid dispersion	5 mg isradipine self emulsifying solid dispersion system showed increased oral bioavailability and extended plasma concentration compared to marketed 5 mg dynacirc capsule in humans	Tran et al. (2013)
Anticancer	Vinpocetine	Cyclodextrin multicomponent complexation	The AUC for the complex formulation was 2.1–2.9 times higher than the vinpocetine immediate release formulation.	Riberio et al. (2007)
Antiasthmatic	Theophylline	Theophylline matrix tablets and novel cluster tablets	Comparison between 2 sustained release preparations. Tablet A swelling disintegration matrix tablet consisting of hydrophobic wax granules showed less inter subject variations than tablet B.	Hayashi et al. (2007)
Antibiotic (used for <i>H. pylori</i> eradication)	Amoxicillin	Novel floating in situ gelling system of amoxicillin	Gellan based amoxicillin floating in situ gelling systems (AFIG) showed significant anti <i>H. pylori</i> effect in gerbil model. Required amount of amoxicillin for eradication of <i>H. Pylori</i> was 10 times less in AFIG than from corresponding	Rajnikanth et al. (2007)

(Continued)

Table 15.2 Clinical Trials of Recently Developed Oral Controlled or Sustained Release Dosage Forms *Continued*

Category	Drug	Dosage Form	Remarks	Reference
Antidiabetic	Metformin hydrochloride	Sustained release metformin hydrochloride pellets	amoxicillin suspension due to prolonged gastrointestinal residence time. Metformin sustained release pellets prepared by coating with a blend of Eudragit L30D55 and Eudragit NE30D acquired perfect sustained release properties and good relative bioavailability	Hu et al. (2006)
Antihypertensive	Carvedilol	Laminated sponges as solid hydrophilic matrices for the buccal delivery of carvedilol microemulsion systems	Carvedilol (CVD) suffers from low absolute bioavailability (25%) due to its limited aqueous solubility and hepatic first-pass metabolism. HPMC laminated buccal sponges loaded with CVD microemulsions (CVD-ME) were used to surmount such limitations. In vivo mucoadhesion and absorption studies of laminated sponges were assessed in healthy volunteers. These systems showed improved drug oral bioavailability and in controlling drug release kinetics via buccal mucosa.	Abd-Elbary et al. (2016)

15.6 PRECLINICAL AND CLINICAL STATUS OF ORAL CONTROLLED AND SUSTAINED RELEASE DOSAGE FORMS

Although there are several hurdles in the development of oral controlled release formulations, success has been gained in development of controlled release formulations of many therapeutic moieties. Currently, several oral controlled/sustained drug delivery systems are undergoing preclinical (Table 15.1) and clinical trials (Table 15.2).

15.7 CONCLUSION

In conclusion, oral sustained release and controlled release products are more favorable over conventional dosage forms, owing to augmentation of biopharmaceutical, PKs, and PDs traits of drugs. Oral controlled or sustained drug delivery systems offer reduction in dose-dosage frequency, to an extent that once-daily dose is adequate for maintaining uniform plasma concentration and providing maximum utility of the drug. Additionally, reduction in local and systemic side-effects and high patient compliance are also the lucrative advantages of oral controlled/sustained drug delivery systems. The reasonable cost of oral controlled release formulations has increased the market share, compared to oral conventional dosage forms. Therefore, keeping in mind the various biopharmaceutical and preformulation factors, oral controlled release or sustained dosage forms can be successfully designed, developed, and scale-up for commercial utility.

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Hydrogels: From simple networks to smart materials—advances and applications

16

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CHAPTER OUTLINE

16.1 Introduction	628
16.2 Hydrogels in a Historical Perspective: From Simple Networks to Smart Materials.....	631
16.2.1 First Generation Hydrogels	631
16.2.2 Second Generation Hydrogels	633
16.2.3 Third Generation Hydrogels	638
16.2.4 Fourth Generation: Smart Hydrogels.....	642
16.3 Properties of Hydrogels	644
16.3.1 Factors Affecting Swelling of Hydrogels.....	644
16.3.2 Dynamics of Swelling.....	644
16.3.3 Mechanical Properties	645
16.3.4 Cytotoxicity and In Vivo Toxicity	645
16.3.5 Miscellaneous Properties.....	646
16.4 Classification of Hydrogels	647
16.4.1 Hydrogels Based on Properties.....	649
16.4.2 Stimuli-Responsive Hydrogels.....	649
16.5 Synthesis of Hydrogels	650
16.5.1 Chemically Cross-Linked Hydrogels	650
16.5.2 Physically Cross-Linked Hydrogels	658
16.6 Hydrogels in Drug Delivery	660
16.7 Conclusion	662
List of Abbreviations	663
References	664

16.1 INTRODUCTION

Bacterial biofilms (hydrated extracellular matrix component) and plant structures (ubiquitous water-swollen motifs) are some of the examples which reveals the primal existence of hydrogels in nature since life on earth (Ullah et al., 2015). If traced back in early human history, it can be deciphered that gelatin and agar were being used for various applications long ago, however the modern history of hydrogels depicts the designing of a class of materials which can be employed for biomedical applications (Ullah et al., 2015). Hydrogel research traces back its origin to the early 1960s with the publication of a novel paper by Wichterle and Lim on poly(2-hydroxyethyl methacrylate) (Wichterle and Lim, 1960). The mechanism behind the net repulsion (between a polymer and poor solvent), phase transition, and change in the swelling degree was successfully predicted in the year 1968. In the early 1960s hydrophobic gel for biological use had been introduced (Tavsanli and Okay, 2016). Remarkable interest from the scientific community has been gained on these materials since the first report of hydrogels were published by Wichterle and Lim. Later on, efforts were made in order to advance and extend the potentials application of hydrogels.

Owing to significant developments in hydrogel technology, a dramatic advancement has been observed in the pharmaceutical and biomedical era. Typical applications of hydrogels include tissue engineering, soft-contact lenses, wound healing, sensors, muco-adhesives, and bioactive factory delivery systems (Nguyen et al., 2015). Because of the high degree of hydration, these hydrogels are the subject of investigation of researchers interested in fundamental aspects of swollen polymeric networks, and have also found widespread application in different technological areas (Hennink and Nostrum, 2012). Starting from their very early and relatively simple application as contact lenses, hydrogels have paved the way in much more complicated applications such as tissue engineering and controlled delivery systems. An example of this can be depicted by drug-loaded hydrogels where the gel matrix allows the drug to become encapsulated into the corona, thereafter releasing it within a therapeutic range (Nguyen et al., 2015).

By definition, hydrogels are three-dimensional (3D) polymeric networks capable of imbibing high amounts of water or biological fluid (Hamidi et al., 2008). In other words, it can be said that a 3D network of polymers made up of natural or synthetic material possessing high degree of flexibility due to large water content is called a hydrogel (Ullah et al., 2015). Hydrogels can also be described in a rheological way as “aqueous solutions of hydrophilic polymers at low or moderate concentrations, where no substantial entanglement of chains occurs, and normally demonstrates Newtonian behavior” (Hennink and Nostrum, 2012). Recently, hydrogels have been defined as two- or multicomponent systems consisting of a three-dimensional network of polymer chains and water that fills the space between macromolecules (Ahmed, 2015).

Their affinity to absorb water contributes to the presence of hydrophilic groups, such as $-\text{OH}$, $-\text{CONH}$, $-\text{CONH}_2$, and SO_3H in polymers forming hydrogel structures (Hamidi et al., 2008; Peppas and Khare, 1993). This higher affinity or degree of hydration (more than 90%) may be attributed to the presence of these hydrophilic groups. The nature of the aqueous environment and polymer composition also plays a vital role in deciding the unique physiochemical characteristics of hydrogels (Hamidi et al., 2008). Compared to other class of synthetic biomaterials, these chemical moieties possess vital physiochemical characteristics viz. high water content, soft and rubbery consistency, and low interfacial tension with water or biological fluids, by virtue of which these structures resemble the living tissues (Blanco et al., 1996). The inert nature of hydrogels often lead to minimized nonspecific interaction with proteins and cells, which makes hydrogels ideal candidates for numerous bio related applications (Buenger et al., 2012).

Despite the fact that these gels retain a higher degree of hydration, they tend to swell rather than dissolve in the surrounding aqueous environment (swell without dissolution up to 90% (w/w) water of their dry weights) (Buenger et al., 2012). This might be attributed to the presence of a critical cross-link in their parent structure. The cross-links in the polymer network are provided by covalent bonds, hydrogen bonding, van der Waal interactions or physical entanglements (Hamidi et al., 2008). Depending on the type of cross-linking, hydrogels can be broadly classified into two major categories:

Reversible or physically cross-linked hydrogels (entanglements or crystallites): These hydrogels are formed by dynamic cross-linking of synthetic or natural building blocks, based on noncovalent interactions such as hydrophobic and electrostatic interactions or hydrogen bridges (Buenger et al., 2012). The network of these hydrogels is held together via molecular entanglements or by secondary forces including ionic, hydrogen bonding, and hydrophobic forces. These hydrogels are not homogenous, owing to the fact that they comprises of clusters of molecular entanglements, hydrophobically or ionically associated domains. Due to the presence of free chain ends or chain loops transient network defects are also represented and formulates a characteristic properties of these hydrogels (Hoffman, 2012).

For example, in the hydrolysis of polyacrylonitrile (PAN) to form amide and acid groups from the nitrile groups, if the nitrile groups remain in sufficient concentration and association, they can stabilize the hydrogel by hydrophobic interactions accordingly forming a physical hydrogel (Hoffman, 2012).

Permanent or chemical cross-linked hydrogels (tie points and junctions): They are formed by covalent networks and do not dissolve in water without breakage of covalent bonds (Buenger et al., 2012). Chemical hydrogels can be prepared in two different ways viz. three-dimensional polymerization (a hydrophilic monomer is polymerized in the presence of a poly functional cross-linking agents) or by direct cross-linking of water-soluble polymers (conversion of hydrophobic polymers to hydrophilic polymers). Like physical hydrogels, chemical hydrogels also depict the phenomenon of heterogeneity. This may be attributed to the fact that

hydrophobic aggregation of cross-linking agents leads to the development of regions of low water swelling and high cross-linking density, called clusters (Hoffman, 2012).

For physical and chemical hydrogels numerous macromolecular structures are possible; some of them are (Hoffman, 2012):

1. Cross-linked or entangled networks of linear homopolymers
2. Linear copolymers
3. Block or graft copolymers
4. Polyion—multivalent ion
5. Polyion—polyion or H bonded complexes
6. Hydrophilic networks stabilized hydrophobic domains
7. interpenetrating polymer networks (IPNs) or physical blends.

Hydrogels may also have different physical forms including (Hoffman, 2012):

1. Solid molded forms
2. Pressed powder matrices
3. Microparticles
4. Coatings
5. Membranes or sheets
6. Encapsulated solids
7. Liquids

Hydrogels can be made responsive to some physical, chemical, or biological stimuli, if their polymeric corona is endowed with certain functional groups. On exposure to respective stimuli, a reversible change is triggered in swelling of the network. This response is determined by the hydrogel composition, cross-linking type, and degree of cross-linking (density of junctions joining the chains into a permanent form). All these properties can be tailored to create hydrogels with adjusted properties (Buenger et al., 2012; Gander et al., 1989). Desired functionality, reversibility, sterilizability, and biocompatibility are some of the characteristic properties which make hydrogel a versatile tool for (Ullah et al., 2015):

- tissue replacement and treatment,
- organ replacement and treatment,
- interaction with biological system,
- treating or replacing the function of living tissues.

In order to circumvent the dissolution of hydrophilic polymer chains in an aqueous environment, it becomes a prerequisite that cross-links must be present. Numerous techniques to establish cross-links have been established and have been employed in the preparation of hydrogels. Furthermore, introduction of labile and biodegradable bonds in the parental structure of hydrogel makes it a versatile tool for varied applications. These bonds can be either incorporated into the backbone or in the cross-links used to prepare the hydrogel (Hennink and Nostrum, 2012). Hydrogels may be chemically stable or they may degrade

eventually disintegrate and dissolve. The unstable linkages may be cleaved in physiological conditions, either chemically or enzymatically; however, hydrolysis plays a major role in the degradation process (Akhtar et al., 2015). It is of great interest to have control over the parameters by which the degradation characteristics can be tailored (Hennink and Nostrum, 2012).

In general, it can be said that hydrogels possess a good biocompatibility; their hydrophilic surface has a low interfacial free energy in contact with the body fluids, which results in a low tendency for proteins and cells to adhere to these surfaces (Hennink and Nostrum, 2012). Moreover, the viscoelastic nature of hydrogels minimizes irritation to surrounding tissues. A rational and proper selection of the hydrogel building blocks will aid in tailoring the nature of the degradation products formed (Hennink and Nostrum, 2012).

16.2 HYDROGELS IN A HISTORICAL PERSPECTIVE: FROM SIMPLE NETWORKS TO SMART MATERIALS

16.2.1 FIRST GENERATION HYDROGELS

It was around the 1900s that the term hydrogel first appeared in scientific literature, where it was used to describe a colloidal gel of inorganic salts (Bemmelen, 1894; Buwalda et al., 2014a). Since the 1900s, the following decades observed an era where hydrogel research focused primarily on the development of relatively simple and chemically cross-linked hydrogels for their application in ophthalmic and drug delivery. These relatively simple gels were deemed to be appropriate for fundamental characterization and modeling of varied physiochemical properties viz. solute diffusivity and cross-link density. The first generation hydrogels are described further, with the representative examples (Buwalda et al., 2014a).

16.2.1.1 Hydrogels prepared by polymerization of water-soluble monomers

This category of hydrogel is prepared chiefly by chain addition reaction employing vinyl monomers. In this method, an initiating free radical species (cross-linking agent) is added to a vinyl monomer. This step is followed by opening of the π bond to form a new radical until the termination of polymerization takes place either by recombination of two radical species or disproportionation. Polymerization of water-soluble monomers possesses an additional edge over the classical bulk polymerization technique:

1. Rapid hydrogel formation
2. Possibility to obtain predefined geometry or configuration (Buwalda et al., 2014a).

An important hydrogel-forming polymer in this category is polyacrylamide (PAM) which found its place in industrial applications as an agricultural gel.

Initially in 1960s, PAMs were being used for the physical entrapment of vital biological moieties viz. cells and enzymes. They were also employed in the covalent attachment of proteins. Now, PAM-based hydrogels have found a huge potential application in augmentation materials and designing of soft tissue fillers (Buwalda et al., 2014a).

Another important polymer is poly(hydroxy-alkyl methacrylate) (pHEMA) which is considered to be among the most extensively employed first generation hydrogels in biomedical applications. Despite its discovery 50 years ago, these hydrogels still remain the basis for the development of contact lenses (Teichoreb et al., 2008). Owing to their characteristic properties such as hydrolytic stability and the possibility to modulate release properties (by altering the cross-link density), these hydrogels have also been employed for controlled drug delivery applications (i.e., antibiotics, contraceptives, antiarrhythmic, ophthalmic, vasoconstrictors, antiinflammatory drugs, and cytostatics) (Buwalda et al., 2014a).

16.2.1.2 Hydrogels based on cross-linking of water-soluble synthetic polymers

Hydrophilic polymers that are covalently cross-linked (by either reaction between functional groups or free radicals) constitute the second major category of the first generation hydrogels. Polyvinyl alcohol (PVA) and polyethylene glycol (PEG) are the two most extensively studied polymers of this category (Buwalda et al., 2014a).

PVA was first described in 1924 as a linear synthetic polymer synthesized by free radical polymerization of vinyl acetate (Lindeman, 1971). Chemical cross-linking method is generally employed for the production of PVA hydrogels. Aldehydes, anhydrides, and isocyanates are some of the most frequently employed cross-linking agents. It was in the 1990s, that PVA hydrogels were eventually recognized and found their application in tissue engineering (Lozinsky and Plieva, 1998). The Sefton group widely used PVA hydrogels in conjugation with heparin (sulphated glycosaminoglycan) for cardiovascular surgery or other blood handling procedures (Goosen et al., 1980). PVA-based hydrogels (swelling-controlled hydrogels) have also been employed for controlled drug delivery applications. Glutaraldehyde-conjugated PVA hydrogels showed near zero-order kinetics for the release of model drug theophylline which was further modulated by altering the cross-linking density of the polymer (Korsmeyer and Peppas, 1981). In another study it was deciphered that model drugs of varying sizes showed controlled and zero-order drug release kinetics, when compressed blends of PVA were complexed with other homopolymers, such as polyvinyl pyrrolidone (PVP) and PEG (Peppas et al., 1983).

It was in the mid-1960s and 1970s that PEG received a considerable amount of attention for its use in hydrogels, owing to its excellent biocompatibility and resistance to protein absorption (Buwalda et al., 2014a; Zhiu, 2010). In the initial phase of hydrogel research, covalent cross-linking methods were generally employed for the preparation of hydrogels from hydrophilic PEG homopolymers.

It was established from numerous studies that gamma or electron beam radiation could be used for the preparation of both linear and star-shaped hydrogels from the aqueous solution of PEG (Buwalda et al., 2014a; Ward and King, 1970; Rempp et al., 1990). Other reactions, such as free radical polymerization reaction, condensation reaction, Michael-type addition, click chemistry, native chemical ligation, and enzymatic reactions, have also been employed for hydrogel preparation using PEG as parent excipient. Early PEG-based hydrogels were vastly applied in the fields of controlled drug delivery systems and antiadhesive materials. For sustained release of prostaglandins, caffeine, and morphine, Buwalda et al. (2014a) designed PEG di-isocyanates cross-linked hexanetriol hydrogels. In the beginning of 1980s a contraceptive sponge containing nonoxynol phenol (spermicide) based on cross-linked PEG di-isocyanates was commercialized and is still available on the market (Edelman et al., 1984).

16.2.1.3 Hydrogels based on cellulose

Apart from numerous synthetic polymers, such as PEG, PVA, and pHEMA, cellulose (a semisynthetic derivative of the natural polymer) attained attention for its use in biomedical applications, especially in the designing of controlled drug delivery systems. In the context of sustained release, a compressed matrix of hydrophilic cellulose ether hydroxypropyl methylcellulose has been widely employed. However, it was observed that the combination of diffusion and dissolution, along with hydration were the major phenomenon governing drug release (Buwalda et al., 2014a; Huber et al., 1966). Another study depicted that a change in polymer concentration had a significant effect on the drug release rate, along with varying the hydrophilicity of polymer, it helped in attaining a zero-order release kinetics for some hydrophilic drugs, such as diazepam (Ford et al., 1987; Ford and Hogan, 1985).

16.2.2 SECOND GENERATION HYDROGELS

It was in the mid-1950s and 1960s that studies conducted by Katchalsky demonstrated the possibility of transferring chemical energy into mechanical energy. With this discovery came an era of 1970s where the focus of hydrogel research shifted from relatively simple networks to stimuli-based hydrogels. These hydrogels were capable of responding to change in external stimuli (environmental conditions) viz. pH, temperature, and concentration of molecules. Specific events can be evoked using these environmental triggers for example, sustained drug release or hydrogel formation (Buwalda et al., 2014a).

16.2.2.1 Temperature-sensitive hydrogels based on PEG–polyester block copolymer

Temperature-sensitive hydrogels are the most widely studied environmentally responsive systems. In these characteristic systems, the cross-links are constituted predominantly by physical interactions (hydrogen bonding and hydrophobic

interactions). The thermosensitive gelation behavior of such systems is found to be governed by the temperature-dependent equilibrium of physical interactions (Buwalda et al., 2014a). Their applicability to in situ-forming systems makes them a versatile tool for biomedical applications. Prior to gelation, these fluids can be injected into body tissues, cavities, or organs in a minimally invasive manner (Ward and Georgiou, 2011). PEG–polyester block copolymers and poly(*N*-isopropyl acrylamide) (pNIPAAm) are some of the polymers which have been most frequently investigated in this class of hydrogels (Buwalda et al., 2014a). An example of a temperature-sensitive hydrogel is the aqueous solution of Pluronic or Poloxamers, which, when used at a concentration above the critical gel concentration (CGC), exhibit the phenomenon of phase transition viz. sol to gel (at low temperatures) and gel to sol (at high temperatures) (Buwalda et al., 2014a). It was in the late 1970s where controlled delivery of drug was accomplished using Pluronic-based hydrogels. Controlled delivery of anesthetics, antimicrobials, ophthalmic, cytostatics, and hormones were attained using Pluronic F127-based hydrogels (Buwalda et al., 2014a).

In the early 1990s, numerous linear di-block and tri-block copolymers having an hydrophilic poly(lactic) acid (PLA) block were synthesized by Kim et al. It was deciphered that, despite keeping the concentration and molecular weight of PEG constant, the tri-block copolymers generally yielded hydrogels at a very low polymer concentration, compared to their counterpart's viz. di-block polymers. It was also highlighted that tuning the hydrophilic to hydrophobic balance (HLB), block length, and the stereo regularity of the PLA block could in turn effect the thermo-responsive properties of the hydrogels (Choi et al., 1999; Jeong et al., 1999). A simultaneous decrease in CGC from 20% to 12% w/v was observed for PEG–PLLA–PEG when the poly(L-lactic acid) (PLLA) block length was increased from 2 to 5 kg/mol. Fluorescein iso-thiocyanate-labeled dextran was used as a model drug for conducting the release studies. Drug release followed zero-order release kinetics and a total of 40% of the initial drug load was released in 12 days (Buwalda et al., 2014a). An inverted tri-block copolymer (BAB) was synthesized via ring opening polymerization of lactide. It was important to learn that, on increasing the block length of PLA from 10 to 15 lactyl units at room temperature, the constrained geometry complex (CGC) of tri-block decreased from 80% to 15% w/v. Poly(lactic acid) block length also has a pronounced effect on the gelation behavior, compared to the ABA-type tri-block copolymers (Zhong et al., 2005).

In the late 1990s, PEG–PLA based systems were replaced by hydrogels based on PEG–PLGA–PEG tri-block copolymers. These hydrogels possessed an innate tendency to transform from sol to gel at lower temperature and gel to sol at higher temperatures. Jeong et al. observed that a transparent hydrogel was formed in situ when an aqueous solution of PEG–PLGA–PEG was administered subcutaneously into rats (Jeong et al., 2000). These hydrogels demonstrated good mechanical properties and were found to be stable for 1 month. Controlled release of several bioactive molecules including model drugs (viz. ketoprofen and spironolactone)

and transforming growth factor- β 1 (TGF- β 1) were accomplished employing PEG–PLGA–PEG-based hydrogels (Buwalda et al., 2014b).

BAB-type copolymers (PLGA–PEG–PLGA) were subsequently introduced as new hydrogel-forming materials and were commercialized under the brand name of ReGel (D.S. Lee et al., 2001). These polymers were synthesized via ring opening polymerization of D,L-lactide and glycolide using PEG as an initiator. Compared to PEG–PLGA–PEG (ABA-type copolymers)-based hydrogels, these hydrogels showed a lower CGC and sol-to-gel transition temperature, consequently specifying a different mechanism of gelation for BAB-type polymer-based hydrogels (Shim et al., 2002).

In a different experiment, the release pattern of paclitaxel (model drug) was studied using ReGel. Diffusion-controlled release pattern was observed for the first 2 weeks, followed by a combinational release (diffusion and degradation) up to 5 weeks in vitro. However, paclitaxel release from pluronic hydrogel was completed within 1 day. In vivo, paclitaxel-loaded ReGel showed a higher tumor efficacy and fewer side effects than the clinically used formulations of paclitaxel (Buwalda et al., 2014b; Zentner et al., 2001). Controlled release of several other bioactive molecules such as interleukin-2, insulin, and testosterone have been accomplished by employing ReGel formulations (Buwalda et al., 2014b). Both ABA- and BAB-type block copolymers were prepared by Bae and coworkers using poly caprolactone (PCL) and PEG as parent excipient (Jeong et al., 2000). Copolymers having an appropriate HLB showed a lower sol-to-gel and gel-to-sol transition temperature with increasing temperature. Similar to PLGA–PEG–PLGA tri-block copolymers, a lower CGC temperature and a larger window was exhibited when aqueous solutions of PCL–PEG–PCL were employed. It was also perceived that a lower CGC and larger window was attained in the case of solutions comprising of PCL–PEG–PCL compared to the PEG–PCL–PEG copolymers. It is ascribed to the probable formation of inter-micellar bridging in PCL, which further lead to more facile aggregation (Jeong et al., 2000; Buwalda et al., 2014b). Vitamin K, honokiol, and albumin were used as model drugs for conducting the drug release studies. PCL–PEG–PCL hydrogels predominantly showed a diffusion-based mechanism for drug release owing to the high in vitro hydrolytic stability of the PCL blocks (Gong et al., 2009).

Recently, poly(ϵ -caprolactone-co-lactide)-b-poly(ethylene glycol)-b-poly(ϵ -caprolactone-co-lactide) (PCLA–PEG–PCLA) tri-block copolymers were employed for synthesizing temperature-responsive hydrogels. The parameters viz. crystallinity, rheological profile, and degradability of the hydrogel were profoundly affected by the molecular weight of PCLA, the caproyl/lactoyl ratio and the nature of the terminal groups present in the copolymer (Petit et al., 2012, 2013). In vitro studies on erythrocytes and chondrocytes revealed that the PCLA–PEG–PCLA hydrogels offers good cyto-compatibility. In vivo studies using micro CT imaging technique and histology exhibited an enhanced intra-articular biocompatibility. No change in the quality or quantity of the cartilage was also shown (Sandker et al., 2013). Poly(3-methylglycolide), poly

(δ -valerolactone), poly(propylene fumarate), and poly(trimethylene carbonate) are some other examples of hydrophobic blocks which have been used for the preparation of amphiphilic PEG-co-polymeric hydrogels (Buwalda et al., 2014b).

16.2.2.2 Temperature-sensitive hydrogels based on poly(*N*-isopropyl acrylamide)

In the late 1950s, poly(*N*-isopropyl acrylamide) (pNIPAAm) was initially used for the synthesis of rodent repellent (Schild, 1992). However, it did not gain much significance. It was in 1968 that Heskins and Guillet (1968) reported that the solutions of pNIPAAm were capable of demonstrating temperature-dependent phase transition in water. After this report, an interest sparked for employing these solutions as an injectable material. This may be ascribed to the fact that these phase transition occurs between body and room temperature, which makes them an excellent vector for biomedical applications (Buwalda et al., 2014b). However, a major hindrance is syneresis (phase transition from hydrated swollen state to collapsed dehydrated state), which hampers the application of pNIPAAm in hydrogel formulations (Schild, 1992). In order to circumvent this drawback, covalent networks were formed by carrying out subsequent radical copolymerization of pNIPAAm with monomers viz. dihydroxyethylene-bis-acrylamide (Wu and Yager, 1992). Since the 1980s cross-linked pNIPAAm hydrogels were studied for the controlled delivery of various active biomolecules of paramount importance viz. vitamin B12, myoglobin, and progesterone (Buwalda et al., 2014b). Owing to a higher drug release rate these pNIPAAm hydrogels were replaced by interpenetrating polymeric networks (IPNs) of pNIPAAm. These IPNs proved to be superior and offered some desirable properties, such as enhanced mechanical stability and sustained release (Zhang and Chu, 2004). Additional efforts were made to increase or decrease the lower critical solution temperature (LCST) by copolymerizing pNIPAAm with hydrophilic or hydrophobic monomers (Feil et al., 1993). These hydrogels provided numerous unique properties, while their applicability in the field of biomedicine is limited owing to their nondegradability. A potent solution to this problem is provided by the introduction of biodegradable segments in the core structure of pNIPAAm, which will ultimately result in the formation of nontoxic low molecular weight degradable by products (Zhang et al., 2009b; Ankareddi et al., 2008).

Manipulation of LCST is the most striking approach for the formation of bioresorbable pNIPAAm-based hydrogel. Copolymers of NIPAAm and 2-hydroxyethyl methacrylate monolactate (HEMA-lactate) were developed by Hennink et al. Neradovic et al. (1999). It was deciphered from the studies that when these copolymers were incubated in aqueous solutions, hydrolysis of their lactate ester side groups along with dissolution of polymer took place at a LCST above 37°C (Buwalda et al., 2014b). Due to its high hydrophilicity and immunogenicity, HEMA was later replaced by *N*-(2-hydroxypropyl) methyl acrylamide (HPMA). With this slight modification in the formulation, a highly tunable degradation rate was obtained (Neradovic et al., 2004). Using a similar

hydrolysis-sensitive approach, pNIPAAm has also been copolymerized with dimethyl- γ -butyrolactone acrylate (DBA) that initially resulted in a decrease in LCST with increasing DBA content (Buwalda et al., 2014b; Cui and Vernon, 2007). On hydrolysis above 37°C, an increase in LCST was observed.

16.2.2.3 *In situ*–forming hydrogels based on other stimuli

Besides temperature another trigger for hydrogel formation is pH. These pH-sensitive hydrogels mainly consist of moieties (acidic or basic) which tend to become ionized at respective high or low pH. A promising controlled release of the hydrogel content can be established by making use of the variations in pH in different body parts (Buwalda et al., 2014a). Interest has emerged in recent years for the preparation of *in situ*–forming materials using polymers which can respond to the concentration of biomolecules. A change in the concentration of biomolecules can, in turn, induce a conformation change of the hydrogel. Consequently, it is credible to state that these biomolecule-sensitive hydrogels are the perfect mimics of nature (Miyata and Nakamae, 2002).

Glucose is the most illustrious biomolecule in this category. In an experiment, glucose oxidase, insulin, and pH-sensitive moiety-loaded hydrogels were fabricated. After the gradual diffusion of glucose peroxidase in these hydrogels, it was observed that it was readily converted into gluconic acid, ultimately leading into a decrease in the pH, followed by a subsequent increase in the swelling of the hydrogel. This unusual trend generated is due to the protonation of amine functionalities present in the polymeric network. However, it was established that these hydrogels possessed a sustained release. Accordingly, it becomes evident that these hydrogels can act as self-regulating systems and consequently offer potential application for the controlled delivery of biomolecules (Ishihara et al., 1984; Vermonden et al., 2006).

16.2.2.4 *Other thermo-responsive systems*

Hydrolytically degradable polymers viz. HPMA lactate monomers have found wide application in designing numerous thermo-responsive hydrogels. Buwalda et al. synthesized hydrogels using an ABA tri-block polymer poly(*N*-(2-hydroxypropyl) methacrylamide lactate) and PEG (Buwalda et al., 2014b). These tri-block copolymers possessed an innate tendency to formulate hydrogels at 37°C (Buwalda et al., 2014b; Vermonden et al., 2006, 2009). It was observed that a change in concentration of the polymers and thermosensitive block length had an adverse effect on the storage modulus, which can be further used for the higher tenability of such hydrogels. Vermonden et al. observed in their experiments that formulation parameters (viz. polymer design, concentration and temperature) were governing the release rate and diffusion of dextran labeled fluorescent dye (viz. fluorescein iso-thio cyanate (FITC)) (Vermonden et al., 2009; Hoth and Lutz, 2005).

Atom free radical polymerization (ATRP) of copolymers, 2-(2-methoxyethoxy) ethyl methacrylate and oligo(ethylene glycol) methacrylate (P

(MEO₂MA-co-OEGMA)), was done by [Hoth and Lutz \(2005\)](#) in order to synthesize a di-block copolymer abbreviated as pOEGMA. This di-block copolymer was tested for LCST in water; however, they were compared with pNIPAAm. It was observed that, like pNIPAAm polymers, these polymers had an analogous independence of LCST on pH and polymer concentration and were consequently used for formulating microgels ([Hoth and Lutz, 2005](#); [Slager and Domb, 2003](#)). These microgels were of particular interest as these system possess additional advantages such as ([Buwalda et al., 2014b](#)):

1. higher tenability depending on monomer feed,
2. smaller difference in transition temperature,
3. lesser dependence of the LCST on polymer chain length,
4. higher biocompatibility.

16.2.3 THIRD GENERATION HYDROGELS

Hydrophobic and ionic interactions were mainly employed for cross-linking the temperature- and pH-responsive physical hydrogels described in the previous section. In order to enhance and formulate finely tuned hydrogels it becomes prerequisite that mechanical, thermal, and degradation properties have to be looked at. It was during the mid-1990s that other physical interactions were recognized and used as novel cross-linking methods which offered the possibility of hydrogel formation in situ ([Buwalda et al., 2014b](#)). The following section will shed light on the third generation hydrogels and the novel methods used for cross-linking (viz. stereo-complexation, inclusion complex formation, metal ligand coordination, and peptide interactions).

16.2.3.1 Stereo-complexed hydrogels

Stereo-selective interaction between two complementing stereo-regular polymers resulting in the formation of a new composite possessing altered physical property is known as polymer stereo-complex ([Buwalda et al., 2014b](#); [Slager and Domb, 2003](#)). It was in 1953 that Pauling and Corey reported stereo-complexation for a polypeptide ([Pauling and Corey, 1953](#)). Ikada et al. was the first to report stereo-complexation between enantiomeric poly lactides (viz. PLLA and PDLA) in 1987 ([Ikada et al., 1987](#)). These enantiomeric poly lactides form the stereo-complex at shorter block lengths of PLA accordingly creating an operation window. As a result, hydrogels can be formulated on mixing the aqueous solutions of block copolymers (PLLA and PDLA) followed by cross-linking through stereo-complexation ([Buwalda et al., 2014b](#); [Ikada et al., 1987](#)). From 2000 onward injectable hydrogels were prepared via employing stereo-complexation between enantiomeric PLLA and PDLA copolymers ([Buwalda et al., 2014b](#)).

In an another experiment, the influence of the architecture of stereo-complexed block copolymer (PEG–PLA) on gelation properties was investigated by Kimura et al. ([Mukose et al., 2004](#); [Mukose et al., 2001](#)). It was established

that BAB-type tri-block copolymers (PLA-PEG-PLA) showed thermo-reversible gelation, while irreversible sol-gel transition phenomenon was exhibited when aqueous solutions of these enantiomeric copolymers were mixed at an incrementing temperature (Buwalda et al., 2014b; Mukose et al., 2001, 2004). Whereas, hydrogels formulated via employing high concentration of ABA (PEG-PLA-PEG) tri-block copolymers showed temperature-dependent reversible gel-sol transition (Mukose et al., 2001, 2004). Further experimentation showed that hydrogels were formulated on mixing the enantiomeric solutions of AB di-block copolymers; however, these hydrogels, unlike the ABA tri-block system, exhibited an irreversible gel-sol transition phenomenon with incrementing temperature (Buwalda et al., 2014b; Mukose et al., 2001, 2004).

In order to study and compare the release kinetics of an immunostimulant thymopentin (used in early years to improve the immunological condition of AIDS patient), Grijpma et al. formulated hydrogels by employing stereo-complexed mediated gelation of di-block (PEG-PLA) and BAB tri-block (PLA-PEG-PLA) copolymers to study and compare the release kinetics of thymopentin (Grijpma and Feijen, 2001). It was revealed from the experimental studies conducted that these systems exhibited a comparatively slower release of the model drug from their counterpart viz. single enantiomer-based hydrogels (Buwalda et al., 2014b; X. Wang et al., 2010a).

In an alternative approach, di-block copolymers viz. PEG-(PLLA)₈ and PEG-(PDLA)₈ having star-shaped geometry and capable of possessing stereo-complex mediated gelation were investigated for their potential application in formulating hydrogels (Zhang et al., 2006). It was presented that these stereo-complexed PEG-(PLA)₈ star block copolymers formed hydrogels with enhanced mechanical strength and the gelation rate was faster (Zhong et al., 2005; Zhang et al., 2006). Rheological study showed that an increase in the storage modulus (from 0.9 to 7.0 kPa) along with a decrease in gelation time (from 40 min to less than 1 min) was obtained, when the average block length of PLA was increased from 12 to 14 lactyl units at a polymer concentration of 10% w/v (Buwalda et al., 2014b). The kinetic study showed that the release of drug (small protein lysozyme) followed a first-order kinetics and approximately 90% of the drug was released in the first 10 days (Zhang et al., 2007).

In a different experiment, stereo-complexed hydrogels were used for the delivery of protein immunoglobulin G (IgG). Here, it was found that the drug release followed nearly zero-order kinetics and almost 50% of the drug was released in 16 days. Stereo-complexed hydrogels having improved mechanical and controlled degradation properties were obtained by replacing the ester linkages between the PEG core and PLA arms by amide linkages (Buwalda et al., 2014b; Buwalda et al., 2012; Forte et al., 2011). Nagahama et al. coupled mono-carboxylated PEG with star-shaped PEG-PLLA or PEG-PDLA di-block copolymers to prepare enantiomeric 8-armed BAB-type tri-block copolymers (PEG-PLA-PEG) by EDC chemistry using *N,N'*-dicyclohexylcarbodiimide as cross-linking agent (Nagahama et al., 2008). It was observed that an aqueous solution comprising of

this copolymer at a lower concentration resulted in the formation of sol at room temperature; however, an irreversible transition to gel takes place with increasing temperature. A faster molecular weight reduction for these tri-block copolymers was observed by in vitro degradation experiments (Buwalda et al., 2014b; Nagahama et al., 2008).

Apart from PEG, numerous other polymers in conjugation with PDLA or PLLA have been utilized for the preparation of stereo-complexed hydrogels (Buwalda et al., 2014b). Dextran grafted with mono disperse L-lactic acid and D-lactic acid oligomers has been utilized for the synthesis of hydrogels by Dejong et al. (2000). It was revealed from the rheological studies that the degree of polymerization (DP) of lactic acid has an innate effect on the formation and strength of the hydrogel. In order to formulate hydrogels, the DP of lactic acid must be greater than or equal to 11 (Buwalda et al., 2014b). An increase in the DP of lactic acid resulted in the formation of much stronger gels. In vitro studies depicted that the number, length, polydispersity, and hydrophilicity of the lactate grafts have an adverse effect on the degradation time of the hydrogels (Buwalda et al., 2014b; Dejong et al., 2001). Lysozyme and IgG were encapsulated in these hydrogels and were tested for their drug delivery efficacy. Release of lysozyme was achieved in 5 days by diffusion, whereas the release of IgG was achieved in 8 days and the release was governed chiefly by diffusion, swelling, and degradation of hydrogel. These stereo-complexed hydrogels showed biocompatibility and have been found to be efficient vectors for the delivery of active biomolecules in vitro (Bos et al., 2004; Bos et al., 2005).

In an alternative approach, a mixture of cross-linked dextran microspheres and substituted L- or D-oligo-lactates were employed by van Tomme et al. to prepare highly stiffened macroscopic hydrogels containing lysozyme protein (van Tomme et al., 2008). The microgels showed a sustained release of lysozyme protein up to 30 days and preserved its enzymatic activity (Buwalda et al., 2014b; van Tomme et al., 2008). Poly[N-(2-hydroxypropyl)-methacrylamide-lactate] (pHPMAM) coupled with oligo-lactic acid possessing side chains of different chirality were utilized by van Nostrum et al. to prepare stereo-complexed hydrogels (van Nostrum et al., 2004). Comparative studies revealed that the hydrogels offer a significantly longer degradation time than dextran stereo-complexed hydrogels (Buwalda et al., 2014b; van Nostrum et al., 2004). Other polymers viz. pluronics, pHEMA, and 2-methacroyloxyethyl phosphorylcholine have also been utilized for the preparation of stereo-complexed hydrogels of biomedical importance (Buwalda et al., 2014b).

16.2.3.2 Hydrogels cross-linked by other physical interactions

Owing to their hydrophobic activity cyclic oligosaccharides (cyclodextrins (CDs)) offer an inherent capacity to encapsulate a majority of molecules. A self-assembled supramolecular hydrogel can be formulated by formation of a CD polypseudorotaxane and subsequent polypseudorotaxane aggregation (Buwalda et al., 2014b). Li et al. (1994) recognized the unique property of formulating

self-assembled physically cross-linked hydrogel. Previous systems employing PEG as guest molecules were often engulfed with disadvantages such as low stability and longer gelation times (Buwalda et al., 2014b).

On mixing CDs with amphiphilic block copolymers such as pluronics, reverse pluronics, or PCL-PEG-PCL, hydrogels with improvised properties were obtained (Buwalda et al., 2014b). For example, a 23 wt% hydrogel obtained from PEG-PHB-PEG [poly(ethylene glycol) poly(hydroxy butyrate) poly(ethylene glycol)] tri-block copolymer and an α -CD released model compound FITC-labeled dextran for over a period of 30 days (Buwalda et al., 2014b). However, a similar formulation prepared from high molecular weight PEG was found to be stable for a period of only 6 days (Buwalda et al., 2014b; Li et al., 2006). Numerous PEG-grafted natural polymers such as dextran, chitosan, and heparin were employed for the preparation of stiff and stable CD hydrogels. Zhang et al. prepared supramolecular hydrogel by employing heparin and α -CD for the controlled delivery of albumin (Ma et al., 2010). These supramolecular hydrogels demonstrated a sustained release of model protein albumin. Changing the concentration of heparin and α -CD aided in tailoring the release of the model drug accordingly (Buwalda et al., 2014b).

Interestingly, reports by Zhang et al. showed that a sustained dual drug release profile of heparin and enhanced anticoagulant property and biocompatibility was exhibited by the bioactive supramolecular hydrogels (Buwalda et al., 2014b; Ma et al., 2010). Another recent alternative approach being used in this field is the preparation of stimuli-responsive hydrogels (viz. pH, photo, and dual responsive systems) (Zhou et al., 2013). For example, a combination of adamantly end-functionalized PEG and star-shaped pNIPAAm having a β -CD core was utilized for the synthesis of thermo-responsive hydrogel (Zhang et al., 2008). van de Manakker et al. (2009a, b) utilized 8-armed cholesterol functionalized PEG with a β -CD core to prepare hydrogels. These hydrogels exhibited enhanced mechanical strength, degradation by surface erosion, and zero-order release kinetics of various model drugs viz. IgG, albumin and lysozyme.

Not much emphasis has been given on publishing the data pertaining to the formation of metallohydrogels. The earliest examples of metallohydrogels were reported by Chujo and Segusa (1993a, b). They prepared redox reversible and thermally stable cobalt (III) and iron (II) bipyridyl-branched polyoxazoline complex hydrogels (Chujo and Segusa, 1993a, b). Subsequently, metallohydrogels were also prepared from polymer functionalized ligands (viz. ter-pyridine, bi-pyridine) in presence of transition metal such as manganese (II) and nickel (II) (Buwalda et al., 2014b). A recent development in this field is the synthesis of PEG-based metallohydrogels by coordinating metal ions with bis-phosphonate and histidine groups (Nejadnik et al., 2013; Fullenkamp et al., 2013).

Hydrogels have also been prepared using an alternative approach, where the self-assembled configuration (α or β helix sheet structure) of the natural building blocks namely peptides has been employed. Two different approaches were employed for designing macromolecules which could self-assemble into

hydrogels. In context to the first approach, Tirrell and coworkers formulated reversible hydrogels by self-assembling the genetically engineered tri-block copolymers prepared from flanking a two coiled-coil forming block with a random coil block (Buwalda et al., 2014b; Petka et al., 1998). In the second approach, Kopecek and coworkers synthesized hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains (Wang et al., 1999). Kopecek and coworkers were the first to evince hybrid hydrogels by noncovalently cross-linking an HPMA copolymer with an assembly of genetically engineered coiled-coil protein motifs (Buwalda et al., 2014a, b; Wang et al., 1999). Nonreversible temperature induced hydrogel was formulated when a structural transition of the coiled-coil domains from an elongated helix to an unfold state takes place (Buwalda et al., 2014b). In a recent approach smart hydrogels containing adenylate kinase capable of translating a specific substrate recognition (viz. enzyme–substrate recognition) into macroscopic motion was designed by Yuan et al. (2008).

16.2.4 FOURTH GENERATION: SMART HYDROGELS

A variety of chemically cross-linked hydrogels have been developed, owing to an encroachment in the field of organic chemistry. Fourth generation hydrogels are also referred to as smart hydrogels. Smart hydrogels are formed by covalently cross-linking polymers with complimentary functional groups. This cross-linking takes place in situ under physiological conditions with minimal toxicity. These hydrogels allow the user to tailor properties such as mechanical stability and release kinetics according to their desired application (Buwalda et al., 2014b).

16.2.4.1 Double-network hydrogels

The application of hydrogels in biomedical applications such as load-bearing tissues, is generally hampered. It is due to the fact that hydrogels formed solely by physical interactions offer weak mechanical properties (Buwalda et al., 2014b; Drury and Mooney, 2003). In situ, chemically cross-linked hydrogels are accompanied by tribulations, such as slow gelation kinetics and early dissolution rate (Buwalda et al., 2014b). A potent solution to these problems is offered by smart hydrogels. In 1992 Hoffman and coworkers designed dual stimulus (viz. thermal and pH-sensitive)-triggered macroporous hydrogels for controlled delivery of digestive enzyme amylase (Qi et al., 1992). Temperature and pH-sensitive moieties, such as *N,N'* diethylaminopropyl-methacrylamide, acrylic acid, and NIPAAm, are generally utilized for the synthesis of multistimulus-triggered hydrogels. Insulin, co-enzyme A (CoA), and indomethacin are some of the model drugs which have been delivered using such hydrogels (Buwalda et al., 2014b). Hydrogels possessing remarkable synergistic effects and varied properties can be designed by combining ionically and covalently cross-linked networks (IPNs) (Matricardi et al., 2013). Importantly, combining physical and chemical cross-links allow the administration of polymer as liquid formulations that undergo

quick gelation due to physical interactions triggered by stimuli such as temperature and are stabilized by chemical reactions (Buwalda et al., 2014b).

Thiol and vinyl-functionalized pNIPPA_m in accordance with hyaluronic acid and thermosensitive polymer pluronics have also been utilized to design dual gelling hydrogels (Z.C. Wang et al., 2010; Lee et al., 2010). Thermo-responsive pNIPAA_m macromers bearing epoxy functionalities were cross-linked in situ with polyamidoamines (PAMAM) to prepare dual stimulus-triggered hydrogels. In a similar approach, Vermonden et al. designed thermo-responsive hydrogels which were initially stabilized by physical cross-links (Buwalda et al., 2014b). Depending on the molecular weight of hydrogel and size of the model drug, a diffusion-controlled release of active molecules, such as lysozyme, bovine serum albumin (BSA), and IgG, was observed from these systems. It is fascinating that during gelation the integrity of the secondary structure and enzymatic activity of the lysozyme is not affected (Censi et al., 2011).

16.2.4.2 Composite hydrogels

The mechanical property and bioactivity of hydrogels can be enhanced by incorporating small inorganic molecules viz. calcium phosphate and hydroxyapatite into the structure of the hydrogels. One fine example of such systems is ceramic-hydrogel composite materials which have been exhaustively utilized for the purpose of controlled drug delivery in tissue engineering (Buwalda et al., 2014b; Habraken et al., 2007). In a different approach, alternative deposition of thermoplastic polymer fibers and hydrogels were employed for constructing hybrid and mechanically strong hydrogels (Schoorman et al., 2011). A significant decrease in burst release (from 20% to 7%) of BSA was observed when it was encapsulated in hydrogel-electrospun composite (Han et al., 2012). In another study, embedded cells were protected using hydrogel composite reinforced with a 3D Ormocomp framework (Kang et al., 2014). An increase in the cell viability along with a sustained release of dopamine was observed with these hydrogel constructs (Buwalda et al., 2014b; Kang et al., 2014).

Recently, it is in limelight that the mechanical properties of hydrogels can be increased by reinforcing them with carbon nanotubes. In an experiment, a reversible gel possessing π – π transitions was prepared by mixing single-walled carbon nanotubes (SNTs) with CDs (Ogoshi et al., 2007). In an alternative approach, supramolecular carbon nanotubes were combined with naturally derived polymers such as gelatin, cellulose, and cyclodextrin, etc. to enhance the strength and stability of hydrogels (Li et al., 2003). Using this approach, hydrogels with threefold higher dynamic modulus were obtained by incorporating 2 wt% carbon nanotube hydrogels cross-linked with divinyl sulfate (Bhattacharyya et al., 2008). An electronic simulation triggered release of ^{14}C -sucrose was attained by incorporating pristine multiwalled carbon nanotubes in a polymethacrylic acid hydrogel (Servant et al., 2013).

Tabata and coworkers developed composite hydrogels of oligo(poly(ethylene glycol)fumarate) loaded with gelatin microparticles (Young et al., 2005). On

further loading, these microparticles with TGF- β 1, release kinetics could be tailored from 13 to 170 pg TGF- β 1/day for days 1–3 and from 7 to 47 pg TGF- β 1/day for days 6–21 (Buwalda et al., 2014b; Tabata et al., 2003). A high control over the release kinetics of active molecules had also been obtained from these hydrogels (Buwalda et al., 2014b; Tabata et al., 2005).

16.3 PROPERTIES OF HYDROGELS

16.3.1 FACTORS AFFECTING SWELLING OF HYDROGELS

One of the paramount factors affecting the swelling of hydrogels is cross-linking. Cross-linking may be defined as the ratio of moles of the cross-linking agent to the moles of polymer repeating units (Peppas et al., 2000). The ratio of cross-linking has a direct correlation with the mechanical strength and swelling of the hydrogel structure. It means hydrogel structures having a higher cross-linking ratio will subsequently have a highly compressed, tightly bound structure and will tend to have a lesser swelling aspect ratio. A vice versa trend is observed in the case of hydrogels having a lower cross-linking ratio. This decrease in the swelling ratio is attributed to cross-linking, which hinders the mobility of the polymeric chains present in the hydrogel structure (Peppas et al., 2000).

The second factor affecting the swelling ratio of hydrogels is the chemical structure of the polymer. It was observed that hydrogels possessing hydrophilic groups tend to swell more compared to those hydrogels having a hydrophobic corona. This is ascribed to the fact that on exposure with water these hydrophobic groups tend to collapse resulting in minimized exposure of these groups with the water molecule (Peppas et al., 2000). Stimuli is another factor which has been found to have a direct influence on the dynamics of (swelling) environmentally sensitive hydrogels. For example, a change in the temperature of the swelling media can affect the swelling aspect ratio of temperature-sensitive hydrogels. Another example is the change in pH and ionic strength of the media which can have an innate effect on the swelling of ionic and pH-sensitive hydrogels, respectively (Peppas et al., 2000).

16.3.2 DYNAMICS OF SWELLING

The aspect ratio of swelling or swelling kinetics can be chiefly classified into two major categories viz. Fickian (diffusion-controlled) and non-Fickian (relaxation-controlled) swelling (Peppas et al., 2000). Hydrogels in which the relaxation of polymeric chains takes place at a much slower pace than the diffusion of water portrays diffusion-controlled or Fickian swelling kinetics. Whereas hydrogels in which relaxation of polymeric chains takes place at a much faster pace than the diffusion of water portrays relaxation-controlled or non-Fickian swelling kinetics (Peppas et al., 2000).

16.3.3 MECHANICAL PROPERTIES

In relation to pharmaceutical applications, mechanical properties of hydrogels play an important role. For instance, in order to obtain approval from US Food and Drug Administration (FDA), the integrity of the drug delivery system during the lifetime of the application becomes prerequisite. This can be explained on the basis that the prime concern of designing a drug delivery system is to protect and maintain the integrity of sensitive therapeutic agent (e.g., protein or antibody) until it is released out of the drug delivery system (Peppas et al., 2000).

Desired mechanical property of the hydrogel can be attained by changing the degree of cross-linking. A stronger gel will be formulated along with a change in the degree of the cross-linking of the system. However, the downside of this enhanced cross-linking is the generation of a more brittle structure. Accordingly, in order to achieve a relatively strong and elastic hydrogel it becomes prerequisite that an optimum degree of cross-linking must be present (Peppas et al., 2000).

In a relatively different approach, copolymerization has also been utilized as a comprehensive tool for enhancing the mechanical properties of hydrogels. The strength of the hydrogel can be increased by incorporating a co-monomer in the polymeric backbone of the hydrogel (Peppas et al., 2000).

16.3.4 CYTOTOXICITY AND IN VIVO TOXICITY

The cytotoxicity of hydrogels can be evaluated using cytotoxicity methods (cell culture methods). Extract dilution, direct contact, and agar diffusion are three assays which have been most frequently used to evaluate the toxicity of hydrogels. The full scale utilization of these hydrogel-based drug delivery carriers have been hampered due to the leaching out of unreacted monomers, oligomers, and initiators during application. This calls for urgency where improved understanding of the various monomers used as the building blocks of the hydrogel will result in the designing of an improvised and more dynamic system (Peppas et al., 2000).

The correlation between chemical structures, the cytotoxicity of acrylate, and methacrylate monomers have been studied exclusively by Yoshi (Peppas et al., 2000; Yoshi, 1997). Modification of polymerization kinetics and extensive washing reduces the toxicity of hydrogel. In order to eliminate the problem of residual initiator, alternative approaches have been employed, where hydrogels are formulated without using any initiator (Peppas et al., 2000). The most commonly used technique is gamma irradiation (Akkas et al., 1999). PVA-based hydrogels have also been formulated without the presence of initiators by using thermal cycle to induce crystallization (Peppas et al., 2000; Hickey and Peppas, 1995). The crystals formed acted as physical cross-links and aided in absorbing the load applied to the hydrogels.

16.3.5 MISCELLANEOUS PROPERTIES

A direct correlation is found to exist between the water solubility of the polymer and temperature. Water solubility of most polymers increases with an increase in the temperature. However, a decrease in water solubility along with a shrinkage in the polymer is seen for hydrogels chiefly comprising of LCST polymers. This type of behavioral pattern is commonly referred to as inverse or negative temperature dependence. Such types of hydrogels exhibiting this phenomenon are found to possess either moderately hydrophobic groups or amphiphilic segments. The hydrogen bonding between the hydrophilic segments of the polymeric chain and water molecules starts to dominate at lower temperatures, which results in an enhanced dissolution in water. With an increase in temperature the reverse trend is observed, where hydrophobic segments strengthen up and hydrogen bonding tends to become weaker. The net cumulative result of this is the shrinkage of hydrogel due to inter-polymer chain association through hydrophobic interactions (Park and Qiu, 2001). In general, as the polymeric chain contains more hydrophobic constituents, LCST becomes lower (Schild, 1992). If the polymeric chains of such hydrogels are not covalently cross-linked, these hydrogels may prefer to undergo sol–gel phase transitions instead of undergoing swelling-shrinking transitions. The thermo-reversible gels exhibit the phenomenon of temperature dependence become sol at higher temperatures (Park and Qiu, 2001).

Depending on the pH of the environment, a significant difference in the swelling properties of cross-linked poly-electrolytes based hydrogels is observed. Depending on the pH, ionization of the pendant acidic or basic groups present on the poly-electrolytes takes place. It has also been observed that the comprehensive ionization on poly-electrolytes is very difficult. This might be attributed to electrostatic effects exerted by other neighboring ionized groups (Park and Qiu, 2001). Compared to nonelectrolyte polymer-based hydrogels, electrolytic polymer-based hydrogels possess a swelling index beyond significant consideration. This is chiefly achieved due to the presence of ionizable groups on polymeric chains. The electrostatic repulsion occurring among the charges present on the polymeric chain are mainly responsible for the swelling of polyelectrolyte hydrogels. The extent of swelling is found to be influenced by any condition (viz. pH, ionic strength, and type of counterion) which reduces electrostatic repulsion (Park and Qiu, 2001; Firestone and Siegel, 1991).

Electrical impulses can also be employed as external stimuli to induce responses of hydrogels. Like pH-sensitive hydrogels, electro-sensitive hydrogels are mainly composed of poly-electrolytes. In the presence of an externally applied electric field these hydrogels prefer to undergo shrinking or swelling. In some cases, a multivariate approach is seen, where these hydrogels show swelling on one side, while on the other side deswelling is experienced. As a result of this multivariate approach, these hydrogels also tends to undergo the phenomenon of bending. The geometric configuration (viz. swelling, shrinking, and bending) of hydrogel is found to be dependent upon a number of conditions. If the surface

of the hydrogel is placed in close proximity of the electrode, the result of applying electric field to the hydrogel may be different from systems where the hydrogel is placed in water or water–acetone mixture without touching the electrode (Park and Qiu, 2001). However, varied results were obtained when the aqueous phase contained electrolytes.

A perfect example of the previously mentioned phenomenon is depicted by partially hydrolyzed PAM hydrogels. When these hydrogels were brought in contact with both the electrodes (i.e., cathode and anode) and an alternatively varying infinitesimal potential is applied across the gel, the hydrogel volume ultimately collapsed. Although hydrogels do not contain any salts, on application of alternating potential, hydrated H^+ ions are generated (Park and Qiu, 2001). These hydronium ions start migrating toward the cathode, resulting in the loss of water at the anode side. Conversely, a uniaxial stress is generated along the gel axis predominantly at the anode side. This might be attributed to the fact that electrostatic attractions are generated as a result of negatively charged acrylic acid toward the anode (Nitta et al., 1994).

Shrinkage of hydrogel particles takes place when microspherical hydrogels placed in water without any salt are exposed to an externally applied electric field. This shrinkage of hydrogels is mainly attributed to the migration of water (electro-osmosis) and charged ions (electrophoresis) from the hydrogel to the cathode (Park and Qiu, 2001; Sawahata et al., 1990). This unique property has been deliberately used for modulating drug delivery by turning on and off the electric field (Park and Qiu, 2001). Table 16.1 highlights the physiochemical properties of hydrogels.

16.4 CLASSIFICATION OF HYDROGELS

Physical properties, nature of swelling, method of preparation, origin, ionic charges, sources, rate of biodegradation, and observed nature of cross-linking are

Table 16.1 Physiochemical Properties of Hydrogels

S. No.	Properties
1.	Degradable or nondegradable
2.	Injectable or prefabricated
3.	Mechanical strength
4.	Ease of handling
5.	Shape and surface/volume ratio (viz. sheets, cylinders, spheres)
6.	Closed or open pores
7.	Water content and character
8.	Chemical modification
9.	Added bioactive molecules (cells, drugs, proteins)
10.	Sterilizability

some of the vital parameters on which the classification of hydrogels depends upon (Ullah et al., 2015; Park and Qiu, 2001). Classification of hydrogels on the basis of different properties has been summarized in Table 16.2.

However, some of the prominent hydrogels, which attract scientists are discussed further.

Table 16.2 Classification of Hydrogels

S. No.	Property	Type of Hydrogel
1.	Chemically responsive	pH responsive Glucose responsive Oxidant responsive
2.	Biochemical responsive	Antigen responsive Enzyme responsive Ligand responsive
3.	Physically responsive	Temperature Pressure Light Electric field Magnetic fluid
4.	Physical properties	Smart hydrogels Conventional hydrogels
5.	Degradability	Biodegradable Nondegradable
6.	Source	Natural Synthetic Hybrid
7.	Ionic charge	Cationic hydrogels Anionic hydrogels Nonionic hydrogels
8.	Cross-linking	Physically cross-linked Chemically cross-linked
9.	Preparation or polymer composition	Copolymeric hydrogels Homopolymeric hydrogels Interpenetrating networks
10.	Physical appearance	Matrix Film Microsphere
11.	Network electric charge	Nonionic (neutral) Ionic (anionic and cationic) Amphoteric (ampholytic) Zwitterionic (polybetaines)

16.4.1 HYDROGELS BASED ON PROPERTIES

Depending on the method of preparation hydrogels may be chiefly classified into four major categories:

1. *Homopolymeric hydrogel*: These gels are produced by cross-linking networks of single type of monomer unit. The monomeric units are generally hydrophilic in nature. Depending on the nature of the monomer used and polymerization technique these hydrogels may possess cross-linked skeletal structure (Ullah et al., 2015).
2. *Copolymeric hydrogel*: They are produced by cross-linking networks of two type of monomer units. Out of these two monomeric units at least one is hydrophilic in nature (Ullah et al., 2015).
3. *Semi-interpenetrating network (semi-IPN)*: A linear polymer penetrates into another cross-linked network without the existence of any chemical bond (Ullah et al., 2015; Zhang et al., 2009a). Due to the absence of a restricting interpenetrating elastic network, the semi-IPNs can effectively preserve and demonstrate rapid kinetics in response to pH or temperature. They provide several other benefits such as modified pore size and slow drug release (Ullah et al., 2015).
4. *Inter penetrating networks (IPNs)*: IPNs are conventionally described as the intimate combination of two polymers, at least one of which is synthesized or cross-linked in the immediate presence of other (Ullah et al., 2015; Lipatov, 2002). In order to attain this state a pre-polymerized hydrogel is immersed into a solution containing a monomer and a polymerization initiator. IPNs in general possess several attractive properties:
 - a. Thermodynamic incompatibility can be overcome
 - b. Achievement of limited phase separation
 - c. Ensured stability of the bulk and surface morphology
 - d. Stiffer and tougher mechanical properties
 - e. Tunable physical properties
 - f. Enhanced drug loading, efficient drug delivery and tunable drug release kinetics.

16.4.2 STIMULI-RESPONSIVE HYDROGELS

Stimuli-responsive hydrogels are also referred to as environment-sensitive hydrogels. These hydrogels tend to generate specific response when placed in contact with the external (environmental) stimuli (Ullah et al., 2015). It has also been established that, on encountering an external stimuli, these hydrogels tend to undergo unexpected change in their growth actions, network structure, mechanical properties, and permeability consequently the name stimuli-responsive hydrogels (Peppas et al., 2000). The stimuli can be chiefly classified into two major categories viz. (Ullah et al., 2015; Gil and Hudson, 2004).

1. *Physical stimuli*: A change in molecular interaction appears, at critical onset points of physical stimuli. These stimuli include: light, pressure, temperature, electric and magnetic field, mechanical stress, and intensity of energy source.
2. *Chemical stimuli*: It results in change in the interaction between polymer chains and solvent at the molecular level. The stimuli are pH, ionic strength, and chemical agents.

Apart from these stimuli-responsive hydrogel another class of responsive hydrogel exists, which are commonly referred to as dual responsive hydrogels. These hydrogels possess a combination of multiple (two) stimuli mechanisms in a single hydrogel system (Ullah et al., 2015). This phenomenon of dual responsive system is well illustrated by poly(acrylic acid-co-poly vinyl sulfonic acid), where a biochemical response is evoked in relation to multiple stimuli viz. ligand, enzyme, antigen, and biochemical agents (Peppas et al., 2000; Gil and Hudson, 2004). Owing to this, these hydrogels have been employed as biomaterials in various fields such as pharmacy, biotechnology, and medical (Kashyap et al., 2005). Tables 16.3 and 16.4 illustrate the salient features and applications of stimuli-responsive hydrogels (Ullah et al., 2015).

16.5 SYNTHESIS OF HYDROGELS

The characteristics and potential applications of hydrogels are described in relation to their preparation, including cross-linking methods (Hennink and Nostrum, 2012). This section will illustrate the advantages and disadvantages of these methods (Table 16.5).

16.5.1 CHEMICALLY CROSS-LINKED HYDROGELS

In chemically cross-linked hydrogels, covalent bonds are present between different polymer chains (Hennink and Nostrum, 2012). Chemical cross-linking can be further of four major types:

1. By radical polymerization
2. By chemically reacting complimentary groups
3. By high energy radiation
4. Using enzymes.

16.5.1.1 Cross-linking by radical polymerization

Radical polymerization of low molecular weight monomers in presence of cross-linking agents can pilot the generation of chemically cross-linked hydrogels (Hennink and Nostrum, 2012). Edman et al. pioneered the research on polymerization by reacting aqueous solution of dextran with glycidyl acrylate (Edman et al., 1980). Among all the studied hydrogels, pHEMA is the most renowned and

Table 16.3 Salient Features of Stimuli-Responsive Hydrogels

Type of Hydrogel	Salient Features/Properties	Limitations	Improvements
pH-responsive hydrogel (pRHs)	1. Defined by their ability to exhibit swelling and deswelling depending on the pH of the surrounding media. 2. Properties of polymers (viz. concentration, cross-linking density, ionic charge, hydrophilicity or hydrophobicity, and degree of ionization) and property of the swelling medium (viz. pH, ionic strength, counterion) are the two most predominating factor which tends to affect the swelling and deswelling of these polymers. 3. A dramatic change in the degree of ionization (pK_a or pK_b) is attained at specific pH. 4. Can be chiefly classified in two major categories viz. a. Anionic hydrogels i. Possess carboxylic or sulfonic acid pendant groups. ii. Deprotonation occurs at an environmental pH above pK_a. iii. Ionization of pendant groups leads to an increase in the swelling rate. b. Cationic hydrogels 5. Possess amine pendant groups. 6. Deprotonation occurs at an environmental pH below pK_a. 7. Increased swelling rate due to an increase in the electrostatic repulsion.	1. Nonbiodegradable. 2. Nonbiocompatible. 3. pH-sensitive variation in degree of swelling. 4. Variation in swelling rate with particle size. 5. A comparatively faster release of active molecules at basic pH (7.4).	1. Development of biodegradable hydrogels. 2. Development of biocompatible hydrogels.

(Continued)

Table 16.3 Salient Features of Stimuli-Responsive Hydrogels *Continued*

Type of Hydrogel	Salient Features/Properties	Limitations	Improvements
Temperature-responsive hydrogels	1. Defined by their ability to exhibit swelling and deswelling Depending on the temperature of the surrounding media. 2. Can be chiefly classified in three major categories viz. a. Positive temperature hydrogels i. Renowned by their upper critical solution temperature (UCST). ii. Demonstrate maximum swelling at a temperature higher than the UCST. This might be attributed to the fact that at higher temperature, dissociation of the hydrogel structure along with the cleavage of hydrogen bonds take place. iii. At lower temperature complex structure formation by the hydrogen bonds takes place as a result of which these gels depicts shrinkage. b. Negative temperature hydrogels i. Renowned by their upper critical solution temperature (LCST). ii. Comprised of two parts; 1. Hydrophilic (–CONH) 2. Hydrophobic (–R–) iii. Compared to positive temperature hydrogels, they tend to swell at a temperature lower than LCST. This might be	1. Nonbiodegradable. 2. Nonbiocompatible. 3. Toxicity. 4. Carcinogenicity. 5. Teratogenic effects.	1. Development of biodegradable hydrogels. 2. Development of biocompatible hydrogels.

	attributed to the formation of hydrogen bonds (due to the interaction of water with the hydrophilic part). Consequently, leading to an improvised degree of swelling. iv. Whereas, these gel shrinks at a temperature higher than LCST. This might be attributed to the inter-polymer chain association. c. <i>Thermo-reversible hydrogels</i> (TRHs) d. These hydrogels share similar structural content as that of PTHs and NTHs. e. The polymeric chains in these thermosensitive hydrogels are covalently cross-linked. f. Instead of swelling and deswelling these gels exhibit sol–gel phase transition. g. Owing to certain molecular interactions (viz. hydrophobic interactions and hydrogen bonding) these hydrogels undergo abrupt volume changes at critical solution temperature (CST).		
Glucose-responsive hydrogels (GRHs)	1. Employed as vectors for delivering insulin. 2. They acts as glucose-sensing carriers and spikes the triggered release of insulin for treating diabetes. 3. They comprises of glucose oxidase mixtures tethered covalently onto the matrix of the hydrogel system. 4. Glucose oxidase, Concanavalin A, PVA-based hydrogels are some of the other sub class of GRHs.	1. Often accompanied with slow release. 2. Nonbiocompatible. 3. Stimulation of undesirable immune response	1. Development of hydrogels which respond to every change in glucose concentration. 2. Development of hydrogels with excellent reproducibility. 3. Development of hydrogels capable of illustrating a sustained release.
Protein-based hydrogels (PHs)	1. Designed by employing recombinant DNA technology (RDT). 2. Designed with a predefined geometrical configuration and possess specific molecular weight.		

(Continued)

Table 16.3 Salient Features of Stimuli-Responsive Hydrogels *Continued*

Type of Hydrogel	Salient Features/Properties	Limitations	Improvements
Antigen-responsive hydrogels (ARHs)	- Coiled-coil technique is used for designing PrHs. In this technique, the residual amino acids of the coiled-coil protein act as cross-linker. Also, synthetic hydrophilic polymer coiled-coil proteins act as cross-linkers to prepare 3D structured hydrogels. - Employed to attain a sustained release and targeted delivery of biomolecules. - Designed by grafting antigen on the hydrophilic backbone of the polymer. - Swelling can be circumvented by mixing ARHs with hydrophilic polymeric backbone cross-linked with antibody.		
Electro-sensitive hydrogels (ESHs)	- Defined by their ability to exhibit swelling and deswelling, depending on the presence of an externally applied electric field. - Exhibit dual phenomenon (viz. swelling on one end and deswelling on other) resulting in the bending of hydrogels.	- Often accompanied with slow release. - Requires a controllable voltage source. - Works in absence of electrolytes.	
Light-sensitive hydrogels (LSHs)	- Defined by their ability to exhibit swelling and deswelling Depending on the presence of light stimulus. - Exhibit instantaneous sol–gel phase transition. - Chiefly classified in two major categories, i.e., UV LSHs and visible LSHs.	- Slow response of hydrogel toward instantaneous illumination source. - Leaching out of chromophores during swelling–deswelling cycles.	
Pressure-sensitive hydrogels (PSHs)	Defined by their ability to exhibit volume phase transition (sol–gel) depending on the change in pressure of the surrounding media.		

Table 16.4 Stimuli-Responsive Hydrogels in Drug Delivery

Stimulus	Polymer	Drug
Magnetic fluid	Ethylene-co-vinyl acetate (EVAc)	Insulin
Ultrasonic radiation	EVAc	Zinc bovine insulin, insulin
Electric field	Poly(2-hydroxyethyl methacrylate) (PHEMA)	Propranolol hydrochloride
Glucose	EVAc	Insulin
Urea	Methyl vinyl ether-co-maleic anhydride	Hydrocortisone
Morphine	Methyl vinyl ether-co-maleic anhydride	Naltrexone
Antibody	Poly(ethylene-co-vinyl acetate)	Naltrexone, ethinyl estradiol
pH	Gelatin-PEO	Riboflavin
Temperature	Poly(<i>N</i> -isopropyl acrylamide)	Heparin
pH temperature	Poly(<i>N</i> -isopropylacrylamide-co-butyl methacrylate-co-acrylic acid)	Calcitonin

frequently studied hydrogel system. It was introduced by Wichterle and Lim to the scientific community and is obtained by radical polymerization of HEMA in presence of ethylene glycol dimethacrylate (Tavsanli and Okay, 2016).

16.5.1.2 Cross-linking by chemically reacting complimentary groups

Hydrogels can be formulated by utilizing the functional groups (viz. OH, COOH, NH₂) present on the surface of water-soluble polymers. Covalent linkages between polymeric chains can be established by reacting the functional groups with complimentary reactivity such as amine–carboxylic acid or isocyanate–OH/ NH₂ reaction and by Schiff base formations (Hennink and Nostrum, 2012).

16.5.1.2.1 Cross-linking with aldehydes

In order to formulate hydrogels, glutaraldehyde can be used to cross-link water-soluble polymers possessing (e.g., PVA) terminal hydroxyl groups (Dai and Barbari, 1999). In order to establish cross-linking, drastic conditions (viz. low pH, high temperature, methanol added as quencher) have to be applied (Hennink and Nostrum, 2012). Conversely, cross-linking of amine-containing polymers using glutaraldehyde can be achieved by employing comparatively mild conditions along with the formation of Schiff's base (Hennink and Nostrum, 2012).

16.5.1.2.2 Cross-linking by addition reaction

Addition reaction by employing bis- or higher functional groups (as cross-linking agents) can be utilized to convert water-soluble polymers into hydrogels. Varying the concentration of the dissolved polymer and the amount of cross-linking agent can result in the generation of hydrogels with tailored network properties.

Table 16.5 Advantages and Disadvantages of Cross-Linking Methods

Cross-Linking Methods	Advantages	Disadvantages
Self-assembly	Potentially reversible; mild gelation on exposure to an external stimulus	Weak mechanical properties
Photo cross-linking	Spatiotemporal control over gelation	Requirement of potential light source and initiator
Michael reaction	Highly specific; gelation under mild conditions; tunable gelation kinetics	Usage of organic base
Disulfide formation	Highly specific; gelation under mild conditions; tunable gelation kinetics	Requirement of oxidizing agent
Schiff's base reaction	Gelation in mild conditions (room temperature and physiological pH); tunable gelation kinetics	Lack of specificity; unwanted reactions of aldehyde groups with amino groups present in biological matrices
Enzyme mediated reaction	Highly specific; gelation under mild physiological conditions	Instability of enzymes; persistence of active enzymes
Click reaction	Highly specific; gelation under mild physiological conditions	Requirement of toxic catalyst
Ionic cross-linking	Gelation under mild conditions; reversible	Interference in cross-linking due to charged therapeutics
Molecular recognition	Highly specific; gelation under mild conditions	Interference in cross-linking due to charged therapeutics
High energy irradiation	Process can be achieved in water; gelation under mild conditions; use of toxic cross-linking agent is avoided	Potential damage of biologically active molecules by the radicals formed during irradiation.

It should be ensured that the cross-linking reactions should be carried out in preferable organic solvents so that water molecules might not interfere with cross-linking agent. These gels must be extracted extensively in order to remove the unwanted toxic traces of unreacted cross-linking agents. Hubbell et al. reported a degradable hydrogel in which cross-links were introduced by reaction of PEG-dithiol with PEG-acrylates (Hennink and Nostrum, 2012; Elbert et al., 2001). The model protein (albumin) was incorporated in the hydrogel by mixing with the precursor solution (gel). It was demonstrated that the gel formation took place at nominal conditions (viz. room temperature and physiological pH). There was a hydrolysis of ester bonds in the network, due to which the gels illustrated degradation under physiological conditions (degradation time varied from 5 to 25 days). However, a sustained release of albumin from 5 to 12 days was attained (Hennink and Nostrum, 2012).

16.5.1.2.3 Cross-linking by condensation reactions

Condensation reactions between hydroxy groups or amines with carboxylic acids or derivatives hereof are frequently applied for the synthesis of polymers to yield polyesters and polyamides, respectively (Hennink and Nostrum, 2012). Similar reactions can also be utilized for the preparation of hydrogels. Among all the reagents used *N,N*-(3-dimethylaminopropyl)-*N*-ethyl-carbodiimide (EDC) formulates one of the reagents, to cross-link water-soluble polymers with amide bonds. In order to avoid possible side reactions and to have an edge over the cross-linking density of the gels, *N*-hydroxysuccinimide (NHS) is added during the reaction (Hennink and Nostrum, 2012). Passerini and Ugi condensation reactions have been used to synthesize polysaccharide hydrogels. In a Passerini reaction an α -(acryloxy) amide is yielded by condensing a carboxylic acid, aldehyde, or ketone with isocyanide. While in Ugi reaction an α -(acylamino) amide is formed by adding an amine to the reaction mixture (Hennink and Nostrum, 2012). The whole reaction is carried out at room temperature and slightly acidic pH. These hydrogels obtained by Passerini condition reactions degrade at an ambient temperature and highly basic pH (9.5). This is due to the presence of ester bonds in the cross-link networks of these hydrogels. Depending on the composition of hydrogel, the degradation time may vary from 1 to 8 days. These gels were found to be stable under such physiological conditions due to the presence of amide bonds in their cross-links (Hennink and Nostrum, 2012).

16.5.1.3 Cross-linking by high energy irradiation

Unsaturated compounds can be polymerized using high energy radiation such as gamma and electron beam. This means that high energy radiation can be utilized to convert vinyl groups derivatized water-soluble polymers into hydrogels (Giammona et al., 1999). An alternative approach for synthesizing hydrogel is radiation induced polymerization of a mixture of monofunctional acrylate (e.g., acryloyl-L-proline methyl ester) and a suitable cross-linker (Hennink and Nostrum, 2012; Caliceti et al., 2001). Moreover, high energy irradiation is able to cross-link water-soluble polymers without additional vinyl groups (Hennink and Nostrum, 2012).

When aqueous solutions of polymers are irradiated with gamma or electron beam homolytic scission of C–H bonds present in the polymeric chains takes place along with the generation of radicals. Additionally, macro radicals are formed due to the attacking of polymeric chain by hydroxyl radicals (generated as a result of radiolysis of water molecules) (Peppas and Mikos, 1986). These macro radicals so formed on varied polymeric chains tether together to form covalent bonds and finally gives a honeycomb-like cross-linked structure (Hennink and Nostrum, 2012).

Urry and coworkers reported on poly(amino acid)-based hydrogels which were obtained by gamma irradiation of aqueous solutions of polypentapeptides (GVGVP, where G = lysine, V = valine, and P = proline). The LCST and

swelling ratio of these hydrogels were found to be temperature-dependent. Swelling had been found to be decreased with increasing radiation dose (J. Lee et al., 2001).

16.5.1.4 Cross-linking using enzymes

In an interesting approach, PEG-based hydrogels were synthesized by Sperinde and Griffith (1997) using an enzyme. In their study, they functionalized a tetrahydroxy PEG with glutaminy groups (PEG-Q_a). Addition of transglutaminase to aqueous solution of PEG-Q_a and poly(lysine-co-phenylalanine) resulted in the formation of PEG cross-linked networks. It might be attributed to the fact that this enzyme catalyzes the reaction between the γ -carboxamide group of the PEG-Q_a and ϵ -amine group of lysine to yield an amide linkage between the polymers (Hennink and Nostrum, 2012). However, the properties of such gels could be tailored by altering the ratio of PEG-Q_a and lysine copolymer. Under suitable physiological conditions, gels with an equilibrium water content of approximately 90% were formed (Hennink and Nostrum, 2012; Sperinde and Griffith, 1997).

In another experiment, a triggered gelling system was evinced by Westhaus and Messersmith (2001) using transglutaminase enzyme (Ca²⁺-dependent enzymes). A mixture of Ca-loaded liposomes, fibrinogen, and a Ca²⁺-dependent transglutaminase remained in fluid form when stored at room temperature however, it illustrated a sol–gel phase transition (solidification) when heated at 37°C (Hennink and Nostrum, 2012). Owing to this unique property of exhibiting phase transition, such gels can be used as a matrix for delivering bioactive substances (Hennink and Nostrum, 2012; Westhaus and Messersmith, 2001).

16.5.2 PHYSICALLY CROSS-LINKED HYDROGELS

Physically cross-linked hydrogels have gained considerable attention and have become a topic of interest. This is due to the fact that use of chemical cross-linking agents is generally forbidden in preparation of these hydrogels. The cross-linkers affect the integrity of the active compounds to be entrapped within the matrix or corona of the hydrogel system, and are often accompanied with toxicity (Hennink and Nostrum, 2012). Numerous methods have been investigated for the preparation of physically cross-linked hydrogels, of which some of them are discussed in the following section.

16.5.2.1 Cross-linking by ionic interactions

Amidst all the polymers, synthetic polymer alginate is well known for its cross-linking ability by ionic interaction. Alginate falls into the category of polysaccharide which generates mannuronic and glucuronic acid residues on degradation. Calcium ions mediated ionic interaction at nominal room temperature and physiological pH can be utilized to cross-link alginate (Gacesa, 1988). Owing to this, alginate-based hydrogels are most frequently used as a matrix for encapsulating

active compounds of interest and sustained drug delivery. However, on addition of a chelating agent, the Ca-ions are extracted out as a result of which these gels become destabilized. Grafting the cationic polymers, viz. chitosan or polylysine, on these hydrogels resulted in an enhanced sustained delivery of active biomolecules such as proteins, amino acids (Hennink and Nostrum, 2012). An alternative to alginate is poly-[di-(carboxylatophenoxy)phosphazene] (PCPP) polymer which can be cross-linked with Ca-ions. These ionotropic hydrogels tends to undergo degradation under physiological conditions (Hennink and Nostrum, 2012). However, incorporation of hydrolysis-sensitive glycinate groups in the polymer resulted in an increased rate of degradation (Andrianov et al., 1994).

Cross-linking by addition of ions does not necessarily require the presence of ionic groups in the polymer for hydrogel formation (Hennink and Nostrum, 2012). Surprisingly, dextran, which lacks ionic binding sites for cations, forms a hydrogel in the presence of potassium ions. The mechanism was elucidated by Watanabe et al. who showed that the ionic radius of the potassium ion perfectly fits into the cage established by six oxygen atoms of glucose units of three polymer chains, thereby forming a microstructure (Hennink and Nostrum, 2012; Watanabe et al., 1996). Amidst all the advantages offered, this potassium/dextran hydrogel lacks practical utility in drug delivery due to its instability.

16.5.2.2 Cross-linking by crystallization of homopolymer systems

A gradual development of hydrogel with low mechanical strength takes place when aqueous solution of water-soluble polymer (PVA) is stored at room temperature. Surprisingly, on freeze-thaw cycles, these aqueous polymeric solutions tend to form a highly elastic gel (Yokoyama et al., 1986). However, it has been established that the formulation parameters, viz. concentration of PVA in water, molecular weight of PVA, temperature and time of freezing, number of freezing cycles, have a deliberate effect on the properties of these hydrogels. Gel formation is ascribed to the formation of PVA crystallites which act as physical cross-linking sites in the network (Hennink and Nostrum, 2012; Yokoyama et al., 1986). These gels were found to be stable at a temperature of 37°C for 6 months.

In a similar study, PVA gels loaded with BSA protein were formulated by dissolving BSA in aqueous solution of PVA. This solution was further imposed to undergo repeated freeze-thaw cycles. PVA also preserved the integrity of the model active biomolecules, Fickian diffusion-based release kinetic of BSA was obtained from these hydrogels (Peppas and Scott, 1992). It was also demonstrated that further addition of alginate to the PVA solution prior to freeze-thaw cycle resulted in the synthesis of gels with modulated properties (enhanced mechanical strength, sustained drug release) (Takamura et al., 1992).

Hennink et al. formulated dextran 6000–based hydrogels (Stenekes et al., 2001). It was discovered that concentrated aqueous solution of dextran when placed at room temperature results in the spontaneous formation of microspherical hydrogels. The formation of a hydrogel is due to the association of chains through hydrogen bonding, resulting in crystallization. The characteristic property of the gels were polymer concentration-dependent (Hennink and Nostrum, 2012).

16.5.2.3 Cross-linking by hydrogen bonds

It is well known that poly(acrylic acid) and poly(methacrylic acid) form complexes with PEG. These complexes are held together by hydrogen bonds between the oxygen of the PEG and the carboxylic group of the poly(methacrylic acid), while for poly(methacrylic acid) hydrophobic interactions play a very prominent role (Hennink and Nostrum, 2012; England et al., 1994). Hydrogen bonding does not only occur between poly(methacrylic acid) and PEG, but also has been observed in poly(methacrylic acid-*g*-ethylene glycol) (Hennink and Nostrum, 2012; Mathur et al., 1998). The hydrogen bonds are only formed when the carboxylic acid groups are protonated (Hennink and Nostrum, 2012). This shows that the swelling of these hydrogels is totally dependent upon the pH of the surrounding media.

16.6 HYDROGELS IN DRUG DELIVERY

Owing to their unique physical properties, hydrogels have attracted noticeable interest in recent years and have also paved the way in designing drug delivery systems. Many patents and academic papers about possible applications of hydrogels in drug delivery have been published, yet only a few have resulted in commercial products (Calo and Khutoryanskiy, 2015). A characteristic property, such as high porosity, offers several alluring advantages such as efficient drug loading and sustained drug release. Another advantage offered by hydrogels is the maintenance of high local concentration of drug over a longer duration of time (Calo and Khutoryanskiy, 2015). Amidst all proposed drug delivery systems, hydrogels have attracted considerable attention as excellent candidates for controlled release kinetics, bioadhesive devices, and targetable devices of therapeutic agents (Peppas et al., 2000). Hydrogel-based delivery systems have been utilized to deliver drugs at varied sites, viz. ocular, nasal, oral cavity, transdermal, subcutaneous implants, vagina, rectum, colon, small intestine, and stomach (Table 16.6)

Table 16.6 Main Types of Hydrogel-Based Drug Delivery System

Route of Administration	Type of Hydrogel	Observation	Reference
Oral cavity	Bioadhesive tablet (Aftach)	Aphthous ulcer was efficiently treated; efficient local systemic delivery of triamcinolone acetonide was achieved.	Nagai et al. (1980)
	Hydrogel-based ointments (Orabase), Carbopol 934 P, neutralized (MAA-co-methyl methacrylate) (MAA) containing liposomes	Electron paramagnetic resonance (EPR) studies showed highest stability of liposome in MAA; transportation of liposome to oral soft tissues; washing out time from oral mucosa or gingiva.	Petelin et al. (1998)
	Hydrogel ointment containing 17 β -estradiol (E_2)	In vivo studies demonstrated maintenance of E_2 plasma level at over 300 ng/mL/cm ³ for 7 h; no primary change in morphology of buccal membrane.	Machadi et al. (1998)
	Buccal bilayered tablet	Unidirectional delivery of nifedipine and propranolol toward mucosa; loss of drug due to washing out with saliva was avoided.	Remunan-Lopez et al. (1998)
Gastrointestinal tract (GI)	Hydrogel formulations with protease inhibitor activities	The hydrolytic activity of trypsin and its freeze-dried modifications (FNaC934P) was shown to be inhibited; efficient enzyme inhibition.	Akiyama et al. (1996)
	pH-responsive complexation hydrogels	Protection of insulin from harsh and acidic environment of the stomach; In vivo studies demonstrated a strong dose dependent hypoglycemic effects.	Lowman et al. (1999)
Rectal	Mucoadhesive poloxamer-based thermal gel	Increased bioavailability of propranolol.	Ryu et al. (1999)
	Xyloglucan based thermal gel	In vivo studies demonstrated a well-controlled drug (indomethacin) plasma; concentration time profile with reduced bioavailability.	Miyazaki et al. (1996)

(Continued)

Table 16.6 Main Types of Hydrogel-Based Drug Delivery System *Continued*

Route of Administration	Type of Hydrogel	Observation	Reference
Ocular	In situ gelling system comprising of alginate and guluronic acid	Enhanced precorneal contact times; extended the duration of the pressure reducing effect of pilocarpine up to 10 h.	Cohen et al. (1997)
	Rubber/hydrogel composite ophthalmic inserts	Significantly higher ocular retention time; In vivo studies demonstrated a sustained release of oxytetracycline up to several days	Chetoni et al. (1998)
Transdermal	Copolymerized BSA–PEG hydrogels	Felicitated the simultaneous release of hydrophilic and hydrophobic drugs; controlled release in wound dressing	Fotier and Gayet (1996)

16.7 CONCLUSION

This chapter has highlighted the synthesis, properties, and classification of hydrogels on the basis of response and activities. Emphasis has been given to the developing trend of hydrogels from a historical perspective; from simple networks to smart materials. Examples of injectable hydrogels for therapeutic agent delivery have also been detailed. The chapter specifically describes the progress that has been made in the field of hydrogels for biomedical applications in the past 50 years, starting from the pioneering work of Wichterle and Lim in the 1960s.

However, it should be noted that each class of physical, chemical, and environment-sensitive (dual gelling systems) possess its own merits and drawbacks, depending on the particular type of application. Over the past several decades, numerous attempts have been made to design and facilitate hydrogels with the properties desired for specific aims. Fundamental studies greatly contributed to understand of this unique class of materials, in terms synthesis and applications. Great progress has been made in the field of hydrogels and these are under investigation, among others, as matrices for the encapsulation of living cells and for the controlled release of pharmaceutically active molecules viz. protein, DNA, peptides, aptamers, and antibiotics.

Many cross-linking methods (viz. physical and chemical) have been developed and are presently available for the preparation of hydrogels. For the entrapment and encapsulation of labile bioactive substances and living cells, physically cross-linked gels are of great interest. Although a number of interesting cross-linking methods are presently known, there is undoubtedly a need for other sophisticated

methods to be developed. It is expected that principles from the expanding research area of supramolecular chemistry will be applied to design novel types of hydrogels with tailored properties that can be preferably be prepared in an aqueous environment. To give way to the formation of hydrogel systems having a precise command on their microstructure and therefore characteristics, protein engineering may also be useful. Finally, it can be foreseen that systems in which gel formation is induced by a specific trigger (temperature, pH, electric field, and magnetic field) will be further designed and applied for pharmaceutical and biomedical processes. Nevertheless, there have still been some common yet notable concerns warranting further investigation as well as persistent challenges to overcome.

LIST OF ABBREVIATIONS

IPN	Interpenetrating polymeric networks
PAN	Polyacrylonitrile
PAM	Polyacrylamide
pHEMA	Poly(hydroxy-alkyl methacrylate)
PVA	Polyvinyl alcohol
PEG	Polyethylene glycol
PVP	Polyvinyl pyrrolidone
pNIPAAm	Poly(<i>N</i> -isopropyl acrylamide)
CGC	Critical gel concentration
PLA	Poly(lactic) acid
HLB	Hydrophilic to hydrophobic balance
PLLA	Poly(L-lactic acid)
PCL	Poly caprolactone
LCST	Lower critical solution temperature
HEMA-lactate	2-Hydroxyethyl methacrylate monolactate
HPMA	<i>N</i> -(2-hydroxypropyl) methyl acrylamide
DBA	Dimethyl- γ -butyrolactone acrylate
FITC	Fluorescein iso-thio cyanate
ATRP	Atom free radical polymerization
pOEGMA	2-(2-Methoxyethoxy) ethyl methacrylate-oligo (ethylene glycol) methacrylate
IgG	Immunoglobulin G
DP	Degree of polymerization
CDs	Cyclodextrins
CoA	Co-enzyme A
PAMAM	Polyamidoamines
BSA	Bovine serum albumin
SNTs	Single-walled carbon nanotubes
TGF-β1	Transforming growth factor- β 1
IGF-1	Insulin-like growth factor
FDA	Food and drug administration

semi-IPN	Semi-interpenetrating network
EDC	<i>N,N</i> -(3-dimethylaminopropyl)- <i>N</i> -ethyl-carbodiimide
NHS	<i>N</i> -hydroxysuccinimide
GVGVP	G = glycine, V = valine, and P = Proline
PCPP	Poly-[di-(carboxylatophenoxy)phosphazene]
EPR	Electron paramagnetic resonance
UCST	Upper critical solution temperature
NTHs	Negative temperature hydrogels
TRHs	Thermo-reversible hydrogels
CST	Critical solution temperature
RDT	Recombinant DNA technology
EVAc	Ethylene-co-vinyl acetate
PHEMA	Poly(2-hydroxyethyl methacrylate)
PSHs	Pressure-sensitive hydrogels
LSHs	Light-sensitive hydrogels
ESHs	Electro-sensitive hydrogels
ARHs	Antigen-responsive hydrogels
PHs	Protein-based hydrogels
GRHs	Glucose-responsive hydrogels
pRHs	pH-responsive hydrogels

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Recent advances in understanding of blood–brain tumor barrier (BTB) permeability mechanisms that enable better detection and treatment of brain tumors

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CHAPTER OUTLINE

17.1 Introduction	674
17.1.1 Blood–Brain Barrier Permeability	674
17.1.2 Blood–Brain Tumor Barrier Permeability	675
17.1.3 Biochemical Modulation of the Blood–Brain Tumor Barrier	676
17.2 Functional Ion Channels on Blood–Brain Tumor Barrier	677
17.3 Nanomedicines and Nanoimaging	678
17.4 Assessment of Brain Tumor, Tumor Vasculature and Permeability	678
17.4.1 Clinical Applications of Perfusion Magnetic Resonance Imaging	680
17.5 Nanotechnology for Improved Brain Tumor Detection and Treatment.....	682
17.6 Summary	684
Acknowledgments	685
References	685
Further Reading	688

17.1 INTRODUCTION

17.1.1 BLOOD–BRAIN BARRIER PERMEABILITY

The blood–brain barrier (BBB) is a cerebrovascular permeability barrier that strictly regulates the entry of variety of substances, including therapeutics and imaging agents into the brain. Unlike blood vessels that circulate blood to other areas of the body, the microvessels that perfuse the brain consist of special endothelial cells and pericytes that lack fenestrations and are sealed by endothelial tight junction proteins (TJp) (Ningaraj et al., 2003; Black and Ningaraj, 2004). These tight endothelium, pericytes, and astrocytic foot processes provide a physical barrier that, together with metabolic barriers, form the BBB (Fig. 17.1). At the blood–brain tumor barrier (BTB) the TJp and astrocytic foot processes are

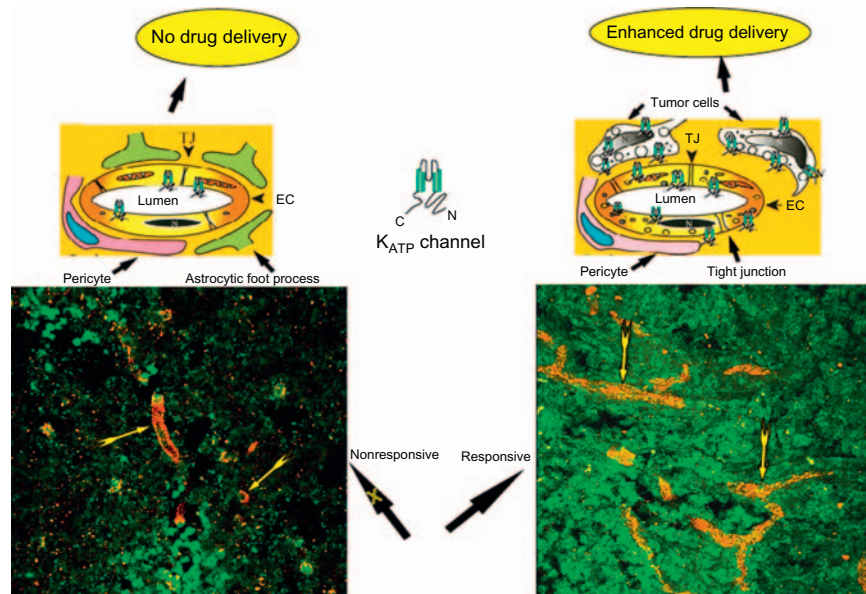


FIGURE 17.1

This figure illustrates the fundamental differences between the BBB and the BTB. The immune staining for K_{ATP} channels (green) and brain tumor vascular endothelia cells (vWF) show the colocalization of K_{ATP} channels and vWF in BTB vasculature but not on the normal BBB vasculature. This suggests that K_{ATP} channels are expressed only on BTB vasculature that we activated with MS to increase BTB permeability without affecting the BBB permeability in glioma models.

Adapted from figure published by Ningaraj, N.S., Rao, M.K., Black, K. L., 2003. Adenosine 5'-triphosphatesensitive potassium channel-mediated blood-brain tumor barrier permeability increase in a rat brain tumor model. *Cancer Res.* 63, 8899–8911.

compromised, leading to a “leaky” BBB. The BBB protects the brain against pathogens and other damaging agents in the circulatory system, including electrolytes that changes the composition of the systemic blood. At the same time the BBB permits entry of certain substances, such as small fat-soluble (lipophilic) molecules that can freely diffuse through the barrier. The BBB also permits entry of essential nutrients, such as glucose and amino acids through receptor-mediated endocytosis to maintain vital brain functions. These nutrients are generally water soluble (hydrophilic), and require more complex mechanisms to cross the BBB, such as carrier-mediated transport, receptor-mediated transcytosis and absorptive-mediated transcytosis.

While protective under normal circumstances, the BBB prevents the delivery of drugs, other therapeutic molecules and imaging agents to the brain. Furthermore, the BBB blocks delivery of more than 98% of central nervous system (CNS) drugs (Pardridge, 2002). Therefore, the challenge posed by the BBB is compelling, particularly as the population ages and the incidence of neurodegenerative diseases such as stroke, Alzheimer’s disease, and Parkinson’s disease increase in prevalence. The problem is particularly acute for patients with malignant brain tumors, who cannot benefit from anticancer drugs effective in treating tumors elsewhere in the body. A solution to drug delivery across the BBB would be to produce an exponential increase in the number of drugs available for the treatment and prevention of CNS disorders. Regulation of BBB/BBB permeability function may involve endogenous nitric oxide production and a cyclic GMP-dependent mechanism (Liu and Sundqvist, 1997).

17.1.2 BLOOD—BRAIN TUMOR BARRIER PERMEABILITY

An ideal strategy for delivery of therapeutic molecules to brain tumors would involve selective opening of only that portion of the BBB that serves the brain tumor. The portion of the BBB that surrounds a brain tumor is known as the BTB. Over the years, many methods have been used to enhance delivery of anti-cancer drugs and imaging agents to brain tumors (Misra et al., 2003). Some of these efforts have focused on changing the therapeutic molecule or encapsulating the drugs in nanospheres or nanoparticles (Amrawy et al., 2016), rather than altering the barrier. Well considered drug design or drug delivery techniques were aimed at “lipidizing” otherwise poorly-lipid soluble compounds, by either developing lipophilic analogs or packing hydrophilic drugs in liposomes. This approach has been limited by the relative instability of lipophilic analogs in the blood, and the rapid removal of these analogs from the blood as a direct result of their increased lipid solubility. Other strategies have focused on circumventing the barrier, for example by directly injecting drugs into the brain or through the use of implantable drug delivery devices, such as Gliadel. These strategies are highly invasive and they do not provide specific delivery to the abnormal brain region. Intracerebral implants, moreover, are ineffective against larger tumors or

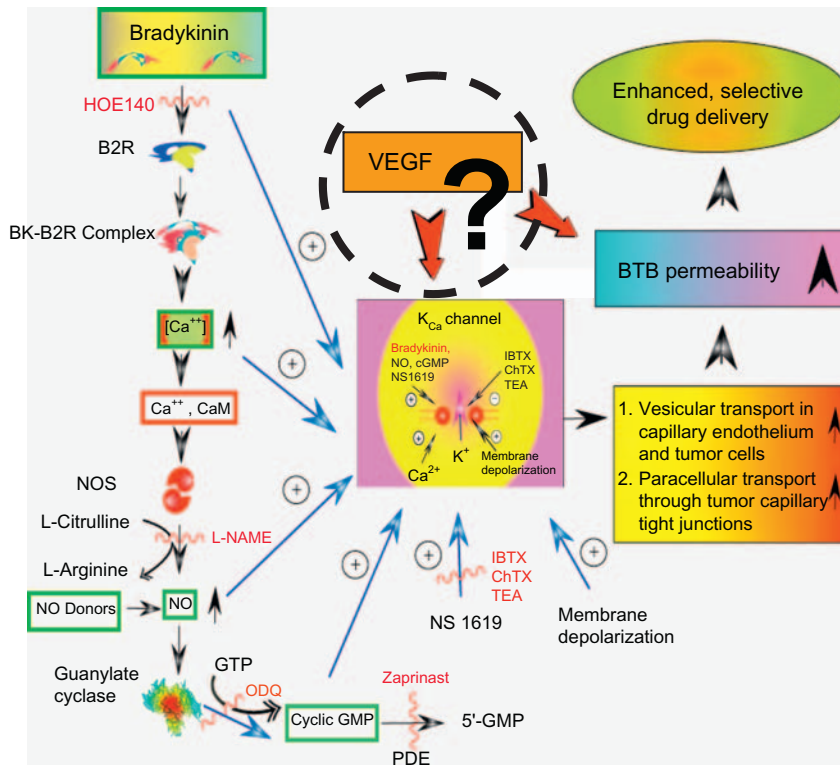
metastatic tumors and highly diffused tumors, such as gliomas [e.g., glioblastoma multiforme (GBM)] that are dispersed across the entire brain region.

One such method sometimes used in brain tumor patients was to disrupt the BBB by “osmotic shock” using Mannitol for enhanced anticancer drug delivery. However, BBB disruption techniques have produced disappointing results with unacceptable toxicity (Misra *et al.*, 2003). Some pharmacological strategies as an adjuvant included coadministration of convulsants and anticonvulsants during the systemic administration of antineoplastic agents (Neuwelt *et al.*, 1983).

17.1.3 BIOCHEMICAL MODULATION OF THE BLOOD–BRAIN TUMOR BARRIER

Biochemical disruption is based on the finding that the permeability of tumor capillaries is enhanced relative to that of normal brain capillaries by administration of certain vasoactive molecules (Fig. 17.2). BTB function is generally impaired in brain tumors because the endothelium-dependent regulation of cerebral blood vessel function is abnormal (Morimoto *et al.*, 2002), which might affect BTB permeability in response to vasomodulators (Cobbs *et al.*, 1995). Accordingly, biochemical opening with vasoactive agents holds promise for increased anticancer drug delivery selectively to brain tumors across BTB, compared to the toxic Mannitol-induced osmotic disruption. This is due to its nonselectivity and unwanted distribution of anticancer drugs to normal brain. We and others have tested a variety of vasoactive compounds, such as leukotriene (LTC₄), bradykinin (BK), cGMP, and certain potassium channel agonists to selectively disrupt the BTB without affecting the BBB, for enhanced anticancer drug delivery in experimental brain tumor models (Sugita and Black, 1998; Hashizume and Black, 2002).

Using immunoblot and immunolocalization studies, we established that BK_{Ca} channels were overexpressed in rat brain tumor capillary endothelium and tumor cells, and demonstrated the functional presence of channels in isolated rat brain tumor capillary endothelial and tumor cells (Ningaraj *et al.* 2002). The BK_{Ca} channel has been shown to be the convergence point of a BK signaling pathway involving nitric oxide, soluble guanylyl cyclase, and cGMP (Fig. 17.1). While BK has been shown to activate BK_{Ca} channels, other known activators of BK_{Ca} do not act as vasodilators; for example, 1,3-dihydro [2-hydroxy(trifluoromethyl)phenyl] (trifluoromethyl)-2H-benzimidazolone (NS-1619) (Holland *et al.*, 1996). Another major class of potassium channels, K_{ATP} channels, have also been shown as overexpressed on BTB and brain tumors (Ruoshlati, 2002; Ningaraj *et al.*, 2003). We have developed methods of increasing delivery of anticancer agents to brain tumor using potassium channel activators, which include compounds that indirectly activate potassium channels, such as nitric oxide, nitric oxide donors, and other activators of soluble guanylyl cyclase. These potassium channel activators selectively increase the permeability of the BBB and, in particular the BTB, for small to large-sized molecules, drugs, and imaging agents (Fig. 17.2).

**FIGURE 17.2**

The figure illustrates the convergence point of BK_{Ca} channels, which mediate the BTB permeability regulation affected by BK_{Ca} channel modulators. We have identified strategies by which intact BTB can be breached that are of greater clinical significance for the treatment of brain cancers. We developed a noninvasive method to modulate BBB/ BTB permeability using certain potassium channel agonists, including VEGF that is highly secreted by glioma cells. Biochemical modulation leads to enhanced anticancer drugs delivery, selectively to brain tumors without affecting the normal brain tissue.

Adapted from figure published by Ningaraj, N.S., Rao, M., Hashizume, K., Asotra, K., Black, K.L. 2002. Regulation of blood-brain tumor barrier permeability by calcium-activated potassium channels. *J. Pharmacol. Exp. Ther.* 301, 838–851.

17.2 FUNCTIONAL ION CHANNELS ON BLOOD–BRAIN TUMOR BARRIER

Several researchers, including us, have shown the importance of ion channels on BTB permeability regulation and their role in anticancer drug delivery (Ningaraj, 2006; Ningaraj et al., 2002, 2003; Black and Ningaraj, 2004). We recently reviewed the role of BTB-associated ion channels in increasing the BTB

permeability for delivering therapeutic, prophylactic, and diagnostic agents to brain tumors (Ningaraj and Khaitan, 2015). We showed that the BTB can be modulated to increase delivery of combination of drugs: trastuzumab with temozolomide in glioma models (Ningaraj et al., 2009). The underlying mechanism is not completely understood, but it involves formation of brain vascular endothelial transcytotic vesicles to facilitate transport of the drugs, as demonstrated by us earlier (Ningaraj et al., 2002, 2003). Recently, we described the role of BK_{Ca} and K_{ATP} channels in brain tumor cell growth. We also showed that modulators of BK_{Ca} and K_{ATP} channels may be utilized to enhance the delivery of antineoplastic drugs and imaging agents to glioma cells in brain tumor models (Ningaraj and Khaitan, 2015).

17.3 NANOMEDICINES AND NANOIMAGING

Despite the challenges faced due to the presence of BBB/BTB, there has been some progress in developing new strategies to treat gliomas. Specifically, gliomas are diffusive with several leading edges, making it difficult to target them with anticancer drugs as well as imaging agents. A recent review article (Juratli et al., 2013) has described the nanomedicine approaches that have been developed and some of the technologies that are being translated to the clinic. It also discusses the hurdles of effective brain tumor treatment and how various nanomedicine techniques that are being explored to overcome BBB using liposomal and polymeric nanoparticles. It is well studied that the BBB becomes compromised both structurally and functionally to transform itself into the BTB, as the primary and/or metastatic tumors grow more than 1–2 mm in diameter. Such tumors also have disorganized or distorted, tortuous blood vessels that are typically referred to as the BTB (Fig. 17.1) (Abbott and Friedman, 2012). It is important to note that BTB capillary ultrastructure is markedly different between a GBM and a brain metastasis (Van Telligen et al., 2015; Hawkins and Davis, 2005). This leads to differential vascular permeability and often requires different strategies to deliver anticancer drugs and imaging agents across the BTB of primary and metastatic brain tumors. Accordingly, BTB permeability should be assessed with advanced methodologies such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) to plan for targeted treatment of different types of brain tumors. In this aspect, we have validated a noninvasive method of BTB permeability measurement using DCE-MRI in brain tumor models (Ningaraj and Khaitan, 2013).

17.4 ASSESSMENT OF BRAIN TUMOR, TUMOR VASCULATURE AND PERMEABILITY

There are four major techniques currently used in both the clinical and research settings: (1) T1-weighted steady-state DCE-MRI method, (2) T2-weighted

first-pass, dynamic susceptibility contrast-enhanced perfusion MRI (DSC-MRI) method, (3) arterial spin labeling method; and (4) vascular space occupancy imaging (Dhermain et al., 2010). Standard imaging technologies such as computed tomography (CT), positron emission tomography (PET), and MRI are routinely used in planning for radiotherapy and monitoring of tumor response to treatment. Recent advances in technologies have also allowed the imaging of the molecular activities of tumors and normal tissues for better localization of invading tumors (Young and Setayesh, 2009). Molecular imaging also helps in assessing the early radiation and or targeted drug treatment response of tumor and normal cells. Importantly, research on methods to increase permeability and permeability assessment of vasculature leakage and clinical validation (Jain et al., 2007) is indeed critical now. The understanding of how each tumor vasculature differs in their permeability for imaging agents and therapies will have profound impact on personalizing the treatment of brain tumors (Fidler et al., 2002; Khayal et al., 2008). In addition, identification and validation of BTB permeabilizing agents and increasing delivery of molecular probes and therapies are also important.

In preclinical experiments, peripheral benzodiazepine receptor (PBR)-targeted probe such as its ligand PK11195 conjugated to a fluorophore was shown to detect breast cancer cells in brain (Manning et al., 2006). Along with DCE-MRI, a study described the use of two scaffolds, a linear polymer and a novel dendrimer, multimeric forms of the PBR-targeted probes (PK11195) to obtain multiple imaging signatures. A fluorescent molecular switch and MRI or PET compatibilities were used for enhanced clinical outcomes (Khayal et al., 2008). Such multimeric molecular imaging strategies and PBR targeting may be used in future for breast cancer metastases detection in brain and for therapeutic efficacy monitoring that might ultimately enhance patient survival rates. Researchers at Vanderbilt University developed a conjugated form of PK11195 coupled to a lissamine dye (Liss-ConPK11195) to target the PBR. The preclinical study (Manning et al., 2006) was validated by a later study that showed that conPK11195 conjugated to the fluorophore retained significant binding to its target cancer cells that express PBR for potential detection of leading edge of brain tumors that typically over express PBR (Shelby et al., 2010).

Besides, brain tumors, application of permeability imaging is critical in cases of ischemic and hemorrhagic stroke and cerebral small vessel disease. In a review of this important area, the application of permeability imaging in ischemic stroke for the prediction of hemorrhagic transformation after infarction, and for the evaluation of newborn secondary and tertiary collateral circulations have been discussed (Hui et al., 2016). Quantitative measurements of BBB disruption by the DCE-MRI have shown the severity of intracranial hemorrhage-induced brain damage, and that the technique has the potential to be used for testing the efficacy of interventions aimed at reducing tissue damage around the hematoma and brain tumors.

17.4.1 CLINICAL APPLICATIONS OF PERFUSION MAGNETIC RESONANCE IMAGING

As the population ages and the incidence of neurodegenerative diseases such as stroke, Alzheimer's disease, and Parkinson's disease increase in prevalence, the drug delivery across the BBB becomes highly critical. In that regard, we used a K_{ATP} channel opener, Minoxidil sulfate (MS) that increased delivery of Gd-DPTA to leading glioma edges and enhances contrast of diffused glioma in a brain tumor xerograft model (Ningaraj and Khaitan, 2013). We validated the DCE-MRI method with QAR (quantitative autoradiography) for noninvasive BTB permeability measurement (Ningaraj and Khaitan, 2013). Following our work, another study proposed the use of perfusion MRI to measure the degree of tumor angiogenesis and capillary permeability, both of which are important biologic markers of malignancy, grading, and prognosis, particularly in gliomas (Miao et al., 2014). Others have shown that perfusion MRI can be used to evaluate hemodynamic properties of the tumor such as tumor blood volume or flow, oxygenation, vessel size, and vascular permeability (Skinner et al., 2016). A generic advantage of perfusion and permeability imaging is that they both incorporate microvascular information (Shiroishi et al., 2011) that can be critical for planning effective treatment.

For instance in GBM, the BTB vessel morphological changes may be attributed to cancer cell induced alteration of the vessel wall that include modification of the basement membrane, endothelial cell fenestrae, reduction of pericytes and smooth muscle, and the formation of blind saccular extension (Fig. 17.1). GBMs are shown to have vasculature of increased diameter, high permeability, thickened basement membrane, and high proliferation of endothelial cells (Dimberg, 2014). We showed that vascular endothelial growth factor (VEGF) increases permeability of BTB in gliomas as cancer cells secrete more VEGF (Fig. 17.2) (Khaitan et al., 2009) factors expressed in gliomas and are known to directly contribute to BBB breakdown in gliomas (Figs. 17.3, 17.4). Donnem et al. (2013) have demonstrated early disease stage cooption process in brain when metastatic cancer cells grow on the outer area of the preexisting blood vessels in early experimental brain metastases and in human clinical specimens reflecting early stages of the disease. Other factors shown to alter the microvascular permeability measurement include the luminal surface area, hydrostatic interstitial, and osmotic gradients across the endothelium (Nagy et al., 2008). In our laboratory, we used a K_{ATP} channel opener, MS that increased delivery of Gd-DPTA to leading glioma edges and enhances contrast of diffused glioma in a brain tumor xerograft model (Ningaraj and Khaitan, 2013). Now drug delivery research is focused on several innovative methods (Figs. 17.3, 17.4), including nanoparticles, microparticles as carriers of anticancer agents, PEG technology, encapsulating anticancer drugs in liposomes, and MABs for the delivery of anticancer drug payloads (Ningaraj, 2006).

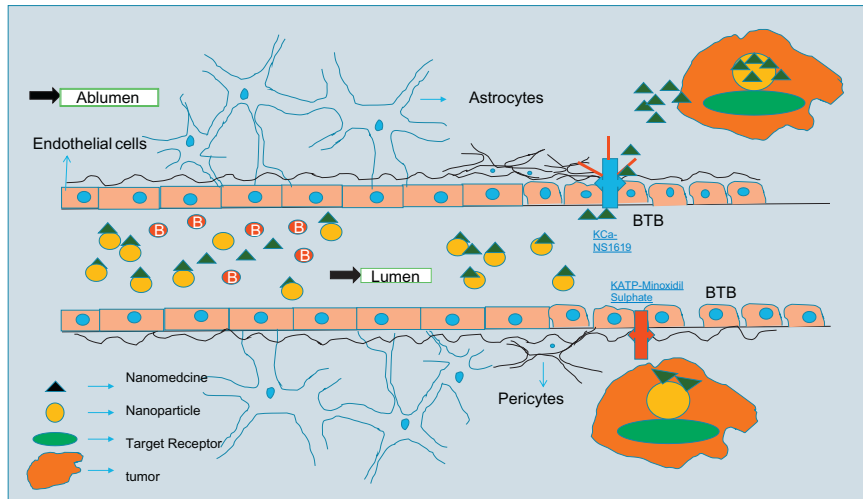


FIGURE 17.3

Representation of challenges and opportunities those are present in brain tumor treatment. As the BBB poses hurdles for drug delivery to brain tumors the molecules (potassium channels) that are uniquely expressed on BTB can be activated to deliver anticancer drugs to brain tumors. This cartoon summarizes the innovative ideas being studied in laboratories across the world to deliver nanomedicines and nanoparticles that carry payloads of targeted therapies for better BBB penetration in order to efficiently detect and treat tumors.

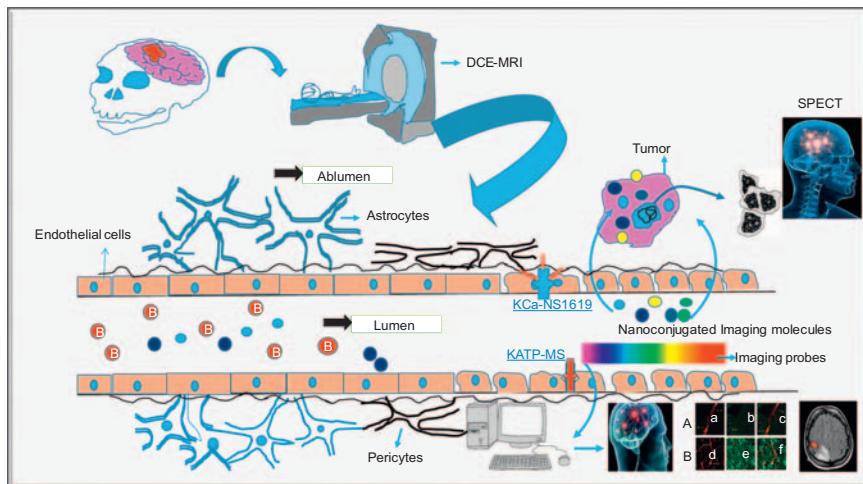


FIGURE 17.4

As the BBB poses hurdles for drug delivery to brain tumors the molecules (potassium channels) that are uniquely expressed on BTB can be activated to deliver nanoimaging molecules to brain tumors. This cartoon summarizes the innovative ideas being studied in laboratories across the world to deliver nanoparticles that carry payloads of targeted imaging molecules and radiotherapies for greater BBB penetration in order to efficiently detect and treat tumors.

17.5 NANOTECHNOLOGY FOR IMPROVED BRAIN TUMOR DETECTION AND TREATMENT

The application of nanotechnology using nanoparticles for increasing drug delivery across BBB has been recently reviewed by [Zhang et al. \(2015\)](#). [Figs. 17.3 and 17.4](#) summarize the innovative ideas being studied in laboratories across the world to deliver nanomedicines and nanoparticles that carry payloads of nanoimaging molecules or targeted therapies for better BBB penetration in order efficiently detect and treat tumors. Nanoparticles have been developed that are able to carry therapeutics across the BBB ([Auffinger et al., 2012](#)). Nanoparticle-tagged drugs have some advantages. They may be administered intracerebrally to release drugs in a sustained manner ([Ren et al., 2012](#)) and prevent degradation when administered systemically ([Lee et al., 2013](#)). To improve drug targeting, some researchers have encapsulated doxorubicin in nanoparticles, which are readily taken up by the BBB/BBB endothelium. Such encapsulation may also prevent drug being effluxed by the endothelial efflux system. [Malatesta et al. \(2014\)](#) showed that chitosan nanoparticles can deliver a hypometabolizing synthetic opioid, D-Ala2- D-Leu5-enkephalin across the BBB and release drugs in the brain. [Amrawaty et al. \(2016\)](#) have extensively reviewed following researching several publications on noninvasive treatment of glioblastoma and other brain tumors using several technologies and patent applications from 2010 to 2015. They concluded that intensive research efforts are necessary develop novel CNS delivery systems that can be used in clinics. Few laboratories have developed nanomedicine techniques employing liposomal and polymeric nanoparticles to deliver drugs to brain tumors ([Tzeng and Green, 2013](#); [Vieira and Gamarra, 2016](#)). Biodegradable polymer-based nanoparticles and gold nanoparticles have shown greater promise for carrying drugs across the BBB to treat glioma ([Auffinger et al., 2012](#)). Specifically, gold nanoparticles permeate the BBB through endothelial cells of the BBB ([Gromnicova et al., 2013](#)). Similarly, [Jensen et al. \(2013\)](#) showed the BBB penetration of RNA interference-based gold nanoparticles following systemic injection. These findings suggest that the nanoparticle-based delivery systems hold great promise in improving specific and efficient intracerebral delivery of anticancer drugs for the treatment of glioma ([Krol, 2012](#)). Interestingly nanoparticles are often integrated with techniques such as hyperthermia or a molecular “Trojan horse,” discussed to improve delivery of anticancer drugs ([Stockwell et al., 2014](#)).

Nanospheres ranging in size from 1 to 100 nm, have shown better binding capacity for specific molecules, allowing varied surface modification and the multivalent binding of targets. Consequently, they have new applications in molecular imaging and nanomedicines ([Lee et al., 2013](#); [Vilella et al., 2014](#); [Hernández-Pedro et al., 2013](#); [Alyautdin et al., 2014](#)). A greater emphasis is given to building nanospheres with their payload therapeutics, imaging agents, or combination of both, that can penetrate BBB and remain in tumor tissue without

being ejected by the efflux pumps (Hernández-Pedro et al., 2013). The nanostructures of 100 nm diameter, whose surface charge and flexibility may be modified to allow increased nanostructure biodistribution (Alyautdin et al., 2014; Chacko et al., 2013; Meyers et al., 2013), could be developed for this purpose. Such flexible nanospheres may be labeled with a radionuclide to develop molecular imaging probes using PET or single photon emission computed tomography (SPECT). The two major strategies for labeling nuclides onto nanostructures depend on whether chelating agents are used. A few studies have shown the chelation free labeling methods by combining nanoparticles such as gold nanoparticles (Meyers et al., 2013; Sheng-Feng et al., 2015), carbon-based nanomaterials (Bhattacharya et al., 2016; Baldrighi et al., 2016), and quantum dots (Zheng et al., 2015; Nair et al., 2008), through a chelating agent, such as tetraazacyclo-dodecanetetra-acetic acid (DOTA) and 1,4,7-triazacyclononane-N, N', N-triacetic acid (NOTA). DOTA now has FDA approval for human application. A recent article has reviewed chelating agent-mediated nanostructures such as gold nanostructures, colloidal gold nanoparticles (AuNPs) with biomolecules (e.g., DNA and peptides) and ^{64}Cu -chelated AuNS, that showed better penetration and accumulation in the squamous cell carcinoma region (Bardhan et al., 2011).

Noninvasive tumor diagnosis, treatment, and monitoring of disease is made possible by molecular imaging techniques such as MRI, CT, PET, SPECT, contrast-enhanced ultrasound, and optical fluorescence (Galanaud et al., 2006). Additionally, various imaging molecules and modalities have been developed, including targeting PBR by PBR ligands (Manning et al., 2006; Shelby et al., 2010), whereby each method and molecular possesses unique strengths. Typically, radionuclide-labeled substances are used as contrast agents in the case of PET and SPECT. Radionuclide-tagged antibodies, antibody fragments, peptides, proteins, and oligonucleotides are used as contrast agents. Generally, radionuclides are linked via a chelating agent to targeting components, such as antibodies, antibody fragments, peptides, proteins, oligonucleotides, or receptor ligands. More recently, exceptional properties of nanostructures have made them a formidable tool for development of accurate and efficient diagnostic agents for multimodality tumor imaging (Meyers et al., 2013). Although the use of nanostructures show promise, the clinical utility of these structures with chelating agents for image guided diagnosis suffers from few limitations such as instability of the complex and detachment of chelating agents from radioligands and nanostructures. Consequently, functionalization methods free of chelating agents have been tried, to overcome those drawbacks (Yousafa et al., 2013). Moreover, molecular imaging tools by themselves or with nanostructures/nanospheres are sufficient, due to their inherent limitations, specifically penetration of tumor tissue across the BTB (Fig. 17.4). Some studies have shown that nanospheres tagged with imaging agents have overcome the BTB problem but more clinical studies are needed to develop this strategy for efficient clinical diagnostic tool.

17.6 SUMMARY

Due to the contribution of many researchers, we now have a better understanding of molecular mechanisms that regulate BBB and BTB regulate permeability. Based on several groundbreaking studies, we are beginning to see a fundamental shift in how brain tumor patients are treated. The advent of high-powered DCE-MRI, PET, and SPEC the detection and resection of brain tumors has improved significantly in the last decade. Brain tumor genomics and the advent of personalized medicine have also contributed to improved brain tumor treatment strategies. Despite these positive developments, the mortality rate of GBMs is still stubbornly high. In order to improve brain tumor patients' survival rate, the key to success is novel technologies for better detection, timely therapeutic intervention, highly targeted drug delivery with enhanced and retained anticancer drugs in the tumor to stop its growth and spreading.

Discovery of nanomedicines and nanospheres that carry a payload of anticancer drugs, combined with radio therapies, are expected to improve the diagnoses and prognoses of brain tumors in the present decade. Earlier, we discussed the same concept with examples of novel agents that are in early clinical development. The “holy grail”, the understanding of BBB permeability regulation in normal and diseases states, has given the researchers a better tool to manipulate the BBB/BTB to gain control over the tumor growth and dispersion in the brain. With the present knowledge of the BBB/BTB permeability regulation, various strategies should be employed to increase BTB permeability in order to enhance selectively delivery of anticancer drugs and nanomedicines employing nanotechnology to brain tumors. In this regard, we employed certain vasomodulators, such as potassium channel agonists, for targeted and enhanced delivery of chemotherapeutics selectively to brain tumor in rodents, and BK or its analog, RMP-7, in humans. The noninvasive biochemical approach to enhanced BTB permeability (Fig. 17.2), has an advantage over other methods because therapeutic drugs are delivered directly to brain tumors with little or no drug delivery to normal brain. Accordingly, BTB modulation should help deliver small to large-sized substances, including contrast-enhancing agents, nanomedicines, antitumor compounds, therapeutic proteins and viral vectors, all of which can be encapsulated with newer delivery vehicles including nanospheres.

In conclusion, a significantly improved diagnosis and prognosis of brain tumors can be achieved by delivering clinically useful nanomedicines, radiotherapies, imaging agents, therapeutic antibodies, and chemotherapeutic drugs tagged to nanospheres through noninvasive biochemical modulation of BTB. More clinical studies are required to develop BTB modulation with potassium channel agonists as adjuvants to enable increased and selective delivery of imaging agents and anticancer agents. Finally, it is hoped that a sustained research effort is maintained in this critical area for potentially increasing the survival rate for patients with debilitating neurological diseases and tumors.

ACKNOWLEDGMENTS

The authors thank Scintilla Bio-MARC, Bangalore, India, Anderson Cancer Institute and Mercer University Medical Center, Savannah, GA, United States, Vanderbilt Ingram Cancer Center, Nashville, TN, United States, Cedars Sinai Medical Center, Los Angeles, CA, United States and American Cancer Society, United States, Georgia Cancer Coalition, Atlanta, GA, United States and NIH for providing opportunity and research grant support.

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Polymeric nanocarriers for site-specific gene therapy 18

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CHAPTER OUTLINE

18.1 Introduction	689
18.2 Site-Specific Gene Targeting	691
18.2.1 Antibody Mediated Targeting	692
18.2.2 Aptamers	692
18.2.3 Other Ligands	693
18.3 Polymeric Nanoparticles.....	693
18.3.1 Chitosan	694
18.3.2 Polyethylenimine.....	696
18.3.3 Poly (Lactic-co-Glycolic) Acid	697
18.3.4 Poly-L-Lysine	699
18.3.5 Cyclodextrins	700
18.3.6 Dendrimers.....	701
18.3.7 Polymethacrylate.....	703
18.4 Clinical Trials of Polymeric Nanocarriers for Gene Therapy	705
18.5 Future Perspectives of Gene Therapy.....	707
References	707
Further Reading	714

18.1 INTRODUCTION

Gene therapy is rapidly growing as a promising strategy for the treatment of both inherited and acquired disorders. In general, the process involves modification or regulation of therapeutic gene expression (DNA or RNA). This is achieved either by replacing, knocking out, knocking down a mutational gene or by introducing a healthy copy of gene to resolve the disease. Gradually, this has emerged as a potential alternative strategy against drug treatment, surgical approach, and enzyme/protein therapy (Ramamoorth and Narvekar, 2015; Choi et al., 2014).

The concept of gene therapy was first initiated by the revolutionary discovery of recombinant DNA technology in the year 1970. Initially, viral vectors (adenoviruses, adeno-associated viruses, helper dependent adenoviruses, retroviruses, lentiviruses), physical methods (such as naked DNA, electroporation, hydrodynamic pressure, ultrasounds, gene gun) and chemical methods (lipid mediated, peptide mediated) were developed for treatment purposes (Niidome and Huang, 2002). However, these were associated with certain limitations such as immunogenicity, inflammatory properties, nonefficient packing of therapeutic gene, lack of specificity and serious off-target effects (Thomas et al., 2003).

With several years of effort, the curiosity for gene therapy gave way to the first successful gene therapy for the treatment of severe combined immunodeficiencies (SCID) on a 4 year old girl, that also gained approval from FDA (Food and Drug Administration) (Massadeh and Alaamery; Rubanyi, 2001; Nagarwal et al., 2010). This further led to a great amount of research in other fields of diseases such as cancer, HIV, and various neurodegenerative diseases (El-Aneedy, 2004a; Herman, 2016; O'Connor and Boulis, 2015). However, researchers faced several difficulties in the course of treatment. These include, inappropriate target identification, nonspecific and nonefficient delivery of therapeutic gene, immune response stimulation, and short half-life of the gene (Misra, 2013). This increased the need of an efficient gene delivery system to carry the gene to the target site. In terms of development of delivery system, the advent of nanotechnology proved to be a benchmark in this field, owing to several inherent properties (Herranz et al., 2011).

Nanoparticles, as the name suggests, concern particles in the range of 1–100 nm. In general terms, nanomaterials with advantageous inherent properties such as biocompatibility, biodegradability, lower cytotoxicity, and tailorability are utilized for the preparation of nanoparticles; they are generated in various forms such as micelles, liposomes, nanocages, and molecular conjugates. Based on the type of material employed, the engineered nonviral vectors are categorized into three groups: (1) lipid based vectors, (2) peptide based vectors, and (3) polymeric nanocarriers. The beneficial properties of nanoparticles are mainly dictated by the small size and high surface area. Furthermore, the valuable properties of nanoparticles make them capable of evading immune reactions as well as overcoming physiological barriers during site-specific gene delivery. Accordingly, this technology can surmount the challenges and assist in the success of targeted gene delivery (Panyam and Labhasetwar, 2003).

Specificity and selectivity are two essential aspects of a delivery system for achieving safer and efficient targeted gene therapy against various diseases. Among different delivery systems available, polymeric nanoparticles are regarded as an ideal platform. They confer target-specific delivery in a single system with high transfection efficiency, ease of modification, and safety. In addition, they have gained much more attention due to their biodegradability, biocompatibility, cost effectiveness, low immunogenicity, less toxicity, and pseudo natural properties (Kirtane and Panyam, 2013). Site-specific delivery of formulation is achieved

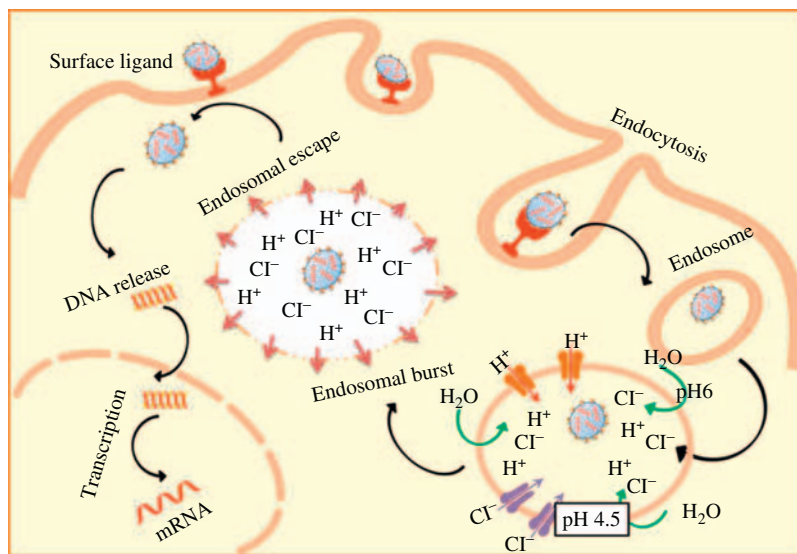
by either passive or active targeting. Passive targeting involves extravasation of nanoparticles through defective vasculature, especially in the case of angiogenic tumor tissues. Whereas, active targeting refers to the targeted delivery of therapeutic molecules in specific cells through nanoparticle-modified ligands, that generate ligand-receptor interaction, followed by effective internalization of nanoparticles (Sakurai et al., 2015).

This chapter summarizes the significance of nanotechnology in gene therapy with a brief introduction, including its types, and hurdles in its success. Furthermore, we have thrown light on various advantages and disadvantages of various vectors along with advantages of polymeric vectors. Additionally, a detailed account of the polymeric nanoparticles, such as chitosan, polyethylenimine (PEI), poly (lactic-co-glycolic) acid (PLGA), etc., and their physiochemical characteristics have been discussed in the subsequent sections. Furthermore, strategies and advances made toward the development of polymeric nanocarriers have also been explored in order to achieve site-specific gene delivery.

18.2 SITE-SPECIFIC GENE TARGETING

The main limitation toward the success of site-specific gene therapy is the complexity and heterogeneity in *in vivo* system. Targeting of nanoparticle formulation to specific tissues and cells is accomplished by two ways: passive targeting and active targeting (Fig. 18.1) (Lin et al., 2015; Sakurai et al., 2015). As mentioned earlier, passive targeting occurs in a tumorigenic microenvironment having leaky vasculature. The increase in the volume of tumor tissue leads to increase in the generation of new blood vessels (called angiogenesis) due to the requirement of nutrition intake, oxygen delivery and waste excretion. This results in leaky vasculature, increased acidity, and poor lymphatic drainage, due to high interstitial pressure (Carmeliet and Jain, 2000). Furthermore, these physiological conditions leads to enhanced permeation retention (EPR) effect, which is responsible for the localization of nanoparticles in the tissue (Maeda, 2001).

In the case of active targeting, specific ligands are conjugated to the nanoparticle system for site-specific delivery through ligand receptor reaction. This ligand modification facilitates distinguishing between target and nontarget tissue or cells (Byrne et al., 2008). Accordingly, in order to achieve better and site-specific targeting of nanocarriers, efforts have been made in the direction of receptor-mediated uptake of the nanovectors. A plethora of work has already been done on covalent conjugation of ligands on DNA-bound cationic polymers. Various ligands used for this purpose are grouped into protein (complete antibody or its fragment), nucleic acid (including aptamers), and other associated ligands. Another class of ligands consists of peptides, vitamins, and carbohydrates. These bind to the receptor overexpressed on the cell surface and enhances cellular uptake of nanoparticles through the process of endocytosis. This effective

**FIGURE 18.1**

Different types of targeting: passive and active targeting of therapeutic molecules and nanovectors during the circulation process in the body.

internalization improves the therapeutic effect in various gene therapies. A few ligands such as antibody, aptamers and other ligands employed for targeted delivery are discussed in the subsequent sections

18.2.1 ANTIBODY MEDIATED TARGETING

Antibodies are a class of protein that are preferentially employed with polymeric nanoparticles for delivery of nucleic acids to a particular site. These are highly specific and stable in systemic circulation ([Attarwala, 2010](#)). However, synthesis and development of the antibody for clinical applications is difficult due to its expensive manufacturing process, requirement of high purification, and quality control ([Yu et al., 2012](#)).

18.2.2 APTAMERS

Aptamers are oligonucleotides consisting of 15–40 nucleotide units, that are specific to the target protein and are extensively used in the field of targeted delivery ([Wilson and Szostak, 1999](#)). They have high specificity and avidity relative to antibodies. In addition, they can be produced by simple and cell-free methods proving it to be more cost effective than antibodies ([McNamara et al., 2006](#)). Moreover, they have several advantages including low immunogenicity, rapid

selection, small size, simpler synthesis, target specificity, and site-specific modification. The main concern of these studies is to generate an ideal vector with modified ligand that results in its high affinity internalization by the cell (Yu et al., 2012).

18.2.3 OTHER LIGANDS

Overexpression of specific receptors are predominant markers of cancers. Ligands commonly used against the receptors are peptides, vitamins, and carbohydrates. For example, transferrin and folate receptors are overexpressed in most of the cancer cells. Accordingly, transferrin/folate-modified nanovectors can be employed for the purpose of gene delivery in metastatic and drug resistant cancer cells (Daniels et al., 2006; Low and Kularatne, 2009). Low density lipoprotein (LDL) has also been used for targeting muscle cells and hepatic cells that express LDL receptors on their cell surface (Yu et al., 2009). From previous studies, it has been discerned that various carbohydrates such as galactose, mannose and lactose can be employed for specifically targeting the cell (Prabhu et al., 2015).

18.3 POLYMERIC NANOPARTICLES

Among all the available polymers, cationic polymeric nanocarriers have gained immense attention in the past decade. These nanoparticles have several advantages over other vector systems in terms of effective encapsulation and efficient transfection (Vasir and Labhasetwar, 2006). This is due to their ability to condense large pieces of nucleic acid, protect DNA from degradation, and generate stable complexes. The polymeric nanoparticles also exhibit a net positive charge value at physiological pH, even after effective encapsulation of nucleic acid. The presence of positive charge mediates endocytosis and endosomal escape of the nucleic acids. It is evident that the properties of the polymers play a crucial role in gene entrapment and ligand conjugation. The cationic polymers differ from one another by relative number, pKa, of protonable amines and the type of amines. The polymers possessing primary and secondary amines exhibit higher pKa value (~ 10.7) with a strong nucleophilic capacity. Whereas, polymers with tertiary amines have a lower pKa value (~ 9.8) that makes them less nucleophilic and less compatible. Conversely, the presence of amine groups is known to confer endosomal buffering, ultimately leading to rupture of endosomes and release of payload in cytoplasm. Furthermore, the effectiveness of gene therapy by polymeric nanoparticles is decided by various other important factors such as polymer concentration, molecular weight (MW), surface charge, and amine-to-phosphate (N/P) ratio (Elsabagy and Wooley, 2012). A number of polymeric nanoparticles have been employed for nucleic acid delivery in gene therapy, including chitosan, PLGA, PEI, cyclodextrins (CDs), dendrimers, etc.

18.3.1 CHITOSAN

Chitosan, a natural cationic polymer, is mainly derived from chitin by N-deacetylation process. Chitin is the second most abundant polymer in nature after cellulose, and forms the basic component of exoskeleton of crustaceans and the cell wall of insects and fungi. This is made of randomly arranged N-acetylglucosamine and glucosamine residue having β -1, 4 linkage, giving rise to a linear conformation. In general, chitosan differs from chitin in terms of degree of deacetylation (DDA), i.e., when the DDA of chitin reaches 40%–50% or beyond (Rinaudo, 2006).

In the last two decades, chitosan has been considered as a promising vector in gene therapy both for in vitro and in vivo studies, attributed to its admirable properties such as biodegradability, biocompatibility, ecological safety, low toxicity, low immunogenicity, and antimicrobial activity. In a quest to find a reliable delivery system, scientists have been focusing on this biopolymer with wider concern. Chitosan has a pKa value of 6.3 that allows it to solubilize under dilute acidic conditions due to the protonation of amine groups at C-2 position. Given its cationic nature, chitosan can easily form complexes with anionic nucleic acid by electrostatic interaction. In addition, the polymer can be tailored for enhancing its stability as well as transfection efficiency, that, in turn, depends on certain important factors such as DDA, MW, N/P ratio of chitosan, pH, and presence of serum. Among all the concerned properties of chitosan, DDA has been considered to play a crucial role in the nanoparticle formation; the higher the DDA, the higher the positive charge on the chitosan and greater will be the complexation ability. Accordingly, for the purpose of synthesizing nanoparticles, chitosan with higher DDA and lower MW (10–50 kDa) is chosen (Yi et al., 2005; Zhang and Neau, 2002; Liu et al., 2007).

The MW of chitosan is also another governing factor for the synthesis of nanoparticles. From previous studies, it is evident that MW of chitosan determines the size, charge, and stability of the nanoparticles for efficient delivery of nucleic acid. It is also established that small-sized nanoparticles with low poly dispersity index (PDI) can be synthesized from higher MW chitosan owing to their better complexation ability. However, higher MW chitosan experiences solubility issues and particle aggregation that could be overcome by several modifications in the backbone of the polymer (Danielsen et al., 2005; Jin et al., 2014). In this context, different experiments were conducted with varying MW of chitosan. In one of the studies, nanoparticles carrying siRNA of size 148 nm were obtained from a higher MW (190–310 kDa) chitosan, which showed 70% inhibition of specific gene expression in human colorectal cancer Lovo cells. In the case of medium MW (110 kDa) and lower MW chitosan, smaller nanoparticles were obtained, depicting a decreasing trend in the size of the particles with decreasing MW of chitosan. Besides the attempt to prepare smaller sized nanoparticles, it is also necessary to monitor the transfection properties and controlled release of the payload. Lower MW chitosan (10 kDa) nanoparticles showed efficient

intracellular release of the payload. A further decrease in the MW of chitosan resulted in inefficient complexation with larger particles. Accordingly, in order to formulate stable and efficient nanoparticles, chitosan with optimal MW and other supporting properties is to be introduced. In this regard, different chitosans with varying properties have been tailored. For example, the cationic properties of chitosan can be modulated to aid in stable nanoparticle formation, which could be performed by several methods. This could also be accomplished by conjugating protamine to the low MW chitosan that imparts positive charge to the polymer. This resulted in the formation of small-sized particles (143.7 nm), lower pDI (0.189), higher transfection and successful gene expression (Patil et al., 2016). Further various hydrophobic and hydrophilic modifications have been carried out for improving cell binding and intracellular DNA release, respectively. This information suggests that MW of chitosan is very crucial for the development of effective delivery system. In addition, the N/P ratio affects the size of chitosan nanoparticles. A range of studies revealed that lower N/P ratio led to the formation of larger size particles. Similarly, higher N/P ratio also resulted in larger size particles due to incomplete complexation. In a similar type of study, it was noted that the size of nanoparticles decreased from 223.6 to 181.6 nm when the N/P ratio was increased from 6 to 71, while the concentration of chitosan is kept low (Howard et al., 2006). Furthermore, in 2007 Liu and coworkers showed higher silencing effect of enhanced green fluorescent protein (EGFP) with higher N/P ratio (50 and 150) over low N/P ratio (2 and 10) (Liu et al., 2007).

The properties of chitosan can also be used for obtaining delivery system for targeted delivery. Jreysaty, et al. prepared nanoparticles with folate-conjugated chitosan of varying MW (5, 10, 25, and 50 kDa) and polyethylene glycol (PEG) spacer for better internalization of VR1412 and pEGFP-C3 in human epidermoid carcinoma cells (KB cells). Among the four types of chitosan, 25 kDa chitosan exhibited better transfection efficiency in the cells (Jreysaty et al., 2012). Lee et al. used thiolated glycol chitosan for TNF- α gene silencing for the treatment of rheumatoid arthritis. The obtained nanoparticles (psi-tGC-NPs) at N/P ratio 10 showed particle size in the range of 370 nm. Furthermore, on increasing the N/P ratio (5, 20, and 40) the size of the particle reached beyond 400 nm. The in vitro transfection efficiency was tested on murine macrophage RAW 264.7 cells. The results showed that thiolated chitosan nanoparticle showed targeted delivery of gene with efficient silencing of TNF- α and suppressed paw swelling (Lee et al., 2014). Recently, Rudzinski used PEGylated chitosan nanoparticles for targeted silencing of β -catenin gene for the treatment of colon cancer. PEGylation provides colloidal stability and increases the circulation time of nanoparticles. The obtained particles showed sizes in the 100 nm range and the efficiency for transfection was the same as of lipofectamine 2000. The transfection results showed decreased level of β -catenine protein along with less toxicity (Rudzinski et al., 2016).

18.3.2 POLYETHYLENIMINE

Among all kind of cationic polymers, PEI has been the most preferred, due to its strong DNA condensation and high transfection efficiency. The reason for higher transfection efficiency of this polymer is its unique buffering capacity called as proton sponge effect (PSE) (Fig. 18.2). The PSE phenomenon of PEI allows effective and controlled release in cytoplasm through osmotic swelling that ultimately results in endosomal burst. The formulation of PEI is prepared using two strategies: nanocomplexation and entrapment. Nanocomplexation facilitates cross-linking between the cationic charged PEI and anionic charged DNA. The entrapment strategy involves formulation of PEI nanoparticle followed by loading of DNA (Nimesh *et al.*, 2006, 2007).

Various properties, such as MW and N/P ratio, govern the rate of success of transfection. Higher N/P ratio (>3) shows higher transfection efficiency, attributed to the PSE due to excess amine groups (Boeckle *et al.*, 2004). Also, MW of PEI plays a critical role toward transfection efficiency. It is evident from previous studies, that higher transfection efficiency is achieved using 25 kDa PEI over low MW PEI. It is noteworthy that PEI of higher MW (>25 kDa) renders greater cytotoxicity, whereas 25 kDa chitosan is less toxic. Due to these properties, 25 kDa PEI emerged as an ideal polymer. Although, PEI of 25 kDa is considered

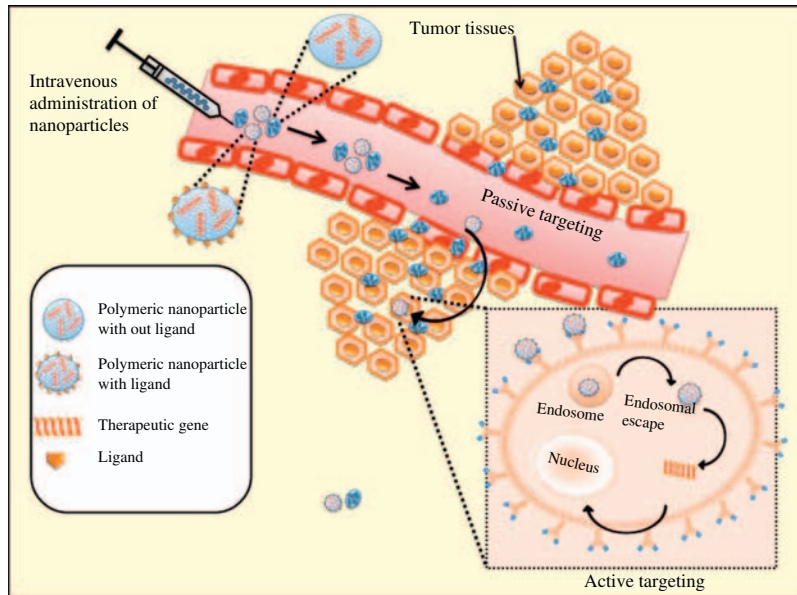


FIGURE 18.2

The proton sponge mechanism of PEI, when the modified nanoparticles are taken up by the cell through receptor-mediated endocytosis.

to be a potent polymer, considerable equilibrium is required to be maintained between lipophilicity and hydrophobicity of the formulation for easier transport across the plasma membrane. Moreover, the distribution of charge due to the presence of secondary amines aids in effective condensation of nucleic acids to form nanoparticles in considerable size range. The major drawback of the formulation is its tendency to form aggregates with the complement in the serum (Deng et al., 2009; Yang et al., 2007; Moghimi et al., 2005; Kawakami et al., 2006; Merkel et al., 2011).

The aforementioned problems can be addressed in two ways: by decreasing the cytotoxicity or by increasing the transfection efficiency by inducing certain modifications in the polymer backbone. The first attempt to reduce toxicity was conducted by Lee et al.; performed by introducing disulfide cross-linked PEI using dithiobissuccinimidyl propionate and dimethyl-3,3'-O-dithiobispropionimide as cross-linking agent. Transfection results with the formulations showed 60% transfection efficiency with 90% cell viability depicting lower cell toxicity (Breunig et al., 2006, 2007; Christiaens et al., 2005).

Alkyl modification by alkyl carboxylate residue on to PEI (25 kDa) also enhances the efficiency of oligonucleotide delivery through carriers. A group of studies demonstrated that, with low degree of carboxylation (~20%), higher buffering capacity could be achieved leading to an efficient endosomal escape. Conversely, it was also stated that the stability of the polymer-DNA complex enhanced with increase in hydrophobicity, due to increase in alkyl chain length. Furthermore, association of negative charge on the PEI reduced interaction potential toward the cell resulting in reduced toxicity (Oskuee et al., 2010). In another study, it was demonstrated that the conjugation of PEI (25 kDa) with PEG (2 kDa) increased the transfection efficiency in gastric carcinoma cells (Wu et al., 2010).

18.3.3 POLY (LACTIC-CO-GLYCOLIC) ACID

PLGA, has been considered as one of the most potent candidates among all available vectors. It is a synthetic co-polymer of lactic and glycolic acid linked through an ester bond. It is evident from the previous studies that the carboxyl-terminated PLGA polymer shows admirable biodegradability under physiological conditions. A PLGA nanoparticle exhibits beneficial properties in gene silencing owing to its efficient cellular uptake, rapid endosomal escape, and sustained release of the therapeutic molecule (Moghimi et al., 2001; Makadia and Siegel, 2011). In addition, the PLGA nanoparticles are easy to synthesize, nontoxic, protect the pDNA from nuclease degradation, and are safer for clinical trials. From the earlier studies, it has been found that PLGA nanoparticles are efficiently taken up by the cells either specifically or nonspecifically through the endocytic process. The lysosomal escape also depends on the surface charge of the polymeric nanoparticle in acidic environment. PLGA is the only FDA-approved biodegradable polymer that has been explored as delivery vector for in vivo system due to

its versatile nature, such as ease of tailorability and derivatization. Although PLGA based nanoparticles have been widely investigated for their delivery purposes, certain modifications are required to overcome the challenges for an efficient gene silencing (Gentile et al., 2014; Blum and Saltzman, 2008). In a study, PLGA-entrapped CaPi-DNA (DNA-calcium-phosphate) particles were synthesized through double emulsification technique, release and physicochemical characterization of the formulated microparticles was determined. It was demonstrated that CaPi-DNA-loaded PLGA microparticles showed considerable protection from nuclease degradation and an increased loading efficiency when compared to the conventional PLGA delivery system (Li et al., 2004). Whereas, the large size of the particles resulted in low transfection efficiency in the cultured cells (Walter et al., 2001; Aral and Akbuga, 2003).

Surface charge modifications have also been done to improve the nucleic acid adsorption capacity. In this regard, chitosan-modified PLGA nanoparticles were developed for better adsorption of siRNA with the polymer. The electrostatic interaction between the positively charged chitosan and negatively charged siRNA, resulted in the formation of highly condensed siRNA-chitosan-PLGA nanoparticles. However, it was observed that chitosan-PLGA nanoparticles formed large size particles of 400–1000 nm with a higher PDI. The possible reason for the large size particles could be the concentration and MW of chitosan and also the type of PLGA used. Therefore, with the lowering of chitosan MW, the nanoparticle size decreases (Katas et al., 2009).

Similarly, chitosan-modified PLGA nanospheres were prepared by solvent emulsion method and smooth, homogenous particles were obtained of size 300 nm, with a positive zeta potential. The modified PLGA nanoparticles showed relatively high loading efficiency, compared to the native PLGA nanoparticles. An enhanced silencing of luciferase gene was also observed in A549 cell lines. Among the various nanoparticles, the highest knockdown efficiency was observed with chitosan-PLGA nanoparticles (Tahara et al., 2009, 2010).

The properties of PLGA can be further fabricated by grafting a PLGA side chain with poly vinyl alcohol (PVA). Amine-modified PVA-PLGA nanoparticles were developed from solvent displacement method and siRNA was entrapped with the polymer (amine-modified-PVA-PLGA/siRNA). The size of the obtained nanoparticles were in the range of 150–200 nm with zeta potential of +15 and +20 mV in PBS. The formulated nanoparticles were transfected in human lung epithelial cell line, H1299 having luciferase gene. The result dictated an increased knockdown efficiency of luciferase gene with 80%–90% suppression (Nguyen et al., 2008). Poloxamers have now been employed for the delivery of plasmid DNA (pDNA). Poloxamers in conjugation with PLGA have been used for the transfection of pDNA in pancreatic BxPC-3 cells. The size of the formed nanoparticles were 198–205 nm. The results showed an efficient gene silencing against methi-CpG binding domain protein I in the cultured cells (Luo et al., 2014; Csaba et al., 2004, 2006).

Furthermore, PEI was introduced into the matrix of PLGA, to improve the encapsulation efficiency. This complexation resulted in two-fold increase in encapsulation efficiency of the particles as well as it maintained the stability during transfection. This PEI-based formulation of PLGA have shown less cytotoxicity and an increased serum stability (Patil and Panyam, 2009).

18.3.4 POLY-L-LYSINE

Poly-L-lysine is the first polymer used for gene delivery. Structurally, it consists of repeating units of a single amino acid, i.e., lysine. It is a cationic polymer with suitable biodegradable properties, necessary for in vivo transfection or gene delivery. The degree of biodegradability relies upon the degree of polymerization of lysine ranging from 90 to 450, that ultimately affects the in vivo analysis and its toxicity profiling. The cytotoxicity can be controlled by chain length of polypeptide, as longer chain degrades slowly and leads to toxicity in the cells. Similarly, with the increase in MW, toxicity increases (Martin and Rice, 2007).

Furthermore, the complexation or encapsulation efficiency of DNA depends on the number of ϵ -amino acids present in the polymer. The larger the number of ϵ -amino acid group, greater will be its encapsulation efficiency. This is due to the protonability of these amino groups which provides considerable cationic charge to the polymer in acidic environment. This in turn results in an effective condensation of the polymer with negatively charged nucleic acids. It has been investigated that PLL has comparable cellular uptake efficiency as of PEI but its transfection efficiency is quite low due to the absence of PSE. Various modifications have been done for the improved delivery of gene and an enhanced endosomal escape activity (El-Aneed, 2004b).

Benns et al. synthesized N-Ac-poly (L-histidine)-grafted poly-L-lysine nanocarriers (PLH-g-PLL). The size of the obtained polyplex (complex of respective polymer with nucleic acid) was in the range of 117 ± 6 to 306 ± 77 nm. The in vitro transfection studies were assessed by the introduction of formulated nanoparticles in 293 T cells. The PLH-g-PLL showed higher transfection efficiency in 293 T cells compared to the native polymer (Benns et al., 2000). Additionally, PLL have been widely employed for gene therapy with different modifications including the dendrimers and co-polymers. Yamagata et al. explored sixth generation dendritic PLL with 128 amine group on its surface (KG6) for the delivery of gene in CHO cells. Although the condensation of DNA in KG6 form were 4 times lower than the linear PLL, the gene expression was 100-fold higher than the native PLL polymer (Yamagata et al., 2007). In another study, Inoue et al. used KG6 as an efficient carrier for siRNA delivery. Furthermore, modification of KG6 with Endo-Porter (weak base amphiphilic peptides) (KG6/EP) resulted in an increased uptake efficiency (>90%) in comparison with HiperFect, DoFect-G1, lipofectamine 2000 and low cytotoxicity. An effective knockdown of GAPDH was observed in the transfected cells (Inoue et al., 2008).

Ma et al. developed a co-delivery agent for drug and gene delivery against cancer. The nanosystem consisted of PLL dendron with porphyrin and arginine moiety attached to the polymer for co-delivery of docetaxel and MMP-9 shRNA plasmid. The in vitro studies showed an effective transfection of MMP-9 shRNA plasmid in HNE-1 cells. The results showed a significant decrease in the expression of MMP protein when compared with the individual docetaxel and MMP (Ma et al., 2014).

Furthermore, Kamaruzaman et al. developed two different forms of delivery systems. The first included various generations of PLL dendron with six histidine attached and the second system consisted of PLL dendron with cell penetrating peptide TAT(48-60). The results showed that the higher generation PLL dendron are compatible carrier in terms of binding, condensation and uptake in comparison to lower generation and hex-L-histidine (Kamaruzaman et al., 2016).

18.3.5 CYCLODEXTRINS

CDs are naturally found cyclic oligosaccharides. Chemically, these are composed of (α -1,4) linked glucopyranose repeating units. CDs possess a basket-shaped geometry with a central hydrophobic core and an outer hydrophilic surface. These are soluble in water, owing to the presence of extensive hydroxyl group. Among many forms of CDs, α -CD, β -CD, and γ -CD are the most commonly used consisting of six, seven, and eight glucopyranose units, respectively. CDs have been extensively used in drug delivery formulation due to their structural property of having both hydrophilic and hydrophobic groups. Agrawal et al. used CDs and their analogues for the delivery of oligonucleotide in immune modulation, in both in vivo and in vitro studies (Habus et al., 1995; Zhao et al., 1996). Similarly, Hwang et al. used linear cationic β -CD for gene therapy in both in vitro and in vivo systems. The polymer was synthesized by condensation reaction of diamino CD monomer A with diamidate monomer B. The polymer was complexed with pDNA pGL3-CV. The size of the obtained complex were in the range of 124–152 nm. The study revealed that the polymer could be a promising agent for in vivo gene delivery applications (Hwang et al., 2001). Furthermore, ligand-grafted CDs were produced to develop target-specific nanovectors for the delivery of nucleic acid. In order to increase the stability of the synthesized nanoparticles in the biological fluid, PEG and adamantane were conjugated onto the surface of CDs. The obtained particles were in the range of 100–15 nm and were transfected in PC-3 (human prostate carcinoma) and K562 (chronic myelogenous leukemia) cells. It was evident from the results, that the nanoparticles remain stable and transfect efficiently at low percentage of transferrin modification, when compared to unmodified particles, whereas excess of transferrin modification decreased the transfection efficiency. This could be due to the inappropriate condensation of nucleic acid in the complex. The results demonstrated that optimum amount of transferrin modification is required for the systemic delivery of nucleic acid, for targeting metastatic cancer cell (Bellocq et al., 2003).

Dandekar et al. synthesized cationic polyrotaxanes that were formed by temperature activated threading of cationic CDs on the positively charged polymer ionenes. The synthesized polyrotaxanes efficiently encapsulated pDNA and siRNA and the obtained size of the particles were in the range of 140–220 nm. The uptake of the complex was studied in A549 cell line. The complex proved to be a promising carrier for transfection of siRNA into mammalian cell (Dandekar et al., 2012).

Furthermore, Yin et al. developed a new conjugated system for targeted delivery of luciferase reporter gene and wild type p53 in CD44-positive and CD44-negative cells. The system consisted of star-shaped cationic polymer, having core of β -CD and multiple branched oligoethylenimine (OEI) covalently attached with hyaluronic acid (HA)(β -CD-OEI-HA). The delivery system was synthesized by reductive amination process between low MW HA and cationic star-shaped polymer β -CD-OEI, followed by condensation of pDNA. The size of nanoparticles were obtained in the range of 100–200 nm. The particles were targeted to CD44 positive breast cancer cell (MDA-MB-231) and the data showed reduced cytotoxicity with efficient condensation efficiency when compared with PEI (25 kDa). The HA binds specifically to CD44, thereby avoiding the off-targeting effects (Yin et al., 2015).

18.3.6 DENDRIMERS

Dendrimers are synthetic macromolecules having multivalent branched globular structures. These were first described in early 1980s, and were illustrated as a three-dimensional (3D) structure having three main domains: (1) central core molecule that comprised of either a single or group of atoms which facilitates linking of branches; (2) layer of generation (a highly branched repeating unit arising from the core); and (3) terminal multifunctional groups that helps in complexation of nucleic acid. These dendrimers can be synthesized by two strategies: divergent (starts with the formation of dendrimers from the central core of molecule) and convergent (starts from the periphery and moves toward the interior) (Chaplot and Rupenthal, 2014). Due to their application as transfection agents, these dendrimers have been used as a gene delivery vector. Dendrimers exhibit limited toxicity and improved transfection efficiency, compared to other linear and branched polymers. They effectively condense the nucleic acid and forms dendriplexes (complex of respective dendrimer with nucleic acid). It has been observed that dendriplexes, when subjected to transfection in *in vitro* condition, produced the same level of transfection efficiency as in the case of lentivirus vectors. Under *in vivo* conditions, however, these dendriplexes shows off-targeting effects due to the interaction of these dendriplexes with the vascular compartments.

To improve the *in vivo* efficiency of these dendriplexes, various functional groups have been grafted onto the dendrimers. These functional group modified dendrimers led to the increase in biocompatibility in *in vivo* conditions in addition to the improved targeted delivery. Currently, more than 50 families of

dendrimers are present which differ in their physiochemical properties and many of them are commercially available. The most common types of dendrimers used for gene delivery are PAMAM, polypropylenimine (PPI), glucodendrimers, and carbosilane dendrimers (CBSs).

18.3.6.1 Poly(Amido Amine) Dendrimers

Poly(amido amine) dendrimers are one of the most widely studied and commercialized dendrimers for gene delivery. The structure comprises of primary and tertiary amino groups, present on the surface and inside, respectively. The surface primary amine assists in nucleic acid complexation, while the tertiary amines present inside induces endosomal release through PSE. PAMAM dendrimers are generally prepared by divergent growth strategies, and have advantages in gene delivery owing to their water solubility and lack of immunogenic properties. The presence of amide group in the backbone of PAMAM leads to hydrolytic degradation. Furthermore, the impartial degradation of higher generation of PAMAM leads to toxicity, when introduced in the cell (Zhou et al., 2006). To overcome these issues, a number of modifications have been employed that increase dendrimer degradability. In a study, Takayoki et al. synthesized CD conjugated PAMAM dendrimers for both in vivo and in vitro delivery systems. The study evaluated the comparison between the dendrimers conjugated with different forms of CDs. Three modified complexes were prepared including 6-O- α -(4-O- α -D-glucuronyl)-D-glucosyl- β -CD (GUG- β -CDE (G2)), α -CyD (α -CDE (G2)), and β -CyD (β -CDE (G2)) in conjugation with PAMAM starburst dendrimers. The size of the obtained dendrimer/ pDNA complexes were in the range of 189–201 nm. Furthermore, to analyze the in vitro gene transfection efficiency, the complexes were subjected to transfection in A549 and RAW 264.7 cells. Results demonstrated that out of three, (GUG- β -CDE (G2)) showed enhanced gene transfer activity both under in vitro and in vivo conditions, when compared with nonconjugated PAMAM dendrimers (Anno et al., 2012). Furthermore, Cheng et al. generated arginine-modified PAMAM for the delivery of interferon beta (IFN- β) gene for both in vitro and in vivo studies. The apoptotic effect was observed in the tumor cells when the formulated dendrimer was introduced into the cells. The results dictate that PAMAM-R/IFN- β could be used as a potential carrier for the treatment of malignant gliomas (Bai et al., 2013). Mastorakos et al. covalently attached the PEG group with the hydroxyl terminated PAMAM dendrimers. It has been observed that the modification provided colloidal stability to the particles, for delivery of transgene to human retinal pigment epithelial cells (Mastorakos et al., 2015).

18.3.6.2 Polypropylenimine Dendrimers

The PPIs are hardly in use, because of their low transfection efficiency and high cytotoxicity. To overcome these challenges, several modification have been done (Chaplot and Rupenthal, 2014). In this regard, Hongmei et al. used fluorinated PPI dendrimers (G3, G4, and G5). The transfection efficiency of these dendrimers

were checked against six representative transfecting agents, such as lipofectamine 2000, SuperFect, PolyFect, JetFect, branched polyethylenimine, and arginine-modified dendrimers on HEK293 and HeLa cells. Among all these transfecting agents, fluorinated PPI dendrimers showed enhanced transfection efficiency with low cytotoxicity (Liu et al., 2014).

18.3.6.3 Glycodendrimers

Dendrimers with carbohydrate moiety attached to their structure are termed glycodendrimers. These can be categorized as: (1) surface saccharides, (2) core saccharides, and (3) saccharide building blocks. These dendrimers are internalized through protein–carbohydrate interactions, which mainly occur in many intercellular recognition events. Among various forms of glycodendrimers, surface-associated saccharides are of special interest for targeted delivery (Cloninger, 2002). Arima et al. synthesized α -CD (possessing lactose with different degrees of substitution) conjugated PAMAM (G2) starburst dendrimers for the delivery of pDNA into hepatocytes. Different complexes were formed by varied degree of substitution of lactose (DSL) as 1.2, 2.4, 2.6, 4.6, 6.2, and 10.2. It was evaluated that highest transfection efficiency was found with lac- α -CDE (DSL 2.6) in HepG2 cell line when compared with other formulated dendrimer (Arima et al., 2010).

18.3.6.4 Carbosilane Dendrimers

CBSs consist of an interior carbon-silicon bond that have been recently proposed as an efficient vector for gene therapy. Initially, it was found that these dendrimers undergo fast hydrolysis in physiological conditions. It has been shown that after modification or insertion of a phenyl group, these dendrimers result in a controlled release of nucleic acid. This also provides stability and sustainability of the complex under physiological milieu (Weber et al., 2008). Perise-Barrios et al. (2014) successfully used the CBS dendrimers as siRNA delivery agents for the treatment of HIV infection. The results showed highest inhibition of HIV infection and reduced phagocytic activity on human peripheral blood mononuclear cells (Perise-Barrios et al., 2014).

18.3.7 POLYMETHACRYLATE

Polymethacrylate is a vinyl-based cationic polymer that has been used for gene delivery in both in vivo and in vitro model systems. The polymer has undergone various modifications to improve its biodegradability, gene delivery efficiency and lower its toxicity (Mintzer and Simanek, 2009). van de Wetering et al. (1997) synthesized a water soluble cationic polymer, poly [2-(dimethylamino)ethylmethacrylate] p(DMAEMA) by radical polymerization of 2-(dimethylamino) ethyl methacrylate. To demonstrate the transfection efficiency and cytotoxicity of the plasmid/polymer complex, varying MW p(DMAEMA)s were synthesized. Furthermore, the pDMAEMA complexed plasmids were subjected to transfection

in COS-7 and OVCAR-3 cell lines. Highest transfection efficiency and least toxicity was obtained by p(DMAEMA) having more than 300 kDa MW. The polymer binds efficiently with the pDNA due to the electrostatic interaction between the positively charged polymer and negatively charged DNA. This facilitates effective destabilization of endosome and its release followed by the dissociation of pDNA from p(DMAEMA)/plasmid complex. The particle size were obtained in the range of 170 and 210 nm, and showed that high MW polymer condensed the DNA effectively. The polymer/plasmid complex showed both clatherin and caveolae dependent endocytic pathway. The uptake would be feasible, even when either of these pathways was blocked with an inhibitor (van de Wetering et al., 1997).

In another study, the group synthesized p(DMAEMA) co-polymers, by the addition of hydrophilic and hydrophobic structures such as N-vinyl-pyrrolidone (NVP) and methyl methacrylate (MMA) respectively. Conversely, copolymerization of ethoxytriethylene glycol methacrylate (triEGMA) (exhibits both hydrophobic and hydrophilic moiety) with p(DMAEMA) was done. Both p(DMAEMA)-NVP and p(DMAEMA)-triEGMA formulations showed increased transfection efficiency with reduced cytotoxicity. Whereas, p(DMAEMA)-MMA co-polymer showed lower transfection efficiency compared to the other two. Furthermore, in p(DMAEMA)-NVP formulation, it was revealed that NVP segment and grafting of PEG prevents the aggregation of particles (van de Wetering et al., 1998).

Additionally, several covalent modifications have been done to deliver the therapeutic molecule to the specific cell. For tumor cell targeting, a first attempt was made by Mastrobattista et al. (2001). The F-ab fragment of mAb (antigen binding fragment of monoclonal antibody) 323/A3 was conjugated with p(DMAEMA) and subjected to transfection in OVCR-3 cells. The results showed that the transfection efficiency improved in conjugated form when compared to unconjugated p(DMAEMA) (Mastrobattista et al., 2001). Other studies include conjugation of folate, galactose, and lactose with the polymer to target-specific tumor cells. The results showed marked increase in transfection efficiency in OVCAR-3 and HepG2 cell lines (van Steenis et al., 2003; Lim et al., 2000; Wakebayashi et al., 2004).

Funhoff et al. synthesized a new polymer, polydiamine methacrylate pDAMP which resembled with p(DMAEMA). The main goal was to enhance the endosomal escape ability of the polymer. The polymer consists of two tertiary amine groups in each of the monomeric unit. One of the amine group exhibits relatively high pKa value (~ 9), which provides positive charge at physiological pH, resulting in an effective condensation of polymer with the nucleic acid. Conversely, second amine has lower pKa value (~ 5), which attributes to an increased endosomal buffering capacity. The nanoparticles were obtained in the size range of 94–105 nm and showed reduction in toxicity (Funhoff et al., 2004a). Moreover in 2004, they prepared guanidinium modified polymethacrylate (pGuaMA) nanoparticles. The nanoparticles were obtained in the range of 110 nm. The results

showed an improved transfection efficiency in COS-7 cells, under serum free condition. The highly basic nature of guanidinium gets fully protonated at physiological pH and is able to condense negatively charged DNA effectively. Aggregation of nanoparticles and protein adsorption on their surface were also observed in the presence of serum (Funhoff et al., 2004b).

Furthermore, in order to achieve higher transfection efficiency, Cristiaens et al. conjugated penetratin, a 16-residue water soluble peptide, with polymethacrylate. The penetratin conjugated polymer facilitated in cell internalization through membrane translocation. The modification did not affect the size of nanocarriers and was in the range of 100–120 nm. The transfection efficiency of the system for gene delivery was analyzed in COS-1 cells. The results showed higher transfection efficiency of penetratin conjugated polymer than PEI-DNA complex and lipofectamine transfection agent (Christiaens et al., 2005). To enhance the biodegradability of the polymer, it was conjugated with hydrolysable cationic side chain. pHPMA-DMAE nanoparticles were synthesized as a carbonate functionality of methacrylate. Furthermore, different variants were prepared (pHPMA-DEAE, pHPMA-MPPM, and pHPMA-BDMPAP), possessing cationic side group. It was observed that the transfection efficiency and cytotoxicity of these polymeric nanocarriers were superior to that of 25 kDa PEI (Luten et al., 2006). In addition, PLGA co-polymerized polymethacrylate nanoparticles were synthesized for the delivery of pDNA encoding mouse IL-10, both in in vitro and in vivo. The results showed an efficient and safer delivery of interleukin-10, thereby preventing the generation of autoimmune diabetes (Basarkar and Singh, 2009).

18.4 CLINICAL TRIALS OF POLYMERIC NANOCARRIERS FOR GENE THERAPY

Polymeric nanocarriers for gene therapy have attracted significant attention in the treatment of various diseases such as hypercholesterolemia, hemophilia, and various neurodegenerative diseases (O'Connor and Boulis, 2015). Some of the polymeric nanoparticles have shown significant effects in clinical trials of gene therapy. Despite this, there are still many challenges and limitations to overcome for gene therapy in terms of safety, efficiency and controllable release. Since 1969, PLGA has been the only FDA-approved biodegradable polymer that has been explored as delivery vector for in vivo system (Blum and Saltzman, 2008; Gentile et al., 2014).

The first successful in-human clinical trial was conducted in January 1990 by Anderson et al., on a four year girl against SCID by employing ex-vivo gene therapy (Massadeh and Alaamery; Rubanyi, 2001; Nagarwal et al., 2010). This aroused great attention toward worldwide research on gene therapy. It made the use of retroviral vector system that proved to be a major success. Later in 2000, Fischer and colleagues used this therapy, and it was noted that the two recipient

boys showed the symptoms of leukemia after 3 years of gene therapy. Furthermore, it was attributed to retrovirus gene integration into the proto-oncogene due to the insertional mutation (Hacein-Bey-Abina *et al.*, 2003). Therefore, nanoparticles were investigated to overcome the barriers associated with safety, immunogenicity, cost and controlled release.

Phase-IIA and Phase-IIB have been conducted for PEI-BC-819 complex. The complex consisted of H-19 promoter pDNA with diphtheria toxin-A-chain gene and PEI as transfectant. The safety and efficacy of these complexes were studied toward the treatment of cancer. The PEI facilitated the effective uptake of the complex by the cell. This study was carried out on a group of nine patients with two different doses, i.e., 4 and 8 mg. In the case of 4 mg, it was given to the three participants; out of three, one patient showed stability in pancreatic cancer. Conversely, 8 mg dose was given to six patients, out of whom four showed stability in disease. Few adverse side effects were observed with this therapy (Sidi *et al.*, 2008; Hanna *et al.*, 2012). Another study with PEI-based nanoparticles system (SNSO01-T) was carried out. The system was developed against B-cells cancer for their apoptosis by small interfering RNA. The study was conducted under Phase I and Phase II clinical trials, and its still under process for establishing efficient delivery system (Francis *et al.*, 2014). Another PEI-based study for genetic and metabolic cardiovascular disease was done, using eNOS-pVAX plasmid with L-PEI complex. For the treatment of pancreatic ductal adenocarcinoma, a PLGA based therapeutic drug siG12D LODER was also developed. The complex conferred silencing of KRASG12D under phase 0/I clinical trials (Taljaard *et al.*, 2010).

Davis and colleagues (2009), reported, CALAA-0 as a nanoparticle-based delivery system in Phase-I clinical trial (Davis, 2009). The delivery system included transferrin as a targeting ligand, PEG, as steric stabilizer, cyclodextran polymer, and RRM2 siRNA for targeting M2subunit of ribonucleotide reductase. In the clinical phase trial, the delivery system showed systemic administration of siRNA in the melanoma cells and resulted in the potent antiproliferative activity among multiple types of cancer (Davis *et al.*, 2010). In addition, PEI has already been explored for clinical trials in the treatment of various types of cancer, but its application is limited because of its cytotoxicity issues. Furthermore, modifications with PEG and cholesterol have demonstrated increased expression of cytokine interleukin-12 for immunotherapy against epithelial ovarian cancer (Alvarez *et al.*, 2014).

Nowadays, researchers are paying attention toward the combinational approach of drug, radiotherapy, photodynamic therapy, and immunotherapy along with the gene therapy approach. GEN-1 (IL-12 plasmid) was formulated with PEG-PEI and cholesterol lipo-polymer under Phase-I clinical trial to analyze its biological activity and safety concern. It was administered intraperitoneally along with the standard nonadjuvant chemotherapy. This was done for the diagnosis of epithelial ovarian, fallopian tube and primary peritoneal cancer (Chen *et al.*, 2016).

18.5 FUTURE PERSPECTIVES OF GENE THERAPY

The application of nanotechnology in gene therapy has proven to be a promising technique in the diagnosis, detection, and treatment of various diseases. This revolutionary technology has overcome the challenges faced during target-specific delivery. Polymeric nanocarriers have emerged as an ideal carrier system by achieving low immunogenicity, selective targeting, better biocompatibility, surpassing physiological barriers, and controlled delivery of the therapeutic molecule in *in vitro* systems. Nonetheless, translation of these nanoparticle-based therapies in *in vivo* systems are still limited. Standard measurements on the usage of nanomedicine should also be designed to address the manufacturing processes, favorable pharmacokinetics, toxicity, and safety profiles. Moreover, considerable improvements in these polymeric nanocarrier systems are continuously made to improve the clinical use in terms of efficiency and specificity. Accordingly, nanoparticle-based gene therapy is believed to be a potent delivery system for the treatment of diseases. In future, this could be used as a therapeutic approach after going through different stages of clinical trials prior to its delivery to the clinic.

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Cyclodextrin-based polymeric nanosystems

19

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CHAPTER OUTLINE

19.1 Introduction	715
19.2 Cyclodextrins	716
19.2.1 Natural Cyclodextrins and Cyclodextrin Derivatives	717
19.2.2 Amphiphilic Cyclodextrins	721
19.3 Amphiphilic Cyclodextrin Nanoparticles	724
19.4 Polymeric Nanoparticles Incorporating Cyclodextrins	730
19.5 Cyclodextrin-Based Polymeric Nanomedicines	733
19.6 Polymer-Drug Conjugates	734
19.7 Supramolecular Hydrogels	736
19.8 Polymer-Modified Drug Carriers	737
19.9 Conclusion	739
References	740

19.1 INTRODUCTION

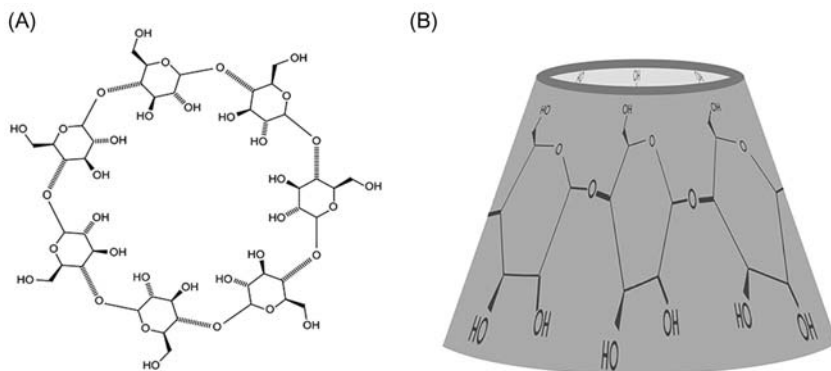
Nanomedicine is defined by the National Institute of Health as a clinical application to nanotechnology, which refers to highly specific medical intervention at the nano-scale for screening, diagnosis, and treatment of biological systems (Moghimini et al., 2005; Fulop et al., 2012; Riehemann et al., 2009). In the case of drug delivery, the main goals of nanomedicines are to prolong release, improve drug bioavailability, target drugs to specific tissues while minimizing adverse effects (Moya-Ortega et al., 2012; Cavalli et al., 2006; Messner et al., 2010). Nanoparticulate carrier systems have been developed to achieve these objectives in drug delivery (Alexis et al., 2010). Cyclodextrins (CDs) are attractive substances that are used as excipients for the design of a variety of drug delivery systems, such as nanoparticles, liposomes, dendrimers, nanosponges, hydrogels, and CD-containing polymers. This is due to their favorable toxicological profile, their potential to form spontaneous nanosized aggregates and their capability to form inclusion complexes with mostly apolar active molecules (Irie and Uekama, 1997;

Loftsson and Duchene, 2007). CDs are macrocyclic oligosaccharides composed of α (1,4)-linked glucopyranose subunits. They are torus-like shaped, having a hydrophobic inner cavity and hydrophilic surface. CDs allow the encapsulation of poorly soluble anticancer drugs in their hydrophobic inner cavity with host–guest complexation and mask the physicochemical properties of the guest molecules. Otherwise, hydrophilic surface of the CD ensures enhanced water solubility for the guest molecule. In addition, CDs and their complexes usually form spherical aggregates with diameters between 50 and 200 nm. In general, however, these self-assembled aggregates are not stable enough to be used as drug delivery systems (Messner et al., 2010). Polymeric systems based on CDs show unique characteristics in the form of mechanical properties, stimuli-responsiveness, and drug release characteristics. Due to their physical structure, these systems are sensitive to external stimuli, such as temperature changes, shear forces, and the presence of competing CD-binding molecules, which can further be used as injectable, in situ gelling devices (van de Manakker et al., 2009). Recently, many different CD-based polymeric nanosystems have been studied in literature. These systems must be evaluated and optimized in preclinical studies in view of their safety and efficacy, making feasible clinical applications of these materials in the pharmaceutical area. Synthesis, properties and pharmaceutical applications of CD based polymeric nanosystems that are formed via inclusion complexes between CD polymers and guest molecules will be described in this chapter.

19.2 CYCLODEXTRINS

CDs, also known as cycloamyloses, cyclomaltoses, or Schardinger dextrins, are a family of macrocyclic oligosaccharides produced by the enzymatic degradation of starch containing (α -1,4)-linked α -D-glucopyranose units (van de Manakker et al., 2009; Zafar et al., 2014). These functional molecules were extensively used in different scientific and technological fields since their discovery in 1891 by French scientist Villiers who isolated a small amount of crystalline substance called “cellulosine” from a culture medium of *Bacillus amylobacter* containing starch (Villiers, 1891). In 1911, Schardinger found a new organism called *Bacillus macerans* that could also produce α and β crystalline dextrins. Later, many CDs were discovered and chemical structures of the CD subtypes were explained. Native CDs were appropriate for pharmaceutical area, they came into use as excipients after development of new CD-based technologies. Currently, more than 30 different pharmaceutical products containing CDs are now in the market worldwide and several CD derivatives with generally recognized as safe (GRAS) status are being produced in large-scale (Loftsson and Duchene, 2007).

The three-dimensional (3D) structure of CDs showed truncated cone shape or torus instead of a perfect cylinder due to the chair formation of the glucopyranose units, as seen in Fig. 19.1. The primary $-\text{OH}$ groups are located at the narrow

**FIGURE 19.1**

(A) Chemical and (B) 3D structure of cyclodextrins.

edge and secondary $-\text{OH}$ groups are extended from the wider edge of CD outer surface (Saenger et al., 1998). This hydrophilic outer surface makes CDs soluble in water due to the formation of hydrogen bonds. The hydrophobic inner cavity gives them the ability to form inclusion complexes with lipophilic small molecules, ions, proteins, and oligonucleotides through host–guest interactions in pharmaceutical science and technologies (van de Manakker et al., 2009; Saenger et al., 1998; Rekharsky and Inoue, 1998; Fuchs et al., 1993; Irie and Uekama, 1999; Lysik and Wu-Pong, 2003). The biomedical applications of CDs are remarkable due to their low toxicity, low immunogenicity, cost-efficient, and easy accessibility (Irie and Uekama, 1997). In addition, pharmacy, analytical sciences, cosmetic, textile, and food industry are the other areas that CDs are frequently used (van de Manakker et al., 2009).

19.2.1 NATURAL CYCLODEXTRINS AND CYCLODEXTRIN DERIVATIVES

Owing to steric factors, CDs having less than six glucose units that are not available. The most commonly used CDs are α -CD, β -CD, and γ -CD (Fig. 19.1), which are composed of 6, 7, and 8 glucose units, respectively. Although larger CDs have been reported, they have limited use in pharmaceutical industry (Ueda, 2002; Larsen, 2002). Natural CDs can be easily purified due to their different aqueous solubilities. β -CD can be removed by crystallization as it has very low water solubility. The other two forms are purified by chromatography or by addition of complexing agents such as toluene and ethanol. β -CD has a lower solubility than α -CD, even though it contains greater number of hydroxyl groups. This is due to the fact that an internal hydrogen bond network is formed between the secondary hydroxyl groups (Zafar et al., 2014).

Table 19.1 Physicochemical Properties of Natural Cyclodextrins; α -Cyclodextrin, β -Cyclodextrin and γ -Cyclodextrin

Property	α -CD	β -CD	γ -CD
Molecular weight of anhydrous compound (Da)	973	1135	1297
Number of glucopyranose units	6	7	8
<i>Number of water molecules present in the stable hydrates of the CD lattices</i>			
Total number	6.4	9.6	14.2
Inside the cavity	2	6	8.8
<i>Approximate dimensions (nm)</i>			
Height (H)	0.78	0.78	0.78
Inner diameter (d_i)	0.50	0.62	0.80
Outer diameter (d_o)	1.46	1.54	1.75
Solubility in water at 25 °C (mg/ML)	129.5 $\pm$ 0.7	18.4 $\pm$ 0.2	249.2 $\pm$ 0.2
$K_{1:1}$ (population mean $\pm$ SD, 25 °C)	130 $\pm$ 8	490 $\pm$ 8	350 $\pm$ 9

Table 19.2 Comparison of Natural Cyclodextrins

Cyclodextrin Type	α -CD	β -CD	γ -CD
Irritation degree	Significant im injection-eyes	Insignificant im injection	Insignificant im injection
Absorption	2%–3%	1%–2%	0.1%
Metabolism	No metabolism in the upper intestinal tract; cleavage only by the intestinal flora of cecum and colon	No metabolism in the upper intestinal tract	Practically no metabolism after intravenous administration
LD ₅₀ oral	Rat > 10,000 mg/kg	Rat > 5000 mg/kg	Rat 8000 mg/kg
LD ₅₀ iv	500–750 mg/kg	450–790 mg/kg	4000 mg/kg

The natural CDs have similar structures that are homogeneous, crystalline, and nonhygroscopic in nature. However, contrary to their physicochemical similarity, they cause different effects in the biological system. The differences in natural CD properties such as irritation degree after intramuscular (im) administration, metabolism, and median lethal dose (LD₅₀) values are shown in Table 19.2. β -CD is the most commonly used CD in pharmaceutical formulations and thus probably the best studied CD in humans. γ -CD has been used as food additive by its main manufacturers. With complexing abilities generally less than those of β -CD and the water soluble β -CD derivatives, its complexes frequently have limited solubility in aqueous solutions and tend to aggregate in aqueous solutions (Del Valle, 2004) (Table 19.1).

Although natural CDs can increase the drug concentration in solution, their aqueous solubility is often limited, especially that of β -CD, due to relatively

strong binding of CD molecules in the crystal state and hydrogen bond between hydroxyl groups, preventing their hydrogen bond formation with surrounding water molecules (Fulop et al., 2012; Coleman et al., 1992; Loftsson and Brewster, 1996). To overcome this problem, a large number of modified CD derivatives have been synthesized. Any substitution of the hydroxyl groups, even by hydrophobic moieties such as methoxy functions, will provide significant improvements in aqueous solubility due to amorphous, noncrystallizable structure of CD derivatives.

Chemically modified CDs have been prepared to increase inclusion capacity and improve the physicochemical properties of native CDs. CD derivatives have also been used for improvement in bioavailability and increase aqueous solubility, physical and microbiological stability, and biological activity of active ingredients. They prevent drug–drug and drug–excipient interactions, reduce ocular or gastrointestinal irritation and parenteral toxicity, and reduce unpleasant taste and smell (Laza-Knoerr et al., 2010). The most widely used CD derivatives in pharmaceutical applications include; hydroxypropyl derivative of β -CD (HP- β -CD) and hydroxypropyl derivative γ -CD randomly methylated β -CD, sulfobutylether β -CD sodium salt (SBE- β -CD) and so called branched CDs such as maltosyl- β -CD (G2- β -CD) (Loftsson and Brewster, 1996; Vyas et al., 2008) (Table 19.3).

Hydrophilic CDs are commonly applied to enhance the solubility and dissolution rate of drugs with poor aqueous solubility as a result of inclusion complexation in oral drug delivery (Sharma and Baldi, 2016). For example, new oral supramolecular systems of furosemide (FUR) polymorphs I and II have been developed with β -CD to enhance solubility and permeability for oral administration. FUR I: β CD and FUR II: β CD solid-state systems were prepared containing FUR polymorphs I and II with the CD derivative β -CD. Phase-solubility studies indicated that drug-CD complexes showed a higher solubility enhancement of FUR in physiological simulated fluids resulting in improved drug absorption (Garnero et al., 2014). In another study, econazole nitrate (EN) interaction with different CD derivatives was investigated in terms of solubilizing effect. SBE- β -CD was found as the optimum complexing and solubilizing agent for EN among HP- β -CD, hydroxyethyl- β CD (HE- β -CD), SBE- β -CD. The EN/SBE β CD complex increases the EN dissolution efficiency approximately 60 times, clearly having the best potential for development of a novel EN delivery system for efficient delivery of the drug to the oral cavity, thus improving the therapy of oral candidiasis (Jug et al., 2014).

Conversely, among the various CDs, HP- β -CD, and SBE- β -CD are mostly used in parenteral delivery due to their high aqueous solubility and minimum toxicity. The advantage of using CDs in parenteral formulation includes solubilization of drug, reduction of drug irritation at the site of administration and stabilization of drugs in aqueous environment (Shimpi et al., 2005). Goodchild et al. studied the effect of SBE- β -CD as a drug-solubilizing excipient and found that SBE- β -CD increased the safety and therapeutic efficacy of alphaxalone compared with commercial product containing 20% nonionic surfactant Cremophor EL in animal studies (Goodchild et al., 2015).

Table 19.3 Derivatives of Cyclodextrins

Derivative	Characteristics	Route of Administration
Hydrophilic Derivatives		
<i>Methylated CDs</i>		
M- β -CD DM- β -CD TM- β -CD DMA- β -CD	Soluble in cold water and in inorganic solvents Surface active, hemolytic Soluble in water, low hemolytic	Oral, ocular, nasal
<i>Hydroxyalkylated β-CD</i>		
2-HE- β -CD 2-HP- β -CD 3-HP- β -CD	Amorph mixture with different derivatives (Encapsin) Highly water soluble (> 50%) Low toxicity	Oral, ocular, rectal, intravenous
<i>Branched β-CD</i>		
G1- β -CD G2- β -CD	Highly water soluble (> 50%) Low toxicity	Oral, parenteral, mucosal
Hydrophobic Derivatives		
<i>Alkylated β-CD</i>		
DE- β -CD TE- β -CD	Water insoluble, soluble in organic solvents, surface active	Oral, subcutaneous
<i>Acylation β-CD</i>		
TA- β -CD TB- β -CD TV- β -CD	Water insoluble, soluble in organic solvents, surface active Mucoadhesive Film formation	Oral, parenteral
Ionizable Derivatives		
<i>Anionic β-CDs</i>		
CME- β -CD β -CD sulfate SBE4- β -CD SBE7- β -CD Al- β -CD sulfate	pK_a 3-4, soluble at pH > 4 pK_a > 1, water-soluble Water soluble Water soluble (Captisol) Water-insoluble	Oral, ocular, parenteral, dermal, pulmonary

Adapted from Erdögar, N., Bilensoy, E., 2014. Cyclodextrins in drug delivery. In: Arias, J. (Ed.), Nanotechnology and Drug Delivery, Volume One: Nanoplatfroms in Drug Delivery. CRC Press.

Recent studies have proved CD derivatives as useful excipients in ophthalmic formulations as they increase the solubility, stability, and bioavailability of ophthalmic drugs, and decrease the drug irritation (Loftsson and Stefansson, 2002). Hydrophilic CDs, especially 2-HP- β -CD and SBE- β -CD, have been reported to be nontoxic to the eye and also are well tolerated in aqueous eye drop formulations. Hydrophilic CDs also increase the ocular bioavailability of lipophilic drugs by providing stable drug molecules in solution and increasing their availability at the surface of the corneal barrier (Sharma and Baldi, 2016). Recent studies demonstrated improvement of the solubility and permeability of many drugs such as riboflavin or cyclosporine A via ophthalmic administration by using α -CD, β -CD, and HP- β -CD (Morrison et al., 2013; Pecchio et al., 2014).

In the case of rectal administration, CD derivatives have seen useful excipients for stabilization, improve drug release and bioavailability and diminish local irritation. CD derivatives improve the rectal absorption of drugs with low aqueous solubility. HP- β -CD with 5-Fluorouracil (5-FU) were reported to increase the solubility, prolong release, and improve local therapeutic efficacy of drug in colon cancer treatment (Wang et al., 2016).

Due to the efficacy and safety of CDs, hydrophobic CD derivatives have proven to be solubilizers and permeation enhancers in nasal drug delivery by increasing solubility of poor-soluble drugs and enhancing nasal absorption (Merkus et al., 1999). Safety levels of the main CD derivatives have been summarized in the recent European Medicines Agency (EMA) report on CDs for different administration routes in adults and children.

19.2.2 AMPHIPHILIC CYCLODEXTRINS

Amphiphilic CDs have been developed to overcome drawbacks of native CDs that limit their pharmaceutical applications. The main objectives for the synthesis of amphiphilic CDs are listed as follows:

- increased CD interaction with biological membranes via external lipophilic structure
- improvement of interaction of CDs with lipophilic drugs
- self-organization of CDs to form nanoparticles spontaneously (Bilensoy and Hincal, 2009).

Amphiphilic CDs can be synthesized by the modification of lipophilic groups at primary and/or secondary face of the CD that gives the amphiphilic character (Sallas and Darcy, 2008). Amphiphilic CD derivatives can form micelles and monolayers at the air–water interface (Parrotlopes et al., 1992). Different self-assembling structures based on amphiphilic CDs with different length of hydrophobic chains, such as nanospheres, solid-lipid nanoparticles, liquid crystals, and vesicles were prepared for their promising properties for pharmaceutical

applications. Amphiphilic CDs are generally classified according to their surface charge as follows and will be discussed accordingly in this chapter:

1. nonionic amphiphilic CDs
2. cationic amphiphilic CDs
3. anionic amphiphilic CDs.

19.2.2.1 Nonionic amphiphilic cyclodextrins

Nonionic amphiphilic CDs are obtained by grafting aliphatic chains of different length onto the primary and/or secondary face of the CD main glucopyranose unit. Different derivatives, explained in Fig. 19.2 (Bilensoy and Hincal, 2009), are named after their structure in the literature.

1. Lollipop CDs are obtained by the grafting a single alkyl chain to 6-amino- β -CD. This structure is poorly soluble so the alkyl chain tends to loop back and enter the CD hydrophobic cavity, leading to intramolecular self-inclusion;
2. Cup and ball CDs are obtained by adding amino protective group at the end of the alkyl chain in order to prevent self-inclusion of the pendant group;
3. Medusa-like CDs are obtained by grafting amino-, amido-, sulfo-, or thio-alkyl chains with length between C10 and C16 to all the primary hydroxyls of the CD molecule;
4. Skirt-shaped CDs consist of α -, β -, and γ -CDs per-modified with alkyl chains through aliphatic ester link (C2 to C14) on the secondary alcohol groups;
5. Bouquet-like CDs result from the grafting one poly(oxyethylene) or polymethylene as well as *O*-alkyl chain on each side of the CD ring molecule. Per(2,6-di-*O*-alkyl) CDs, where the alkyl chain may be propyl, butyl, pentyl, 3-methylbutyl or dodecyl, also take part in the bouquet family. This derivative was designed to mimic transmembrane ion channels after insertion in lipid membranes (Erdoğan and Bilensoy, 2014).

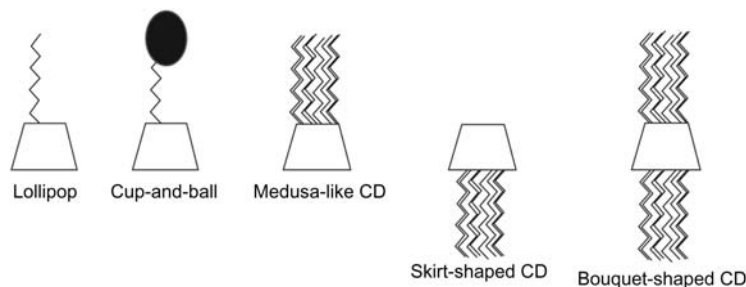


FIGURE 19.2

Different nonionic amphiphilic cyclodextrins reported in the literature (Sallas and Darcy, 2008).

19.2.2.2 Cationic amphiphilic cyclodextrins

Cationic amphiphilic CDs are obtained by the substitution of ω -amino groups to oligo(ethylene oxide) including CDs. They can also be synthesized by modifying amido groups on the primary face of CDs with alkyl chains at positions 2 and 3, thereby per-6-amino- β -CD 2,3-di-*O*-alkyl ethers were prepared (Parrotlopes et al., 1992; Donohue et al., 2002). Structural properties of cationic amphiphilic CDs were derived from the balance between thioalkyl chains and hydrophilic ethylene glycol oligomers. Ethylene glycol chains increased the colloidal stability of nanoaggregates of cationic amphiphilic CDs (Bilensoy and Hincal, 2009).

Recent studies have been carried out on their potential for gene delivery studies (Ortiz Mellet et al., 2010). For example, heptacationic amphiphilic derivatives (HexCD-N and MyrCD-N) were synthesized by replacing the primary hydroxyl groups with different amino-containing moieties (Diaz-Moscoso et al., 2009). In another study, per-azido components were reduced to the per-amino amphiphiles, used as their hydrochloride salts in gene delivery studies (Cryan et al., 2004). Heptakis (2- ω -amino-*O*-oligo-(ethyleneoxide)-6-hexylthio)- β -CD, which carry the oligoethylene glycol group that gives “stealth” property have been used for intracellular drug delivery (Mazzaglia et al., 2003). Cationic amphiphilic CDs have shown a greater ability to bind nucleotides and increase delivery by vectors. The superior properties of polycationic CDs and their nanoparticles is an ability to interact with nucleic acids combined with their self-assembly properties (Cryan et al., 2004).

19.2.2.3 Anionic amphiphilic cyclodextrins

Anionic amphiphilic CDs can be synthesized by attaching a sulfate or carboxyl group that renders anionic property to their structure on the primary/secondary face (Zhang and Ma, 2013). Carboxyl containing CD amphiphiles were prepared by grafting alkyl at the position 6 and carboxymethyl groups at positions 2 and 3 (Kraus et al., 2001). Sulfate-containing amphiphilic CDs can be synthesized through sulfating the 6-hydroxyls of esterified CDs at positions 2 and 3 (Dubes et al., 2003; Sukegawa et al., 2002). These derivatives are able to form supramolecular aggregates in aqueous media (Dubes et al., 2001).

Fluorine-containing anionic β -CDs that are functionalized at the 6-position by trifluoromethylthio groups have also been reported. They show amphiphilic behavior at the air–water interface and are believed to be good candidates for a new class of amphiphilic carriers. The synthesis of new amphiphilic perfluorohexyl- and perfluorooctyl-thio- β -CDs and their alkyl analog, nonanethio- β -CD, has been described in the literature. These derivatives may also be considered to belong to the nonionic class; however, owing to their high negative charge, they have been included in the anionic amphiphilic CDs (Granger et al., 2000).

19.3 AMPHIPHILIC CYCLODEXTRIN NANOPARTICLES

CD nanoparticles are generally prepared to increase the solubility of drugs in aqueous media. However, their external hydrophilicity makes it difficult to contact with cell membrane. To eliminate this problem, nanoparticles which are prepared from amphiphilic CDs are frequently studied in the literature. Regardless, amphiphilic CDs are able to provide a second site for encapsulation of hydrophobic drugs with their long aliphatic chains, unlike other CD derivatives. Another important advantage of amphiphilic CDs is allowing self-assembly of nanoparticles without requiring the use of surfactants or co-solvents, thanks to their molecular structure. Amphiphilic CDs can be in many different forms, such as medusa-like, skirt-shaped, cup-and-ball, lollipop, bouquet-shaped, or cholesteryl as described in the previous section. By using these varieties of amphiphilic CDs, it can be possible to prepare various nanospheres or nanocapsules for drug or gene delivery. There are several methods for preparing amphiphilic CD nanoparticles; however, nanoprecipitation is generally preferred through these methods in literature because it is simple and easy (Bilensoy and Hincal, 2009; Duchene et al., 1999).

Amphiphilic CD nanoparticles have been studied for many years as drug delivery systems, especially through the last 15 years, and the number of studies on this subject are reported to increase each year. Some selected studies about amphiphilic CD nanoparticles are summarized in Table 19.4 (Bilensoy and Hincal, 2009).

Physical stability of colloidal system is an important challenge for pharmaceutical formulations. Amphiphilic CD nanoparticles emerge as promising carriers to solve this problem. Geze et al. prepared amphiphilic β -CDs nanospheres with different particle size varying from 60 to 100 nm by using nanoprecipitation method. Size distribution and morphology of nanoparticle formulations were controlled over 3 years by using dynamic light scattering and cryo-transmission electron microscopy. During that storage time, no significant change in particle size and morphology of nanoparticle suspension were reported. This result demonstrated that an amphiphilic CD is a good candidate to prepare long-term stable pharmaceutical formulations (Geze et al., 2004). In another study of this group, methods to obtain different sizes of nanoparticles by using amphiphilic CD were extensively studied. They developed experimental design methodology and succeeded to prepare amphiphilic β -CD nanoparticles ranging in size from 60 to 400 nm by solvent displacement technique (Choisnard et al., 2005). This study showed that nanoparticles with desired size can be prepared by using amphiphilic CD for passive targeting of tissues or organs.

An amphiphilic CD is suitable to obtain both nanospheres and nanocapsules. Amphiphilic β -CD modified with 6C aliphatic esters on the secondary face was used to obtain tamoxifen citrate-loaded nanocapsules and nanospheres of size varying from 250 to 300 nm in our laboratory. Cell culture studies showed that,

Table 19.4 Selected Literature Examples About Amphiphilic Cyclodextrin Nanoparticles in Pharmaceutical Studies

Main CD	Primary Face	Secondary Face	Active Agent	Structure	References
Amphiphilic α -CD	Perfluoroalkyl propanethiol chains	Permethylated	Acyclovir	100 nm nanosphere	Ghera et al. (2009)
Amphiphilic β -CDs		Hexanoic chains		60–400 nm nanoparticle	Choisnard et al. (2005)
Amphiphilic β -CDs		6C aliphatic esters	Tamoxifen citrate	250–300 nm nanosphere and nanocapsule	Memisoglu-Bilensoy et al. (2005), Memisoglu-Bilensoy and Hincal (2006)
Amphiphilic β -CDs		6C aliphatic esters	Hydrocortisone, testosterone and progesterone	Nanosphere and nanocapsule	Memisoglu-Bilensoy et al. (2006)
Amphiphilic β -CDs		6C aliphatic esters	Paclitaxel Camptothecin	Nanosphere and nanocapsule	Bilensoy et al. (2008a, 2008b), Cirpanli et al. (2009, 2011)
Amphiphilic β -CD	Iodinated	Acyl group		90–110 nm nanospheres	Geze et al. (2007)
Amphiphilic β -CDs	Alkylthio chains	Galactosylthio-oligo-(ethylene glycol)		Vesicles, nanoparticle	Mazzaglia et al. (2004)
Amphiphilic β -CDs	Sulfate groups	Acyl group		29–160 nm nanosphere	Abdelwahed et al. (2008)
Amphiphilic β -CDs	Alkylthio chains, oligo(ethylene glycol)	Alkylthio chains, oligo(ethylene glycol)	Docetaxel	95 nm nanoparticle	Quaglia et al. (2009)
Amphiphilic β -CDs	Butyrate		Lutein	250 nm nanoaggregate	Stancanelli et al. (2012)
Amphiphilic β -CDs	Alkyl, perfluoroalkyl	Per-methylated	Acyclovir	120–220 nm nanoparticle	Perret et al. (2013)

(Continued)

Table 19.4 Selected Literature Examples About Amphiphilic Cyclodextrin Nanoparticles in Pharmaceutical Studies
Continued

Main CD	Primary Face	Secondary Face	Active Agent	Structure	References
Amphiphilic β -CDs	Alkyl chains	Alkyl chains	Paclitaxel	70–275 nm nanoparticle	Erdoglar et al. (2016)
Amphiphilic β -CDs	Hexanoyl chains, ammonium groups	Hexanoyl chains		75–367 nm nanoparticle	Varan et al. (2016)
Amphiphilic β -CDs		6C aliphatic esters	Camptothecin	154–347 nm nanocapsule	Unal et al. (2015a,b)
Cationic Amphiphilic β -CD	Alkylthio chains	w-Amino-oligo (ethylene glycol)	Anionic porphyrins	100–1000 nm colloidal nanoparticle	Sortino et al. (2006)

tamoxifen-loaded nanospheres and nanocapsules have cytotoxic effect against MCF-7 human breast cancer cells and yielded controlled release of tamoxifen up to 6 h (Memisoglu-Bilensoy et al., 2005). In addition, these nanoparticles are also suitable carriers of steroid drugs such as hydrocortisone, testosterone, and progesterone (Memisoglu-Bilensoy et al., 2006a); and anticancer drugs such as paclitaxel (Bilensoy et al., 2008a,b) and camptothecin (Cirpanli et al., 2009, 2011). The cytotoxic effects of the surfactant that is used in the preparation of the nanoparticle have also been investigated. In this study, cytotoxicity of blank nanoparticles prepared from same amphiphilic β -CD was evaluated with cell culture studies, showing that amphiphilic β -CD nanoparticles with increasing surfactant concentration increased cytotoxicity on L929 mouse fibroblast cells and PMN human polymorphonuclear cells. Amphiphilic CDs allow the self-assembly of their supramolecular aggregates into nanoparticles without using any surfactant. Results demonstrated that surfactant-induced toxicity can be prevented and stable nanoparticles can be formed by using amphiphilic CDs without the use of any surface active agent (Memisoglu-Bilensoy et al., 2006b).

Sterilization of the injectable pharmaceutical formulation is another critical issue. Our studies showed that, amphiphilic CD nanospheres and nanocapsules can be sterilized by using gamma irradiation without affecting the particle size, drug loading, or release properties. Gamma irradiation affected the zeta potential of nanoparticles due to chemical changes. However, it was reported that there were no significant changes on nanoparticle properties. Accordingly, it can be said that sterilized injectable drug formulations can be produced by using amphiphilic- β -CD nanoparticles by gamma irradiation as well as autoclaving or membrane filtration, the latter two having a negative effect on nanoparticle yield and final properties (Memisoglu-Bilensoy and Hincal, 2006). For the purpose of active targeting, folate conjugated amphiphilic β -CD nanoparticles were developed for delivery of paclitaxel by Erdoglar et al. Cell culture studies showed that blank CD nanoparticles have no cytotoxic effects on L929 cells. Conversely, paclitaxel-loaded nanoparticles showed significant anticancer effects due to good interaction with the folate receptors on breast cancer cell. Results demonstrated that folate conjugated amphiphilic CD nanoparticles are safe, stable and effective drug carrier systems for paclitaxel (Erdoglar et al., 2016). Amphiphilic β -CD nanoparticles of cationic charge were recently reported to be targeted to cholesterol domains of cancer cell membranes which are significantly higher for cancer cells, when compared to healthy cells suggesting selectivity of the nanoparticles. Varan et al. prepared cholesterol-targeted anionic β -CD (6-OCAPRO- β -CD) and polycationic β -CD (PC β -CDC6) nanoparticles by using nanoprecipitation technique for the treatment of breast cancer. They investigated cytotoxic and apoptotic effects of nanoparticles against different cancer (such as MCF-7, HeLa, HepG2, and MB49) and healthy cells (such as L929 or G/G). Results showed that blank nanoparticles have no cytotoxic effect on healthy mouse fibroblast cells (L929). Interestingly, these blank nanoparticles have cytotoxic effect on cancer cell lines (MCF-7, HeLa, HepG2, and MB49). Cell culture results and TUNEL assay showed that

both blank CD nanoparticles have apoptotic effect on MCF-7 cancer cells. It was reported that anionic and polycationic amphiphilic CD nanoparticles effect membrane integrity by removing cholesterol from cell membrane. Blank amphiphilic CDs have the ability to form the inclusion complex with cell membrane lipids, especially cholesterol. This effect caused cell membrane disruption of cancer cells and resulted in apoptosis through the mitochondrial pathway. In addition, according to the findings of gene expression studies, the BAX/BCL-2 ratio was increased in human breast cancer cells especially, when treated with polycationic amphiphilic CD nanoparticles (Varan et al., 2016).

Quaglia et al. researched the intracellular effects of amphiphilic CD nanoparticles for the delivery of anticancer drug docetaxel. For this purpose, they prepared docetaxel-loaded nanoparticles with a size of 95 nm by using heptakis (2-Oligo (ethyleneoxide)-6-hexadecylthio-) β -CD. Entrapment efficiency of docetaxel was found as 98% and release of entrapped drug continued for about 8 weeks, thanks to crystalline nature of this amphiphilic CD derivatives. The most important feature of this nanoparticle formulation was the absence of hemolytic activity on red blood cells, in contrast to a commercial docetaxel formulation (Taxotere) (Quaglia et al., 2009).

Amphiphilic CD nanoparticles can be also used for photodynamic therapy. Sortino et al. prepared anionic porphyrin encapsulated amphiphilic β -CD for the photodynamic therapy of cancer. Photodynamic efficacy of formulations with varying molar ratio were determined against Hela human cervical adenocarcinoma cell line and they showed that molar ratio of porphyrin: CD intimately correlated with photodynamic activity (Sortino et al., 2006).

Now, the biodistribution of nanoparticles is one of the crucial questions for drug delivery systems. Geze et al. prepared amphiphilic β -CD nanospheres by solvent displacement method and investigated biodistribution of nanoparticles in male mice. They demonstrated that, amphiphilic β -CD nanoparticles accumulated in liver and spleen, like other uncoated nanoparticles. Accordingly, they require surface modification for long-term circulation in blood system and site-specific targeting. Conversely, amphiphilic β -CD nanoparticles could be tolerated by mice and did not show any toxicity (Geze et al., 2007).

Amphiphilic CD nanoparticles have the ability to form spontaneously, thanks to their variable chemical structure. For instance, Abdelwahed et al. prepared sulfated and nonsulfated amphiphilic β -CDs nanospheres and determined physico-chemical properties of formulations. It was reported that, there is a direct correlation between structure of CD and ability to form nanoparticle. The hydrophilic–lipophilic balance of CD is a critical parameter for self-assembly nanoparticles. According to their results, if this value is higher than 8, CD can be soluble in water and can create spontaneous nanoparticle. When this value is lower than 7.4, CD can be soluble in organic solvents and nanoparticles can be prepared by using nanoparticle preparation technique (Abdelwahed et al., 2008).

In addition to nanospheres and nanocapsules, amphiphilic CD nanoassemblies are also used as drug carrier systems in literature. Stancanelli et al. prepared

lutein-loaded butanoyl β -CD nanoassemblies by using emulsion-solvent evaporation method for age-related macular degeneration treatment. Although lutein is a water-insoluble drug, this problem was solved by using β -CD nanoassemblies and stable lutein-loaded nanoparticle formulation was successfully obtained (Stancanelli et al., 2012).

Amphiphilic CD nanoparticles are also used to improve oral bioavailability of drugs. Zhang et al. developed tacrolimus-loaded amphiphilic poly(methyl vinyl ether-*co*-maleic anhydride)-*graft*-hydroxypropyl- β -CD nanoparticle by solvent evaporation method to improve oral bioavailability. Structural integrity of nanoparticles could be protected in simulated gastric fluid for 2 h. Regardless, release of nanoparticles was delayed up to 24 h in the simulated intestinal fluid. In vivo results demonstrated that oral bioavailability of tacrolimus for nanoparticles increased 20 times when compared with tacrolimus solution (Zhang et al., 2015). Unal et al. prepared chitosan coated cationic 6-OCAPRO- β -CD nanocapsules to improve oral bioavailability of camptothecin, which is an effective anticancer drug with severe stability problems under physiological conditions that result in clinical ineffectiveness. In vivo and in vitro characterization of amphiphilic CD nanocapsules were realized and compared with camptothecin-loaded PCL nanocapsules. Results showed that chitosan coating provided the cationic surface for CD and PCL nanocapsules. The cationic surface improved both the drug-loading capacity and the interaction ability with cellular membranes. Moreover, amphiphilic CD nanocapsules were demonstrated to improve oral bioavailability of camptothecin (Unal et al., 2015a,b). Accordingly, these studies showed that amphiphilic CD nanoparticles can be considered a good candidate for oral drug delivery.

Amphiphilic CD nanoparticles are prepared generally from β -CDs, however there are some studies about amphiphilic α -CD or γ -CD nanoparticles in the literature. For instance, Ghera et al. prepared and characterized acyclovir-loaded amphiphilic α -CD nanoparticles. It was shown that perfluoroalkyl α -CDs are able to form self-assembly nanospheres with small particle sizes (100 nm), and they were physically stable for 9 months (Ghera et al., 2009). Yaméogo et al. used γ -CD nanoparticles and decorated the nanoparticle surface with different polymers for the encapsulation of artemisinin. They investigated the particle behavior toward the immune system and in vivo biodistribution of nanospheres and nanoreservoirs. Results showed that surface decoration of γ -CD protects against plasma proteins and improves circulation of nanoparticle in the circulatory system. Their study suggested that surface decorated γ -cyclodextrin nanoparticles are good candidate for artemisinin delivery (Yameogo et al., 2014).

In addition to drug delivery with small molecules, amphiphilic CD nanoparticles especially polycationic derivatives, are also used in nonviral gene delivery studies. There are several studies about polycationic amphiphilic CD-pDNA as CDplexes in literature (Diaz-Moscoso et al., 2009, 2010, 2011; Mendez-Ardoy et al., 2009, 2011; O'Neill et al., 2011; Bienvenu et al., 2012). Aranda et al. prepared polycationic amphiphilic CD-DNA nanoparticle with folate conjugation for

active targeting. In order to determine in vitro and in vivo characterization of nanoparticles were performed in human cervix adenocarcinoma. Results showed that, folate conjugation increased transfection efficiency and caused high accumulation in the lung and liver, when compared with naked DNA. The developed system can be considered as a nonviral vector for gene therapy and can be modified according to the target organ (Aranda et al., 2013).

Amphiphilic CD nanoparticles were also evaluated in gene silencing studies for cancer therapy. Evans et al. prepared and characterized anisamide targeted and untargeted cationic amphiphilic CD (heptakis[2-*O*-(*N*-(3''-aminopropyl)-1'*H*-triazole-4'-yl-methyl)-6-dodecylthio]- β CD) nanoparticles to treat metastatic prostate cancer. According to results of in vitro characterization studies, mean particles sizes of nanoparticles were found less than 200 nm with cationic surface charge. When they were compared to PEGylated and unPEGylated formulations, it was reported that PEGylation reduced the surface charge of amphiphilic CD nanoparticles, and also reduced nonspecific binding to serum proteins. Two-dimensional and 3D cell culture studies developed to determine the transfection effect of nanoparticles. However, there were no significant differences on the levels of polo-like kinase 1 (PLK1) mRNA knockdown between targeted and untargeted amphiphilic CD nanoparticles. According to 3D cell culture studies, PLK1 mRNA knockdown levels of targeted nanoparticles were found significantly higher than untargeted nanoparticle formulations. It was emphasized that anisamide-targeted amphiphilic CD nanoparticles in especially 3D cell culture model provide pioneering work for further studies (Evans et al., 2017).

19.4 POLYMERIC NANOPARTICLES INCORPORATING CYCLODEXTRINS

The polymeric nanoparticle drug delivery systems have been attracting attention for many years. Nanoparticles have various properties that make them indispensable as drug delivery systems. In order to improve the existing properties of nanoparticles, several approaches have been adopted, such as changing surface charge, increasing drug-loading capacity, changing drug release profile, and enhancing interaction with biological membranes. For this reason, CDs are also used to improve properties of other polymeric nanoparticles, especially in oral administration of drugs, gene delivery, and increasing solubility of hydrophobic drugs.

The oral administration of drugs is preferred because of the convenience to the patient. Nevertheless, this administration route is not feasible for every drug or active molecules, such as peptides. Orally administered drugs face many difficulties during oral absorption because of their physicochemical properties and biological barriers along with the harsh environment of the gastrointestinal tract and the wide pH range encountered through the tract. To overcome these

biological barriers and improve the oral bioavailability in oral route, CD derivatives are used in the formulation of active molecules. Agüeros et al. prepared paclitaxel inclusion complex with HP- β -CD and then encapsulated this complex in poly(anhydride) nanoparticles (Agueros et al., 2009). It was reported that drug-loading capacity was increased 500 times when a nanoparticle was incorporated with HP- β -CD. In addition, permeability of paclitaxel nanoparticles was 12-times higher than when compared with Taxol. In addition, it was determined that, relative oral bioavailability of paclitaxel was 80% for paclitaxel-loaded β -CD or HP- β -CD nanoparticles after in vivo oral administration (Agueros et al., 2010). Some CDs are known as inhibitors for P-gp and cytochrome P450 that play important role in oral absorption and bioavailability of drug molecules (Unal et al., 2015a). Moreover, it was known that HP- β -CD can be incorporated with poly(anhydride) easily. Thank to this property, HP- β -CD was often incorporated in poly(anhydride) nanoparticles, especially for oral administration of anticancer drugs. Camptothecin is another highly effective anticancer drug that has a stability problem of active form under physiological conditions. Combination of the camptothecin-loaded poly(anhydride) nanoparticles with different CD derivatives has been successful in protecting the active form. According to the result of in vivo studies, HP- β -CD increased the oral bioavailability of drug by sevenfold compared to the control (Huarte et al., 2016).

The Trapani group researched the potential of chitosan nanoparticles and CD-incorporated chitosan nanoparticles for oral delivery of peptide glutathione (Trapani et al., 2010). In this case, α -CD and sulfobutyl ether- β -CD were used for nanoparticle formulations. The particle size of drug-loaded nanoparticles were determined 500 and 190 nm for α -CD and sulfobutyl ether- β -CD, respectively. The nanoparticles were prepared with sulfobutyl ether- β -CD increased peptide loading capacity fivefold compared to the α -CD nanoparticles. Interestingly, CD incorporating changed release profile of glutathione in different pH. As a consequence of the protection of peptide by CD, only 30% of the peptide was released in gastric medium in 3 h. However, the peptide was released completely from nanoparticles in absence of CD in same conditions. Chitosan is a nature polysaccharide and has mucoadhesive effect (Anitha et al., 2014; Nagpal et al., 2010). In addition, it is well known that chitosan increases bioavailability of drugs by opening the tight junctions (Erdogor et al., 2014). Incorporating of chitosan with CD is seen as a promising approach for oral administration of low molecular weight peptides.

CDs can be used as coating material for surface of nanoparticles as an alternative approach. CDs allow the encapsulation and protection of hydrophobic molecules in their cavities. Ahmed et al. prepared nanoparticles from poly(ϵ -caprolactone) (PCL) and poly(ethylene glycol) (PEG), their copolymer (PCL-PEG-PCL), and coated nanoparticles with poly(acrylic acid) conjugated β -CD (PCDAA) (Ahmed et al., 2015). They compared PCDAA-coated and uncoated PCL-PEG-PCL nanoparticles in terms of mean particle size, drug encapsulation

efficiency, and drug release profile for paclitaxel. The particle size of nanoparticles was reported between 100 and 200 nm and accepted as appropriate size for anticancer drug delivery systems. In this study, drug release profile was especially emphasized. In order to mimic the acidic environment of tumor site, in vitro release studies were performed in PBS (pH 5.0). More than 50% of paclitaxel was released from PCL-PEG-PCL nanoparticles within 12 h. However, coating of nanoparticles with PCDA changed drug release profiles. The burst effect was observed for PCL-PEG-PCL nanoparticles in the first 3 h; however, coating with CD overcame burst release. It was reported that paclitaxel was encapsulated into CDs that were on the surface of nanoparticles and therefore released slowly from nanoparticles.

Varan et al. also investigated the effect of coating nanoparticle surfaces with CD in another study (Varan and Bilensoy, 2014). They designed HP- β -CD-coated polymeric nanoparticulate system for controlled delivery of model anticancer drug docetaxel. Poly(ϵ -caprolactone) (PCL) and its derivative poly(ethylene glycol)-*block*-poly(ϵ -caprolactone) (mePEG-PCL) were used to prepare nanoparticle. Blank or drug-loaded nanoparticles were prepared with nanoprecipitation method. For coated nanoparticles, CD was added in aqueous phase during preparation. The particle size was evaluated respectively as 74 and 126 nm for PCL and mePEG-PCL nanoparticles. Coating of the nanoparticles with CD increased the particles size as well as drug-loading capacity for docetaxel as expected. According to results of in vitro cell culture studies, it was reported that docetaxel-loaded nanoparticles have cytotoxic effect on MCF-7 human breast cancer cell line, compared with docetaxel solution.

Combination drug delivery systems have attracted interest in recent years especially for site specific drug release. Esparza et al. designed a novel nano-sized drug delivery combined system with fluorescent dye, rhodamine, and bodipy (Ruiz-Esparza et al., 2014). They used rhodamine-loaded poly(lactic-co-glycolic acid) (PLGA) as a core and bodipy complexed with cationic CD (QA β -CD) as shell in this system. This group hypothesized that following intravenous administration, nanoparticles arrive at tumor site through the enhanced permeability and retention effect, the drug complexed with CD in the shell is released and next the drug encapsulated at the inner PLGA core is released. There was an electrostatic interaction between PLGA and cationic CD. It was reported that, in 12 h 50% of bodipy was released from nanoparticles and at the same time, only 10% rhodamine was released. The examination of nanoparticle internalization was evaluated on MCF-7 human breast cancer cell lines with confocal microscopy. After 3 h, bodipy was observed in MCF-7 breast cancer cells and after 96 h, the fluorescence in MCF-7 cells was due to rhodamine having extremely slow release profile. In vivo studies were performed on MCF-7 breast tumor xenografts from mice after intravenous administration. Both dyes were imaged for 3 h after administration in tumor tissue. Moreover, it was reported that after 24 h, there was a decreased intensity of bodipy, in contrast with rhodamine. It was determined that fluorescence intensity of rhodamine increased at 96 h.

19.5 CYCLODEXTRIN-BASED POLYMERIC NANOMEDICINES

Cyclodextrin-based polyrotaxanes (CDPRs), also called “molecular necklaces,” in which CD molecules are threaded on a PEG chain, were prepared by capping the polymer chain in the complexes with bulk groups (Harada, 1996; Higashi et al., 2007, 2008, 2009a, 2009b). These supramolecular systems generally comprise of α -CD, PEG, and a biodegradable moiety, which exists at both terminals of the PEG chain. The most commonly used polymers are poly(ethylene oxide) (PEO) or PEG, poly(propylene oxide) (PPO) or copolymers having blocks of PEO and/or PPO, solely such as PEO–PPO–PEO (Pluronic), or combined with other blocks such as poly[(*R*)-3-hydroxybutyrate] or poly(caprolactone) (PCL) (Ooya and Yui, 1999). Formation of polyrotaxanes using CDs can be classified in three types: the “threading” approach, “slipping” approach, and “inclusion polymerization” approach, as can be seen in Fig. 19.3 (Harada et al., 2009).

Polypseudorotaxanes have been prepared to incorporate CDs using an axis polymer such as polyethers, cationic polymers, polyamines, inorganic polymers, i.e., in aqueous media called the threading method. CD inclusion complexes are formed with hydrophobic and van der Waals interactions between the inner surface of the CD ring and the hydrophobic sites of the guest molecules. The capping of both ends of the pseudopolyrotaxanes with bulky molecules prevents the

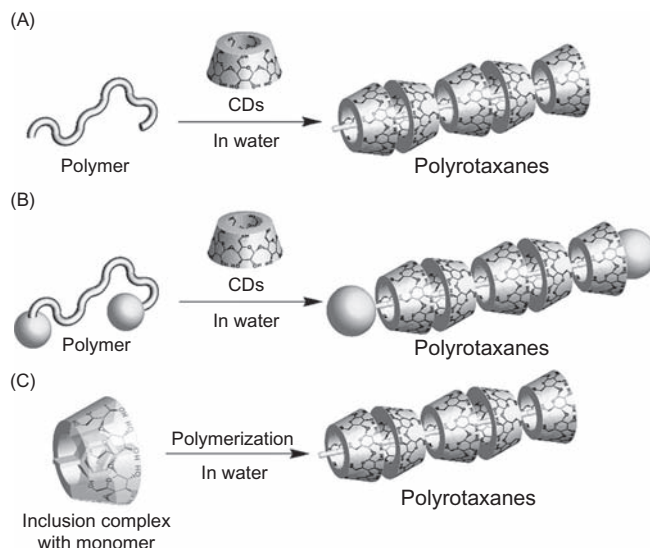


FIGURE 19.3

Schematic illustration of manufacturing methods of polypseudorotaxanes incorporating CDs (Harada et al., 2009). (A) “Threading” approach, (B) “slippage” approach, and (C) “inclusion polymerization” approach.

dissociation of the α -CD rings from the PEG leading “polyrotaxanes” (Osaki et al., 2009; Harada et al., 1992).

Since CDPRs are highly multifunctional and biocompatible molecules, they have been used for the delivery of drugs. Due to their controllable size, low cytotoxicity, and unique structure, CDPRs have potential for a wide range of pharmaceutical applications (Harada et al., 2009). In addition, CDPRs should have principal properties such as aqueous compatibility, biodegradability, biocompatibility, stimuli-responsiveness, targeting, and bioactivity for biomedical applications (Dong et al., 2015; Loethen et al., 2007; Araki and Ito, 2007). These systems possess many advantages such as improved solubility and stability of drug and specific cell uptake in drug delivery (Pourjavadi et al., 2015; Simoes et al., 2015). The drug can be covalently attached to the CD rings of a polyrotaxane. The resulting functional polyrotaxane has to be water-soluble (Wenz et al., 2006). The amount of functional hydroxyl groups on the CD also provides polyrotaxanes the ability of carrying a high dose of active substances for delivery (Moon et al., 2007). They enhance the rate of internalization of the nanoparticles and anticancer drugs into the cells upon attachment to their surface, and accordingly they are seen as promising systems in cancer therapy (Moon et al., 2007, 2008; Huang et al., 2010; Zhang et al., 2014). Polyrotaxanes provide the increased molecular mobility of targeting ligands resulting in enhanced active targeting due to shortened response time between cell-surface receptor proteins and material (Seo et al., 2013).

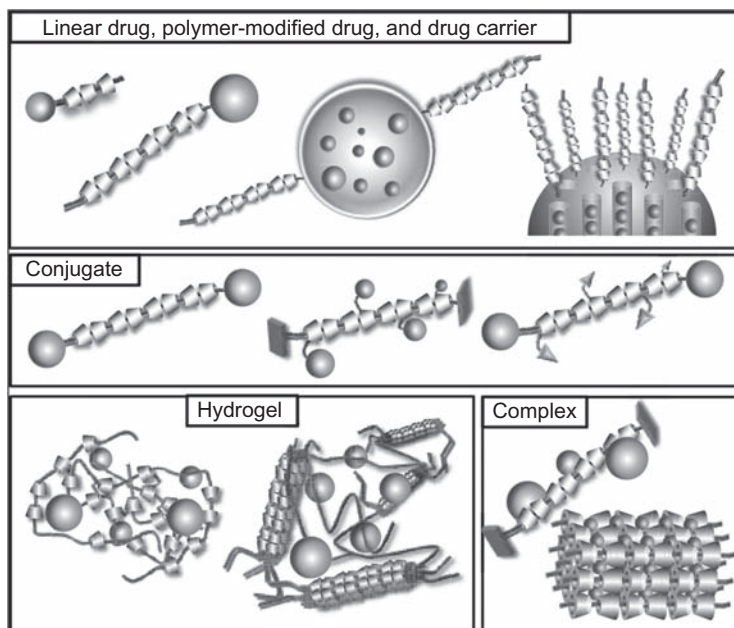
These nanocarriers obtained from CD-based polypseudorotaxanes and polyrotaxanes can be classified as follows:

- direct formation of polypseudorotaxanes with linear drugs and polypseudorotaxanes with polymer-modified drugs or drug carriers (nanospheres, micelles, linear polymer-appended silica particles) (Park et al., 2007);
- conjugates of polyrotaxanes with drugs (Yu et al., 2013; Bilkova et al., 2010, 2011);
- hydrogels including drugs (Abu Hashim et al., 2010; Guo et al., 2013);
- drug complexes with polypseudorotaxanes or polyrotaxanes (Higashi et al., 2009c; Zhou et al., 2012). Illustrated in Fig. 19.4.

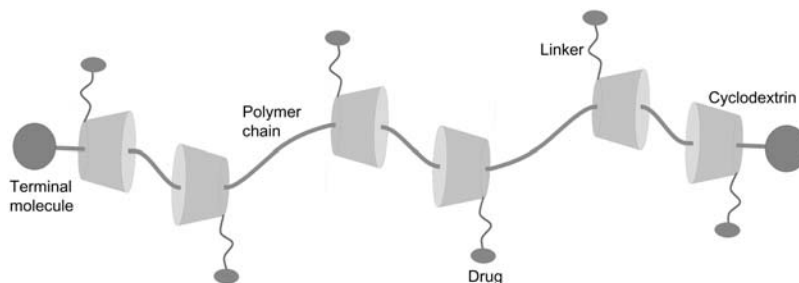
These nanosystems exhibit a large number of hydrophilic/hydrophobic domains and recognition sites; accordingly, they are widely applied nanocarriers for both hydrophilic and hydrophobic bioactive molecules (Zhang and Ma, 2013).

19.6 POLYMER-DRUG CONJUGATES

CDPRs, self-assembled polymers obtained from biocompatible components are potential candidates to form polymer-drug conjugates, especially in cancer

**FIGURE 19.4**

Different drug carriers using polypseudorotaxanes and polyrotaxanes (Guo et al., 2013).

**FIGURE 19.5**

Schematic illustration of polyrotaxane-drug conjugate.

treatment. Anticancer drugs were conjugated on the polyrotaxanes by either nondegradable spacers or degradable linkages with the combination of cleavable bonds in the end-caps of the polyrotaxanes, as seen in Fig. 19.5 (Moon et al., 2007). At the formulation stage, polymer-drug conjugates have been applied, due to many advantages. This includes abundance in hydroxyl functional groups on the CDs renders CDPRs the capability of carrying higher dose of anticancer drugs resulting in high drug loading and provide selective cytotoxicity against tumor tissues. As well as

higher drug loading, targeting efficiency is more important for anticancer drug delivery. In polyrotaxane drug conjugates, both anticancer drugs and ligands were immobilized on CDs via the functional —OH groups. CDs could slide along and rotate around the PEG chains; this flexible motion avoided the mismatch of ligand–receptor interaction and the multivalent interactions between the conjugates and cell membranes could be formed. This active recognition mode provides a promising strategy to enhance targeting efficiency (He and Gu, 2013).

Moon et al. developed and evaluated a new polyrotaxane-based drug delivery system using anticancer drug doxorubicin (DOX) (LMWP-PR-DOX). The LMWP-PR-DOX conjugates were synthesized by threading the α -CD residues onto a PEG chain successfully and DOX showed a sustained-release profile in more than 4 days with hydrolysis mechanism. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium analysis and confocal microscopy method confirmed that LMWP-PR-DOX conjugate internalized and displayed sevenfold increase in cytotoxic effects in A2780 human ovarian cancer cells (Moon et al., 2007). In another study, Moon and colleagues prepared a novel camptothecin conjugated polyrotaxane. Camptothecin has low aqueous solubility and severe side effects including gastrointestinal toxicities, limiting the effectiveness in clinical use. ^1H NMR studies confirmed successful polyrotaxane–camptothecin conjugates synthesis and increased camptothecin solubility, due to hydrophilic nature of polymer components (PEG and CDs) and abundant of carboxyl groups. In 7 days, more than 75% of camptothecin was released from conjugate by hydrolysis. In vitro cytotoxicity results indicated that the ID_{50} of the polyrotaxane–camptothecin conjugates in A2780 human ovarian cancer cells was more than 200-fold higher than that for free drug (Moon et al., 2008). Yu et al. synthesized β -CD polyrotaxane by using poly(propylene glycol) (PPG) terminated by alkynyl groups and mono-(6-azido-6-deoxy)- β -CD (β -CD- N_3) as the end-capping reagent. Following this, the conjugation of anticancer drug paclitaxel and polyrotaxane was performed through a hydrophilic spacer of diaminotri(ethylene glycol) and a biodegradable ester linkage. The cellular uptake, cytotoxicity, biodistribution, tumor penetration, and antitumor performance of the polyrotaxane-paclitaxel conjugate were evaluated. Due to unique in vivo fate, significant antitumor response, and deep intratumoral penetration, polyrotaxane-paclitaxel conjugation has been found superior, compared with commercial product Taxol (Yu et al., 2013).

19.7 SUPRAMOLECULAR HYDROGELS

The (pseudo)polyrotaxanes of CDs have an ability to form higher supramolecular assemblies such as hydrogels via physical or chemical crosslinking. Supramolecular hydrogels based on the self-assembly of the inclusion complexes between CDs with biodegradable block copolymers could be used as promising

injectable drug delivery systems for sustained release of macromolecular drugs. These systems have also been utilized in drug delivery and biomedical applications because of their hydrophilicity, biocompatibility, low inflammatory response, drug-loading capacity, and release properties (Limon et al., 2015; Cheng et al., 2016; Zhang et al., 2016; Hoffman, 2002). These systems composed of PEO, PEO-PPO, PEO-PCL, and Pluronic F127 have been used with α -CD to form hydrogels in drug delivery (Pradal et al., 2015).

Ma et al. studied supramolecular hydrogels comprising of methylated polyethylene glycol-indomethacin and α -CD via a host–guest interaction at room temperature. The *in vitro* release studies showed a sustained-release profile without an initial burst release. The PEGylation of indomethacin provides high aqueous solubility for a hydrophobic drug as well as supramolecular gelation properties in the presence of α -CD (Ma et al., 2011).

Furthermore, multifunctional supramolecular hydrogels based on host–guest inclusion of CD and PEG were used for controlled release of two different anticancer drugs with different release profiles. Ha et al. synthesized a thermosensitive, pseudopolyrotaxane hydrogel system composed of the anticancer drug camptothecin conjugated to a PEG and α -CD to form an amphiphilic prodrug. By varying the length of the PEG and the concentration of α -CD, the structure of pseudopolyrotaxane could be controlled, resulting in tunable mechanical strength, morphology and temperature-responsive gel–sol transition behavior in an independent manner. Meanwhile, another water-soluble anticancer drug, 5-FU was loaded in the formed hydrogel and this system successfully delivered two anticancer drugs via structure-related dual-phase drug-release behavior. This easily prepared supramolecular hydrogel, with excellent biocompatibility and tunable temperature responsiveness, has significant potential for anticancer therapy (Ha et al., 2014). In the same method, He et al. synthesized supramolecular injectable hydrogels for delivery of anticancer drugs (He et al., 2009). The polyrotaxane of α -CDs/mPEG-*g*-PMA was prepared via host–guest interaction between α -CDs and mPEG-*g*-PMA and DOX hydrochloride was loaded in the hydrogel. They found that sustaining release time lasted longer than 3 days and cell viability decreased greatly. Recently, Qing et al. synthesized mPEG1900–LUT–Fe₃O₄ conjugates that formed stable supramolecular hydrogels through α -CD in water. 5-FU was loaded into the formed hydrogels to control its release *in vitro*. The drug release studies showed that introducing Fe₃O₄ particles into the hydrogel improved the sustained release effect (Qing et al., 2016).

19.8 POLYMER-MODIFIED DRUG CARRIERS

Supramolecular polymer micelles (SMPMs) have been used in biomedical applications such as cellular imaging and drug delivery. These SMPMs make their application due to many advantages, such as simple synthesis, structure

controllability and higher drug loading. Micelles were obtained by self-assembly from amphiphilic block copolymers (Dong et al., 2008).

Dong et al. investigated novel SMPMs synthesized from maleic anhydride modified α -CD (MAh- α -CD) and PCL/PLLA-based copolymers which were approved by the Food and Drug Administration (FDA) through host–guest and hydrophobic interactions in terms of potential application in drug delivery. Compared with conventional micelles, the SMPMs display higher entrapment efficacy (37.5%) and sustained drug release of prednisolone acetate (PA), an anti-inflammatory drug with low aqueous solubility (Dong et al., 2011). The Du group first prepared PA-loaded SMPMs by the self-assembly of amphiphilic polyrotaxanes that were formed through the hydrophobic host–guest interaction with α -CD as host molecule and PCL as the guest molecule. They then synthesized SMPMs from β -CD (β -CD)/maleic anhydride modified β -CD (MAh- β -CD) and methylcellulose (MC) in aqueous solution by one-pot self-assembly procedure, in which, β -CD and MAh- β -CD inclusion complexes were used as the hydrophilic shell and the free MC as the core. In vitro drug release studies showed that the MC/MAh- β -CD micelle had a drug-enrichment core and excellent drug released behaviors with a sustaining release time of 30 days (Du et al., 2012). In previous studies, it was found that higher drug loading was observed with polyrotaxanes containing β -CD due to the hydrogen bonding between antifungal drug amphotericin B and β -CD along polymer chains, so polyrotaxanes containing β -CD have been potential carrier with a high drug-loading ability for drugs having many hydroxyls in the molecules. Following this study, folic acid (FA)-conjugated amphiphilic polyrotaxane-based copolymers synthesized using β -CDS and PEG methyl ether methacrylate via in situ ATRP and azide-ethylene click chemistry. The conjugated copolymers enable self-assembly into unique core-shell micelles in aqueous solution and to load DOX into the hydrophobic core. The drug-loading content is increased (25%) due to the hydrogen bond interaction between DOX and β -CDs threaded. These tumor-targeted polymeric micelles also show a slower and sustained DOX release behavior. The cell culture studies confirmed that these micelles are internalized in MDA-MB-231 cells via FA receptor mediated endocytosis; consequently they present enhanced cytotoxicity to the selected breast cancer cells (Jiang et al., 2013a). Jiang et al. also synthesized novel pH-labile micelles containing β -CDs entrapped on the Pluronic F127 block of a polyrotaxane-based triblock copolymer for cancer treatment with DOX. Transmission electron microscopy (TEM) results showed that polyrotaxane-DOX conjugates form self-assembled micelles of 70 nm in diameter in aqueous solution. The release of DOX was 37% over 72 h at acidic pH. The DOX-loaded polyrotaxane micelles showed higher cytotoxicity in HeLa cells (Jiang et al., 2013b, 2014).

Mesoporous silica nanoparticles (MSNs) have been of great interest in drug delivery systems owing to tunable pore size, mesoporous structure, large surface area, and biocompatibility in vitro and in vivo (Ashley et al., 2011; Ambrogio et al., 2011; Tarn et al., 2013; Vallet-Regi et al., 2007). They have been utilized

to release therapeutic agents because of their better encapsulation ability and high sensitivity to external stimuli (Ambrogio et al., 2011). Luo et al. developed a novel (2)rotaxanes into MSNs by using FDA-approved tetraethylene glycol, α -CD and FA. Doxorubicin-loaded rotaxane-mechanized MSNs could selectively induce the apoptosis and death of tumor cells. This biocompatible and efficient system improves the targeting capability to tumor tissues in vitro and inhibited the tumor growth with minimal side effects in vivo (Luo et al., 2013).

Gold (Au) nanoparticles have been greatly used in drug delivery due to their low toxicity, easy surface functionalization, and good biological compatibility. Hybrid nanoparticles which include a nanoparticle core and a polyrotaxane shell reduce adverse effects and kill tumor cells via the photothermal properties of system. They also provide drug transport and release in target tissue. It was found that Au nanoparticles were endocytosed by cancer cells and anticancer drug DOX was released by a photothermal explosion of the polyrotaxane/Au nanoparticles by Adeli et al. (2011).

In another study, (α -CD/PEG) polyrotaxane nanoparticles were prepared through self-assembly method to carry antimetabolite and antifolate drug, methotrexate (MTX). The results showed that drug loading was higher (20%) and most of the drug was released within 8 h. The blank polyrotaxane nanoparticles were nontoxic to cells. The in vitro anticancer activity of the MTX-loaded polyrotaxane nanoparticles was higher than that of free drug (Zhang et al., 2014).

CRLX101 is a first-in-class nanopharmaceutical, currently in Phase IIa development, which has been developed by covalently conjugating camptothecin to a linear, β -CD-polyethylene glycol copolymer that self-assembles into nanoparticles. As a nanometer-scale drug carrier system, the CD polymeric nanoparticle technology, referred to as “CDP,” has unique design properties and capabilities. CRLX101 preclinical and clinical data especially confirm that this system can solve solubility, formulation, toxicity, and pharmacokinetic drawbacks associated with administration of camptothecin, but more importantly, can impart unique biological properties that enhance camptothecin pharmacodynamics and efficacy (Young et al., 2011).

19.9 CONCLUSION

In the last decade, CD or CD-based nanoparticles have drawn attention for safe drug or gene delivery. CDs are considered safe as nano-sized drug or gene delivery systems, thanks to their physicochemical properties, and these properties of CDs are supported by studies in the literature. In addition, there are several approved products containing CD in the market, used clinically through various routes of administration. EMA has published a recommendation report on CDs as excipients in pharmaceutical products. This contributes significantly to the efficacy and safety of CD drug delivery.

Additionally, the ability to change the surface charge during synthesis and make surface modification in accordance with the active targeting provides an advantage for drug delivery and reduction of side effects. The altered surface charge plays an important role in increasing the interaction with cell membranes, especially in gene transport. In order to take advantage of these properties, CDs are also used in modification of polymeric nano-sized drug delivery systems such as surface coating. Amphiphilic CD derivatives are preferred for not only drug delivery but also for gene delivery, as they provide the required properties of a safe and effective nano-sized delivery system. Thanks to their long aliphatic chains, amphiphilic CDs provide high loading capacity for hydrophobic molecules, especially cancer drugs. It is possible that amphiphilic CD nanoparticles can provide more effective cancer treatment with less active molecules.

CD polymers, conjugates, and polyrotaxanes are also emerging drug delivery systems, as they can easily be fine-tuned to respond to external stimuli or to effectively target drug molecules or gene receptors.

In summary, it can be said that CDs are attractive substances that are used as excipients in nano-sized drug or gene delivery systems, such as nanospheres, nanocapsules and nanosponges, directly or in corporation with polymeric nanoparticles due to their unique physicochemical properties.

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Lipid nanoparticles: In vitro and in vivo approaches in drug delivery and targeting

20

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CHAPTER OUTLINE

20.1 Introduction	750
20.2 Preparation of Lipid Nanoparticles	752
20.2.1 High-Pressure Homogenization	752
20.2.2 Microemulsion Technique.....	753
20.2.3 Solvent Emulsification Evaporation Technique	753
20.3 In Vitro Characterization.....	754
20.3.1 Routine Characterization of Lipid Nanoparticles	754
20.3.2 Polymorphism and Degree of Crystallinity	757
20.3.3 Assessment of Coexisting Structures	759
20.3.4 In Vitro Lipolysis	759
20.4 In Vivo Characterization	760
20.4.1 Pharmacokinetics and Bioavailability	760
20.4.2 Biodistribution	761
20.4.3 In Vivo Imaging	762
20.5 Models for Specific Administration Route	766
20.5.1 Evaluation of Lymphatic Absorption	766
20.5.2 Dermal Delivery.....	767
20.5.3 Pulmonary Delivery.....	769
20.6 Cellular Models	770
20.6.1 Endocytosis	770
20.6.2 Translocation	772
20.7 Conclusion	773
Acknowledgments	774
References	774

20.1 INTRODUCTION

Lipid nanoparticles (LNs), such as solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs), are colloidal dispersions composed of a solid lipid phase stabilized by surfactants (Feeney et al., 2016; Naseri et al., 2015; Tseng et al., 2014; Silva et al., 2015). LNs are first developed in the 1990s (Schwarz et al., 1994; Westesen et al., 1993; Morel et al., 1996). The concept was originally derived from oil-in-water (o/w) nanoemulsions, adopting solid lipid to improve long-term stability and drug protection (Silva et al., 2015). Therefore, LNs combine the advantages of both o/w nanoemulsions and solid nanoparticles, including physical stability, protection of the payloads, controlled release, biocompatibility and ease of scaling-up, while circumventing their drawbacks (Das and Chaudhury, 2011; Parhi and Suresh, 2012). LNs have been extensively studied both in drug delivery and cosmetic applications.

SLNs are the first generation of LNs with a matrix composed merely of solid lipids. The lipids referred to herein are of a broad sense: (1) pure triglycerides (e.g., tripalmitin/Dynasan 116, trimyristin/Dynasan 114, tristearin/Dynasan 118); (2) partial glycerides (e.g., glyceryl behenate/Compritol 888 ATO, glycerol palmitostearate/Precirol ATO 5, glyceryl stearate/Imwitor 900); (3) fatty acids (e.g., stearic acid, palmitic acid, and decanoic acid); (4) steroids (cholesterols); and (5) waxes (e.g., cetyl palmitate). Besides lipids, surfactants are essential (Kathe et al., 2014). A sufficient amount of surfactant is required to reduce the surface tension at the lipid/water junctions and provide stabilizing effects. Typical surfactants used include phospholipids (e.g., soybean and egg lecithin, phosphatidylcholine), poloxamers (e.g., poloxamer 188 and 407), bile salts (e.g., sodium cholate and sodium glycocholate) and polysorbate 80. In some cases, coemulsifiers such as butanol are needed. NLCs are the second generation of LNs, developed to overcome the disadvantages associated with SLNs. The composition of NLCs is similar to SLNs, except that a mixture of liquid and solid lipids are used to form the matrix (Fig. 20.1). The solid lipids alone in SLNs present like well-packed bricks, leaving limited spaces for accommodation of drug molecules. Conversely, transition of lipids to more stable polymorphs may expulse loaded drugs out of SLNs. However, addition of liquid lipids leads to imperfect lipid matrix structure within NLCs, providing more spaces to accommodate drug molecules (Fig. 20.1) (Cipolla et al., 2014; Jaiswal et al., 2016). Moreover, liquid lipids in NLCs extend the duration of unstable polymorphic forms of the lipids, leading to improved stability (Muller et al., 2002, 2011). Based on the structure, NLCs can be further divided into three types: (1) imperfect type, in which gap presents between triglyceride fatty acid crystals; (2) amorphous type, which is without crystalline structure; and (3) multiple type, which is a mix of the above two (Naseri et al., 2015).

LNs are versatile carriers to deliver an array of chemically diverse drugs via different routes. With a lipid core, LNs are able to solubilize lipophilic drugs in

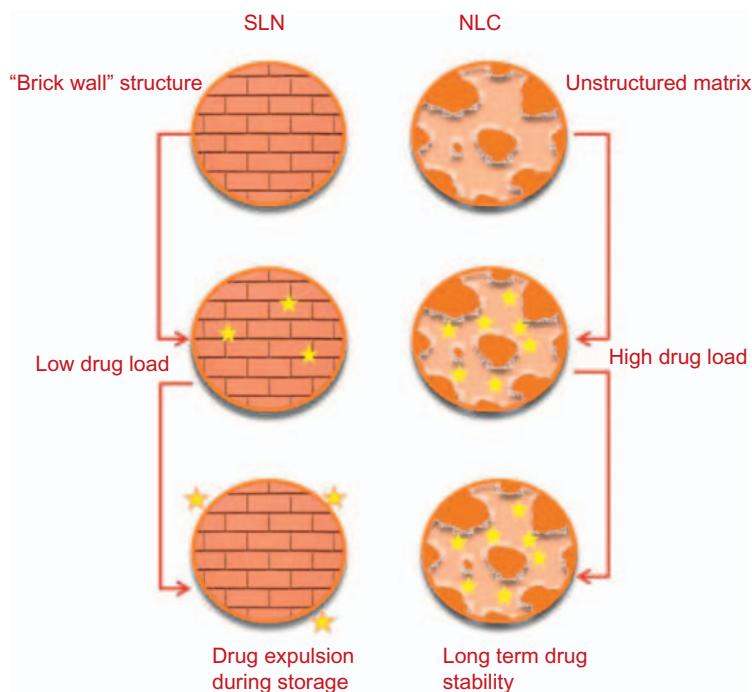
**FIGURE 20.1**

Illustration of structural differences between SLN and NLC structure as well as advantages of NLC over SLN.

Source: Adapted from Beloqui, A., Solinis, M.A., Rodriguez-Gascon, A., Almeida, A.J., Preat, V., 2016. Nanostructured lipid carriers: promising drug delivery systems for future clinics. *Nanomedicine* 12, 143–161.

the lipid phases to improve their solubility and bioavailability. Accordingly, LNs are most commonly applied to lipophilic drugs that cannot be delivered by conventional preparations. Recently, LNs have been applied to improve the delivery of biologicals, such as proteins, peptides, antibodies, and DNAs (Silva et al., 2015). Even highly hydrophilic biologicals can be loaded into LNs by optimizing the preparative technologies. The lipid matrix not only protects biologicals from proteolytic degradation after administration but also modulates release profiles of the payloads (Yang et al., 2011; Zhang et al., 2006; Almeida et al., 1997). Cationic LNs have been developed as novel nonviral vectors to deliver genes since 2000 (del Pozo-Rodriguez et al., 2009b; Zhang et al., 2008b; Olbrich et al., 2001). Surface decoration with specific ligands, cell penetration enhancing peptide or other factors that facilitate cellular uptake has been carried out to enhance the performance of LNs (de Jesus and Zuhorn, 2015; del Pozo-Rodriguez et al.,

2009a). Moreover, LNs have been applied to versatile administering routes including oral, parenteral, dermal, intranasal, and ophthalmologic routes.

This chapter reviews the progress on *in vitro* and *in vivo* characterization of LNs, following a brief introduction of preparation methods and general *in vitro* characterization methods.

20.2 PREPARATION OF LIPID NANOPARTICLES

There are two main methods used for preparation of LNs, that is, high-pressure homogenization (HPH) and microemulsion-based technique. HPH is the most widely used technique. Besides, several other methods have been developed to load different types of drugs, such as solvent emulsification evaporation, solvent emulsification diffusion, melting dispersion, ultrasonication, double emulsion, solvent injection, supercritical fluid, and microchannel/microfluidic techniques.

20.2.1 HIGH-PRESSURE HOMOGENIZATION

The principle of HPH is using high pressure to force lipid suspensions passing through a narrow gap of a few microns at high velocity. During the process, cavitation effects caused by the rapid pressure drop and high-shear forces facilitate disruption of the lipid particles. The mixture also hits the impact valve of the homogenizer, leading to collision of the lipid particles. The lipid concentration in this process can be as high as 40% (Lippacher et al., 2000), in contrast with a normal range of 5%–10%. In addition, HPH can be further divided into hot homogenization technique (HHT) and cold homogenization technique (CHT) according to the processing temperature.

In the process of HHT, the drug is firstly dispersed/solubilized in a molten lipid. The lipid phase is then mixed with isothermal aqueous surfactant solution under high shear forces to form hot pre-emulsions. High pressure homogenizer, maintained at temperature about 5–10°C above the melting point of the lipids, is introduced to decrease the particle size of the pre-emulsions to nanometer scale under a specific pressure for several cycles. The obtained hot o/w nanoemulsions were cooled to form LNs. Since drugs can be dispersed in the lipid phase, this technique is free of organic solvent. Conversely, HHT can be easily scaled-up. Generally, high temperature is advantageous to decrease the particle size of LNs due to decreased viscosity but disadvantageous to heat-labile drugs. Lipid crystallization may be retarded in this process due to the small particle size and presence of emulsifiers, leading to super-cooled melt remaining for several months (Muller et al., 2006). In addition, HHT is not suitable for hydrophilic drugs due to leakage of the drugs into the aqueous phase. Even if adequate amount of hydrophilic drugs are loaded, they may reside at the boundary surfaces between lipid and aqueous phases, leading to burst release (Zhang et al., 2000).

CHT is an improvement of HHT with the purpose of preventing drugs from degradation at high temperatures and increasing loading of hydrophilic drugs. The first step in CHT is the same as HHT. But the lipid phase is rapidly cooled by liquid nitrogen or dry ice to be converted to a solid state instead of mixing with aqueous phases to form pre-emulsions. The solid lipid phase is then pulverized to micron size and suspended in emulsifier solution. The suspension is subjected to HPH for size reduction (You et al., 2007). Although thermal exposure is unavoidable in the first step, the overall exposure time is reduced in CHT compared with HHT, avoiding or minimizing the degradation of the payloads. Partition of hydrophilic drugs into aqueous phase may be retarded by the solid lipid, accordingly increasing the loading of hydrophilic drugs. The loading may be further increased by replacing water with solutions in which the drug is poorly soluble.

20.2.2 MICROEMULSION TECHNIQUE

In this process, the lipids are melted at a temperature above the melting point. Then a mixture solution of surfactants and cosurfactants at equal temperature is added to the melted lipids under stirring to form microemulsions. The hot microemulsions are cooled by mixing with 10–50-folds of cold water (2–3°C) to form LNs (Marengo et al., 2000; Heydenreich et al., 2003). The rapid cooling process as well as the solid lipid construct prevent crystallization of drug molecules. Consequently, drug molecules are dispersed in the lipid matrix of LNS in an amorphous state (Cavalli et al., 1995; Mukherjee et al., 2009). The process is simple, although a large amount of surfactants and cosurfactants are needed to form microemulsions.

20.2.3 SOLVENT EMULSIFICATION EVAPORATION TECHNIQUE

The lipids and the hydrophobic drugs are dissolved in a water immiscible organic solvent, which is mixed with surfactant aqueous solution to form o/w nanoemulsions under mechanic forces. The organic solvent is removed by evaporation at room temperature under stirring or by rotary evaporator at reduced pressure, leading to lipid precipitation to form LNs (Lemos-Senna et al., 1998; Tan and Nakajima, 2005). Although thermal stress can be avoided in this process, the application of organic solvent may bring about environmental hazards. This technique is generally not suitable for hydrophilic drugs, due to partitioning of drug molecules to the continuous aqueous phases during solvent evaporation. To this end, w/o/w double emulsion technique was developed to improve encapsulation of hydrophilic drugs (Cortesi et al., 2002). The hydrophilic drugs are dissolved in the inner aqueous phase, while the lipid phase can prevent partition of the hydrophilic drugs into the outer aqueous phase. This technique is suitable for loading of proteins/peptides due to the absence of thermal stress.

20.3 IN VITRO CHARACTERIZATION

In vitro characterization is essential to control the quality of LNs. Particle size, zeta potential, morphology, drug loading, and drug release are routine issues of characterization for nanoparticles. They are also applicable to LNs. In addition, lipid undergoes physical transformation during formulation and storage, affecting physicochemical properties of LNs and accordingly the performance of the delivery systems. It is also important to evaluate the crystallization tendencies and polymorphic transition of the lipids in LNs (Westesen et al., 1993; Siekmann and Westesen, 1994; Bunjes et al., 1996a).

20.3.1 ROUTINE CHARACTERIZATION OF LIPID NANOPARTICLES

20.3.1.1 Particle size and size distribution

Particle size and size distribution are critical parameters to evaluate the quality of LNs because they have great effects on the performance of LNs (Manolova et al., 2008). Dynamic light scattering (DLS), also called photon correlation spectroscopy (PCS), laser diffraction (LD), and coulter counting are the commonly used techniques to measure particle size and size distribution of nanoparticles. Recently, scanning ion occlusion sensing (SIOS) (Yang et al., 2012) and nanoparticle tracking system (Guo et al., 2015) are also used for the characterization of LNs.

Both DLS and LD provide rapid analysis of particle size for LNs. However, neither of these techniques measures the particle size directly; DLS measures the intensity of the light scattered from a laser that passes through a colloidal solution, while LD measures the diffraction patterns of the laser beam. Light intensity distribution data are accordingly obtained by analysis of various sensing elements. For both techniques, the variation in the scattered light intensity are recorded on autocorrelation function fitted into an exponential function, where the corresponding decay constant(s) are related to the diffusion coefficients. The hydrodynamic size of particles and particle agglomerates can be calculated using standard assumption of spherical size, lower concentration, and known viscosity of the suspending medium using these diffusion coefficients. PCS yields the mean diameter (*z*-average) of the bulk population of the particles and polydispersity index (PDI) value to evaluate the particle size distribution. PDI is equal to the square of the standard deviation/mean diameter, with value of 0.1–0.25 indicating narrow size distribution and value greater than 0.5, indicating broad size distribution. However, the diameters 50%, 90%, 95%, and 99% are used in LD measurement to characterize the size distribution. For example, diameter 50% means that 50% of the particles are below the given size (volume distribution). The suitable size range for PCS measurement is from 3 nm to 3 μ m, while LD is more suitable for particles larger than 3 μ m.

The mechanism for coulter counting is different to DLS and LD, which is based on the measurement of the change in electro-resistance when each particle

passes through an aperture of the sensing zone. Therefore, particles need to be dispersed in an electrolyte solution for coulter counting measurement. Coulter counting can measure the absolute number of particles per volume unit of different size classes. The particle size range for coulter counting measurement is from 0.5 to 1000 μm .

SIOS is a method adopted from coulter counting recently (Yang et al., 2012). The mechanism for these two techniques is similar except that a tunable pore is adopted in SIOS to expand the detectable particle size range. SIOS directly measures the size of individual particle and provides a better analysis on polydispersity than DLS and LD (Vogel et al., 2011; Yang et al., 2012). However, AgCl is required as the conducting electrolyte in this method, which might lead to aggregation of LNs and result in erroneous results.

20.3.1.2 Zeta potential

Surface charge, usually characterized by zeta potential, is an important parameter concerning the physical stability of nanoparticles. Diffusion of adsorbed counter ions from the surface of particles forms double electrical layers, that is, a Stern layer (ions strongly bound layer) and a diffusion layer (less firmly associated layer). Within the diffusion layer is a notional boundary (slipping plane) within which the particle acts as a single entity. Zeta potential is accordingly defined as the potential difference between the dispersion medium and the slipping plane.

Zeta potential can be measured by electrophoretic light scattering (ELS) and electroacoustic determination (O'Brien et al., 1995). ELS is based on the linear relationship between the electrophoretic mobility of the particles and their zeta potential, which provides better resolution and more reliable results than electroacoustic determination. In the process, a capillary cell equipped with electrodes on both sides is used for the measurement. The particle suspensions are filled into the capillary cell and moved under an applied electric field. The speed of the nanoparticles can be monitored by a laser beam to calculate the zeta potential. However, electrolytes may affect the zeta potential, because counter ions in the diffusion layer may be repulsed to the inner layer, leading to decreased zeta potential. With increased concentration of the electrolytes, zeta potential may be decreased to zero, or even be reversed to opposite charges. Therefore, it is important to control the ionic strength during the measurements of zeta potential. In addition, ELS is not suitable for samples with concentration more than 0.01% (w/v) (Xu, 2008). In those cases, an improvement by using a transparent electrode can measure concentrated samples up to 1% (w/v) (Sekiwa et al., 2004).

The stability of LNs can be predicted by the absolute zeta potential value. LNs tend to aggregate due to the extremely large surface area and free energy, resulting from the small particle size. Surface charge provides electrostatic repulsion among individual nanoparticles, preventing aggregation. Generally, LNs with zeta potential higher than 20 mV are regarded as stable and are less prone to aggregate (Freitas and Müller, 1998). Furthermore, zeta potential may affect the

in vivo fate of LNs. LNs with positive zeta potential generally show extended circulation time (Hollis and Li, 2013; Sarmiento et al., 2011).

20.3.1.3 Morphology

LNs are not always spherical and may sometimes present rod or pellet shape (Patel and San Martin-Gonzalez, 2012; Win and Feng, 2005). The morphology may affect the performance of LNs, which has not drawn sufficient attention. Scanning electron microscopy and transmission electron microscope are commonly utilized to observe the morphology of LNs. However, there are some disadvantages regarding these techniques. The electron gun may cause melting of the lipids (Kumar and Randhawa, 2013; McCaffrey et al., 2001), while the high vacuum condition may lead to rupture or shrinkage of LNs (Dubes et al., 2003), producing erroneous results. In addition, the drying process in sample preparation may destabilize LNs (Garti and Sato, 1988). In regard to these, cryo-field emission scanning electron microscopy (Cryo-FESEM) may be a good option. Since samples are observed in the frozen state under Cryo-FESEM, the original morphology and shape of LNs are well preserved. However, all techniques mentioned above only give two-dimensional (2D) figures. On the contrary, atomic force microscopy (AFM) provides 3D topography. Moreover, both the preparation and observation conditions are mild, compared with other techniques, leading to less damage to the integrity of the LNs. Therefore, AFM is more favorable for the determination of the shape and morphology of LNs.

20.3.1.4 Drug entrapment efficiency and drug loading

Drug entrapment efficiency and drug loading are important parameters in formulation optimization. The more drugs encapsulated into LNs, the less the total amount of preparations are needed for administration, increasing patient compliance, and decreasing potential side effects derived from excipients. Drug entrapment is defined as the percentage of entrapped drug to total drug in the preparation, while drug loading is defined as the ratio of the amount of drug in LNs to the total amount of nanoparticle. In the measurement, it is essential to separate loaded drugs from free drugs. Ultracentrifugation is widely utilized to separate nanoparticles from the dispersion medium (Hu et al., 2004). The centrifugation forces and duration shall be optimized and verified for successive separation. Ultrafiltration can also be used for this purpose. Using membranes with proper cut-offs, free drugs are filtered through the membrane while LNs are retained. It is important to choose membranes made of suitable materials to avoid adsorbance of free drugs. Alternatively, size exclusion chromatography can be utilized for separation (Pandita et al., 2011). When LNs suspension flow through a gel filtration column, colloids directly pass the column through gaps among the gel, whereas flow of free drugs are retained by the pores of the gel for separation. The separated LNs are then dissolved in suitable solvent to liberate the loaded drugs for analysis.

Generally, lipophilic drugs can be efficiently loaded into the lipid matrix of LNs, but solubility studies shall be performed in advance for the selection of

lipids to achieve high drug loading (Kalam et al., 2010). Drug partition from lipid matrix to water in different temperatures is another consideration in lipid selection to reduce drug loss. On the contrary, it may be difficult to encapsulate hydrophilic drugs because these drugs migrate and deposit on the surfaces of LNs. Besides selection of suitable excipients, it is important to select proper preparation techniques. For example, insulin SLNs prepared by solvent emulsification evaporation technique obtained encapsulation efficiency of 68%, higher than 38% of microemulsion technique (Na et al., 2006).

20.3.1.5 Stability

Stability, either chemical or physical, is another major consideration in formulation development, especially for nanoparticle-based formulations. The physical stability issues, for example, particle size variation and payload leakage, may significantly change the in vivo performance of nanoparticles. In LNs, features like super-cooled melts and gelation, coexist with pure colloidal lipid dispersions. These features are more sensitive to crystallization–polymorphic transitions and accordingly highly related to the changes in particle size and drug loading (Ram et al., 2012; Heurtault et al., 2003). Lipid composition and storage conditions therefore govern the storage stability of LNs. Exposure to light may induce crystallization of lipids, leading to gelation and increasing of particle sizes, whereas the destabilizing speed is related to the intensity of the light (Thukral et al., 2014). Similarly, temperature may lead to changes in the crystalline structure of lipids causing destabilization of the SLNs, due to reduction in their zeta potential values (Freitas and Müller, 1998). Electrolytes also influence the stability of LNs partly due to the effects on zeta potential (Choi et al., 2014).

20.3.2 POLYMORPHISM AND DEGREE OF CRYSTALLINITY

Lipids used for LNs preparation can exist in polymorphic forms (Clarkson and Malkin, 1948). Although chemically identical, the polymorphs exhibit different properties, that is, solubility and stability. The predominant forms of triglycerides are α - and β -polymorphs. β -Polymorph is the most stable polymorph due to the close chain arrangement and packing. Accordingly α -polymorph tends to convert to β -polymorph through a metastable form of β' -polymorph (Freitas and Müller, 1999; Jennings et al., 2000). During this transition, the arrangement of lipid molecules changes, leading to possible undesirable drug expulsion or alteration of drug release rate. Some lipids like trilaurin can form super-cooled melt (Bunjes et al., 1996b). They usually do not recrystallize until a temperature lower than the melting point (Bunjes et al., 1996b; Westesen and Bunjes, 1995). Formation of super-cooled melt may be ascribed to the presence of emulsifiers, the solubilization of the drug (Langevelde et al., 2001), or the small particle size (Bunjes et al., 2000). It was reported that particle size correlates with the reduction in melting point of lipids (Bunjes et al., 2000). In general, lipid crystallinity strongly affects drug incorporation, release rates, and stability of LNs. Thermodynamic stability and

lipid packing density increase, whereas drug incorporation rates decrease in the order of super-cooled melt, α -, β' -, and β -modification. Crystallinity and polymorphic behavior of LNs can be identified by X-ray diffractometry (XRD), differential scanning calorimetry (DSC), and Raman spectroscopy.

20.3.2.1 X-ray diffractometry

XRD has been widely utilized to study crystallinity by determining the diffraction angles of the monochromatic beam of X-ray. Since the intensity and position of diffraction are unique to a specific crystalline structure, XRD patterns can be used to predict the manner of arrangement of lipid molecules and phase behaviors, and characterize and identify the structure of lipid and drug molecules (Bunjes et al., 2007; Estella-Hermoso de Mendoza et al., 2008). For example, modification with a single scattering reflex of the side chains of glycerol at a Bragg distance of 0.415 nm is called α -modification, whereas modification with two reflexes at Bragg distances of 0.389 and 0.420 nm is called β' -modification, and modification which does not fulfill the above criteria is called β -modification (Muller et al., 2008).

20.3.2.2 Differential scanning calorimetry

DSC can provide information on the type of the lipid modifications and the degree of crystallinity by measuring the melting points and melting enthalpies. Structural variations of materials lead to heat exchanges, that is, endotherm during melting and exotherm during crystallization. Measurement of heat by DSC allows drawing conclusions on polymorphic transitions and changes in lipid physical properties. Thermal cycle (heat-cool-heat) can be performed to measure the enthalpies of melting and recrystallization. Both α and β polymorphic forms have been identified in LNs by this technique (Laura et al., 2013). Most of the lipids used in LNs melt below 80°C, whereas drugs melt at higher temperatures. It is important to set a reasonable temperature range to avoid degradation of lipids. It should be noted that solubilization of drug in melted lipids may lead to disappearance or decrease of melting point. It is reported that gatifloxacin-loaded SLNs showed a sharp endotherm peak that cannot be assigned to either lipids or the drug (Kalam et al., 2010). The peak may be ascribed to either the dissolution of gatifloxacin in lipid matrix or the melting of gatifloxacin-lipid complex.

20.3.2.3 Raman spectroscopy

Raman spectroscopy provides information on polymorphic transitions by detecting molecular vibrations excited by a laser beam (Pratiwi et al., 2002; Tandon et al., 2000). Since water does not interfere with Raman analysis, LNs aqueous suspensions can be directly detected. Raman spectra is sensitive to conformational, packing, and dynamical changes of lipid chains (Wartewig and Neubert, 2005). Accordingly, the required sample amount for Raman detection is small.

20.3.3 ASSESSMENT OF COEXISTING STRUCTURES

Micelles, liposomes, mixed micelles, super-cooled melts, and drug crystals may coexist in LNs. It is essential to characterize and quantify the coexisting structures, because the *in vivo* performance of these colloids is different from LNs. However, the assessment is difficult due to similar morphology and ultra-small size. In addition, sample preparation process such as dilution may result in structural changes. Nuclear magnetic resonance (NMR) and electron spin resonance (ESR) are suitable techniques for investigating dynamic phenomena and the characteristics of the nanocompartments in colloidal lipid dispersions. These techniques are also noninvasive and do not require preparatory steps.

NMR detects the relaxation time for protons, which varies depending on their state and environment. By assigning chemical shifts to molecules, the protons of lipids can be easily identified. NLCs that contain solid and liquid lipids can also be characterized by NMR. Signals from the solid lipids are generally weak and broad, while the liquid lipids present sharp and narrow signals. Paramagnetic spin probes are used in ESR detection. Direct, repeatable and noninvasive measurement of the probe distribution between the aqueous and the lipid phase can be measured. Crystallization of LNs excludes the probes into the aqueous phase from the lipid phase.

20.3.4 IN VITRO LIPOLYSIS

Orally administered lipid formulations are fated to experience digestion in the gastrointestinal tract. Accordingly, the enhanced oral bioavailability by LNs is closely related to lipolysis of lipids. However, the degree of lipolysis in different gastrointestinal segments is different, due to difference in enzymatic activities. Lipid digestion is initiated by gastric lipases in stomach, where up to 25% of total triglycerides are partially digested to diglycerides and fatty acids (Porter et al., 2007). Pancreatic lipases are the main enzymes for lipolysis. In the presence of colipases, triglycerides can be broken down completely by pancreatic lipases to diglycerides, monoglycerides, and fatty acids (Porter et al., 2007). Therefore, the structure of LNs may be destroyed during the lipolysis process and the loaded drugs are released as a consequence. If precipitation of the released drug molecules occurs in the gastrointestinal tract, the oral bioavailability will certainly decrease. However, the presence of exogenous lipids in small intestine stimulates secretion of endogenous biliary lipids (bile salts, phospholipids, and cholesterol) from the gall bladder. In the presence of these biliary lipids, the lipid digestion products (monoglyceride, fatty acid, and lysophospholipid) are subsequently incorporated into a series of colloidal structures, that is, multilamellar and unilamellar vesicles, mixed micelles, and micelles. These species significantly increase the solubilizing capacity of drugs in the small intestine. The *in vitro* lipolysis experiment facilitates prediction of the changes in solubilization capacity that may occur upon digestion of LNs.

Realizing the effects of digestion on in vivo performance of lipid-based formulations, lipid digestion models have been widely adopted for in vitro assessment of lipid-based formulations (Porter and Charman, 2001; Porter et al., 2004; Christensen et al., 2004; Dahan and Hoffman, 2006). The models are composed of a thermostat reaction vessel (37°C), a pH electrode, a pH-stat meter controller and an autoburette. Digestion is initiated by addition of pancreatic lipases. Fatty acid, one of the products of lipolysis, causes a drop in pH, which is quantified by the pH electrode. A pH-stat meter controller and autoburette maintain the pH at a set point by addition of equimolar NaOH to automatically titrate the resulted fatty acids. The digestion speed and extent can be indirectly quantified by the addition rate and amount of NaOH added. After the digestion process, the lipolysate can be separated by ultracentrifugation into three phases, that is, a poorly dispersed oil phase, a highly dispersed aqueous phase, and a precipitated pellet phase. Quantification of the amount of drug distribution in these three phases provides important information on proclivity toward in vivo precipitation and hence prediction on in vivo performance of the formulations.

Besides the gastrointestinal tract, lipolysis of LNs can take place at sites even within cells where lipases are abundant. For example, the endocytosed LNs can be digested by lysosomal acid lipases (Xue and Wong, 2011), and the digestion completes for most lipids within 2 h (Xue and Wong, 2011). In addition, a majority of degraded lipids can be excreted out of the cell within 24 h, with approximately half detected in both urine and feces (Maier et al., 2013).

20.4 IN VIVO CHARACTERIZATION

20.4.1 PHARMACOKINETICS AND BIOAVAILABILITY

Taking advantage of the colloidal properties and the lipid compositions, LNs can improve the pharmacokinetic characteristics and bioavailability of the loaded drugs (Qi et al., 2012; Lu et al., 2012). Lipid composition may retard the release of the loaded drugs, leading to increased MRT and $t_{1/2}$. Moreover, LNs in blood stream may be ingested by macrophages, leading to quick clearance and accordingly decreased MRT and $t_{1/2}$ (Vonarbourg et al., 2006). It is essential to assess the pharmacokinetics as a measure to evaluate the in vivo performance of LNs.

Pharmacokinetics provide a general understanding of the dynamic profiles of the active ingredients. To date, there are many reports on the pharmacokinetics of SLNs administered by different routes. The general procedure is as follows: animals are divided into groups according to the preparations to be tested; blood samples are withdrawn in predetermined intervals post administration; the content of the target molecule in each sample is detected; drug concentration versus time curves are drawn based on the data; pharmacokinetic models are adopted to analysis these data for pharmacokinetic parameters.

Besides the original drugs, quantification of the metabolite is adopted in some cases. For example, the concentration of fenofibric acid is measured in the pharmacokinetic study of fenofibrate SLN (Weng et al., 2014). Fenofibrate is actually the prodrug of fenofibric acid. Mass spectrometry is usually used to achieve high sensitivity to observe concentrations after a low dose and a long time period. The most common instrumentation used in this application is LC-MS with a triple quadrupole mass spectrometer. Tandem mass spectrometry is usually employed for enhanced specificity. Except for drug concentrations, pharmacodynamic effects can be detected, especially for drugs derived from protein, peptides, and genes. Hypoglycemic effect, indicated as the level of blood sugar versus time, is usually detected in the study of pharmacokinetics and bioavailability of insulin-loaded LNs. Although the pharmacokinetic parameters obtained from drug concentrations and pharmacodynamic effects can evaluate the bioavailability of LNs, it should be kept in mind that these data cannot reveal the actual in vivo behavior of integral LNs. Limited by separation, discriminative quantification of integral LNs is still full of challenges.

Bioavailability can be defined as the proportion of the drug that reaches systemic circulation or the site of action. Therefore, intravenous administration provides the highest bioavailability, which is regarded as absolute (John, 2009). Bioavailability is important to evaluate the in vivo performance of LNs via routes other than intravenous administration. Bioavailability can be either absolute and relative, based on the reference administration routes. Absolute bioavailability compares the bioavailability obtained from nonintravenous administration to intravenous administration, while relative bioavailability compares bioavailability of different formulations obtained from nonintravenous administration. Usually, area under the curve (AUC) is used to calculate bioavailability. Once the bioavailability has been measured, it is possible to calculate the changes for its dosage in order to reach the required blood plasma levels. Bioavailability is, accordingly, a mathematical parameter that indicates the improved performance from formulations. The bioequivalence of formulations can also be evaluated.

20.4.2 BIODISTRIBUTION

Similar to other colloidal delivery systems, LNs may change the biodistribution of the loaded drugs and consequently affect their in vivo performance. The effects are more predominant in intravenous route than other routes. Due to the hydrophobic surfaces, LNs in the blood stream are prone to opsonization by the physiological clearance mechanisms and accordingly uptaken by macrophages. LNs are generally taken up by organs or tissues abundant of macrophages, such as liver, spleen, and lung (Du et al., 2010; Tian et al., 2008). Surface modification of LNs with PEG helps LNs evade the recognition and uptake of macrophages, and accordingly prolongs the circulation time of LNs in blood while decreases the accumulation in reticulo-endothelial organs (Wang and Wu, 2006; Li and Huang, 2009; Zhang et al., 2008a). LNs circulating in the blood may then accumulate to

the tumor site, due to the so called enhanced permeation and retention effect (Liu et al., 2015; Suk et al., 2016). Moreover, surface modification with ligands may further improve the tumor targeting efficiency, such as improved liver targeting by mannan or galactoside modification (Wang et al., 2010; Lu et al., 2008; Lian et al., 2008; Yu et al., 2010), improved breast tumor targeting by folate modification (Liu et al., 2009), and improved brain targeting by transferrin modification (Gupta et al., 2007).

For biodistribution study, animals are sacrificed in predetermined intervals and dissected to collect organs and tissues. Drug contents in organs and tissues are then detected to draw drug concentration versus time curves in corresponding tissues and organs. Biodistribution or targeting of preparations can be evaluated through three indicators, that is, relative uptake rate (r_e), targeting efficiency (t_e), and ratio of maximum concentration (C_e). It is noted that the evaluation is still based on the detection of drug molecules, which, however, cannot represent the actual biodistribution of LNs per se.

$$r_e = (AUC_i)_p / (AUC_i)_s$$

where, AUC_i is the area under the curve for tissue i calculated by drug concentration versus time curves; subscript “p” and “s” represent preparation and drug solution, respectively. The value r_e indicates the selectivity of different preparations to the same tissue or organ. Targeting to tissues or organs is confirmed by r_e values larger than 1. The larger the r_e value is, the higher the targeting effects is achieved. An r_e value equal to or less than 1 indicates no targeting effect at all.

$$t_e = (AUC)_{\text{targeting}} / (AUC)_{\text{non-targeting}}$$

where, AUC is the area under the curve for the tested tissue or organ. The value of t_e indicates the selectivity of preparation to targeting tissue or organ. The larger the t_e value is, the stronger the selectivity is achieved. The t_e value larger than 1 indicates that the preparation is selective to the targeting tissue than the nontargeting tissues. By comparing the ratio of t_e values preparation to solution, the enhanced folds of targeting by preparation can be calculated.

$$C_e = (C_{\max})_p / (C_{\max})_s$$

where, $C_{\max}$ is the maximum concentration; subscript “p” and “s” represent preparation and drug solution, respectively. C_e value indicates the selectivity of different preparations to the same tissue or organ. C_e value in each tissue or organ indicate the extent of distribution alteration induced by targeting preparation. The larger the C_e value is, the more significant the distribution is altered.

20.4.3 IN VIVO IMAGING

The in vivo distribution of nanoparticles in one single animal cannot be continuously tracked by the afore mentioned animal experiment, and the labor intensity

for the traditional biodistribution experiment is usually huge. In order to conquer these disadvantages, the concept of molecular imaging was proposed by Weissleder from Harvard University in 1999 (Weissleder, 1999). It enables the visualization of the cellular function and tracking of the molecular process in living organisms without disturbing them. With this technique, the in vivo fate of nanoparticles can be tracked continuously on an individual animal. Interindividual deviation, experimental cost, and labor intensity are significantly lowered. Living imaging has been widely applied for evaluation of biodistribution of nanoparticles.

Living imaging is categorized into radio-nuclear imaging, optical imaging, magnetic resonance imaging, ultrasound transmission tomography, and computed tomography. Radio-nuclear imaging and optical imaging have been used to characterize in vivo distribution of LNs.

20.4.3.1 Radio-nuclear imaging

Radionuclides with gamma (γ) emissions can be used for imaging and quantification of in vivo distribution of nanoparticles, as γ radiation can be detected and recorded by gamma camera through γ -scintigraphy and γ -counting. ^{67}Ga , ^{123}I , ^{131}I , ^{111}In , and $^{99\text{m}}\text{Tc}$ are γ -emitting radionuclides generally used for gamma imaging. $^{99\text{m}}\text{Tc}$ was labeled to etoposide, negative and positive charged tripalmitin nanoparticles, respectively, for evaluation of the pharmacokinetics and biodistribution of LNs after i.v. administration (Reddy et al., 2004). The positively charged LNs present the highest blood concentrations with long-circulating properties among the radiolabeled complexes. The LNs, irrespective of surface charges, show significantly lower accumulation to organs abundant with reticulo-endothelial system than etoposide. However, the positively charged LNs show higher distribution to bone and brain than the negative ones. Similarly, $^{99\text{m}}\text{Tc}$ labeling was used to compare the in vivo behavior of i.v. LNs with different particle sizes (Hirsjarvi et al., 2013b). Smaller LNs (25 nm) were eliminated faster from the blood circulation than bigger ones (50 and 100 nm). However, the biodistribution profiles of these LNs at 24 h were similar. Only low quantities of LNs were detected in tumor sites regardless of the particle size.

Radionuclides with positron emissions can be used for positron emission tomography (PET) imaging. The emitted positron travels a short distance before it encounters an electron. The annihilation converts their momentum and energy into two γ -rays with opposite direction and equal energy of 511 keV. A ring of detectors detects the two γ -rays for PET imaging. PET imaging provides higher spatial resolution and is more sensitive than the conventional imaging techniques. ^{11}C , ^{14}N , ^{15}O , and ^{18}F that emit positive electron can be adopted in PET, but considering the half life, ^{18}F is more manageable than others.

20.4.3.2 Optical imaging

Bioluminescence imaging is one kind of optical imaging, which takes advantage of the process of light emission in organisms. It is a form of chemiluminescence

involving the light-emitting pigment luciferin and the enzyme luciferase. The enzyme catalyzes the oxidation of luciferin to emit light. During the process, target cells are firstly transfected by luciferase gene expression vector and transplanted into target organ of recipient. Exogenous luciferin is then introduced for bioluminescence. Bioluminescence imaging was used to evaluate the abilities of LNs in delivering small-interfering RNA (siRNA) to hepatocytes of live animals via systemic administration (Tao et al., 2010). Luciferase siRNA loaded LNs were injected to mice with liver-specific expression of firefly luciferase. The reduction of luciferase expression as indicated by reduced bioluminescent intensity can be used to characterize the delivery capability of LNs.

Fluorescent imaging is another real-time, noninvasive, and radiation-free optical imaging technology. However, until now, in vivo fluorescence imaging is principally performed in rodents. Fluorescent labeling, either on the payload or the carrier, is required to generate in vivo emission source. The labeled nanoparticles can be detected by in vivo imaging system (IVIS) spectrum imaging system to acquire spatio-temporal information of biodistribution both qualitatively and quantitatively. In order to achieve deep tissue penetration and high spatial resolution, near-infrared fluorescent probes are preferable. However, the biggest challenge lies in the ability to discriminate nanoparticles per se from the signals of free probes that may be misread as integral nanocarriers (Hu et al., 2016a; Qi et al., 2016). Only when traditional probes are securely anchored to the nanocarriers can they work well to represent integral nanocarriers. As discussed previously, in vivo lipolysis of lipid is inevitable, especially in gastrointestinal tract. The labeled fluorescence may escape from LNs due to drug release and/or degradation of the carriers. Indocyanine green (ICG), a cyanine dye, was found leaking quickly from LNs in blood after i.v. administration, leading to discrepancy in biodistribution patterns between integral LNs and fluorescent signals (Mérián et al., 2015).

To accurately identify LNs in vivo, it is crucial to discriminate the nanocarrier-bound signals from those of the free probes. Förster resonance energy transfer (FRET) imaging is an appropriate approach to achieve this goal. FRET is a nonradiative energy transfer process, where donor fluorophores in electronic excited state transfer energy to spatially close (2–10 nm) acceptor fluorophores. It occurs when the emission spectrum of a donor fluorophore overlaps with the absorption spectrum of an acceptor. Yet, since the efficiency is inversely proportional to the sixth power of the distance between donor and acceptor fluorophores, FRET is extremely sensitive to changes in donor–acceptor distance, making FRET imaging a “nanoscale ruler” (Sahoo, 2011). Accordingly, the signal switching provides information on the structural changes of the integrity of the nanoparticles as well as the release of the cargoes. FRET imaging has been utilized to study the in vivo behavior of LNs, such as stability in blood circulation, spatio-temporal degradation kinetics, and biodistribution (Laine et al., 2014; Gravier et al., 2014; David et al., 2012; Hirsjarvi et al., 2013a). For example, octadecyl indocarbocyanines (DiI) and oxacarbocyanines (DiD) were coencapsulated in the core of LNs for FRET imaging (Gravier et al., 2014). It was found that the

particles were destroyed immediately upon incubation with serum, indicated as an instant diminution of 20% FRET fluorescent density. However, LNs were stable in subcutaneously implanted tumors, as FRET signal can be observed in tumor slices 24 h after administration. It should be noted that the released FRET pairs may be relocated to the cell membrane, leading to reappearance of FRET signals (Chen et al., 2008a,b).

Aggregation caused quenching (ACQ) effect is a common phenomenon found for fluorescent probes, which is usually required to be avoided or reduced when designing new fluorescent probes. However, the ACQ probe may serve as excellent environment-responsive probes for smart bioimaging. A series of ACQ probes, bearing BODIPY or aza-BODIPY structure, have been introduced to study the in vivo fate of nanoparticles (Hu et al., 2015, 2016b; Tian et al., 2016; Xie et al., 2016a,b). This kind of ACQ probe emits near-infrared fluorescence if well dispersed in nanoparticles, but quenches immediately upon contact with water through π - π stacking when they are released from nanoparticles (Fig. 20.2). Since water is ubiquitous in living organisms, fluorescent signals from these dyes represent integral nanoparticles throughout the body under any circumstance. It was verified that the quenching of the fluorescence correlated well with both in vitro and in vivo lipolysis of SLNs (Hu et al., 2015). Based on ACQ probes, predominant digestion of SLNs were observed within 2 h and complete digestion within 4 h after oral administration to rats. On the contrary, DiR (nonwater-quenching probe)-loaded SLNs always displayed strong fluorescence, although significant digestion of SLNs had occurred (Hu et al., 2015). The study indicates that the signals of normal fluorescence cannot be used to track the behavior of integral nanoparticles. Furthermore, it was found that the integral SLNs cannot penetrate across the intestinal epithelia (Hu et al., 2016b).

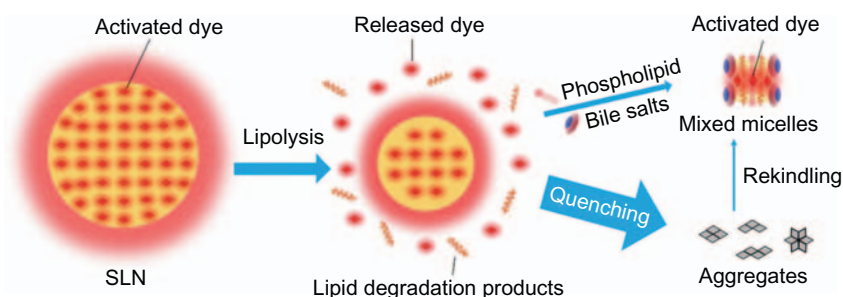


FIGURE 20.2

Schematic illustration of ACQ dyes quenching in water as probes to monitor the integral SLNs.

Source: Adapted from Hu, X., Zhang, J., Yu, Z., Xie, Y., He, H., Qi, J., et al., 2015. Environment-responsive aza-BODIPY dyes quenching in water as potential probes to visualize the in vivo fate of lipid-based nanocarriers. *Nanomedicine* 11, 1939–1948.

20.5 MODELS FOR SPECIFIC ADMINISTRATION ROUTE

20.5.1 EVALUATION OF LYMPHATIC ABSORPTION

Lymphatic transportation plays an important role in oral absorption of lipophilic drugs. Drugs transported via intestinal lymph circumvent first-pass metabolism in liver and directly enter into the systemic circulation. Lymphatic transportation may also improve pharmacokinetics and systemic distribution patterns of drugs (Caliph et al., 2000; Hauss et al., 1994). In addition, the lymphatic system plays an important role in immune responses, tumor metastases and dissemination of infections. Lymphatic transportation of anti-HIV and anticancer drugs will certainly elevate drug concentration in lymph, leading to improved therapy. It is found that lymphatic absorption of drugs can be significantly enhanced by coadministration with high fat diet, because the exogenous lipids facilitate intestinal absorption of lipophilic drugs by enhancing luminal solubilization and stimulating chylomicron secretion for lymphatic transportation. Therefore, it is essential to evaluate the contribution of lymphatic absorption to the bioavailability of LNs.

Various animal models have been developed to evaluate intestinal lymphatic drug transportation (Edwards et al., 2001; Bargoni et al., 1998; Paliwal et al., 2009; Cho et al., 2014). The general principle of these models is to collect lymph by cannulation into either the mesenteric or thoracic lymph duct. The total amount of drugs transported through lymph can then be measured and used to calculate the lymphatic transportation percentage by comparing with the administered dose. Systemic plasma drug concentrations measured by this model can be used to evaluate the extent of absorption from portal blood as well. The contribution of portal blood absorption to bioavailability can be determined by the ratio of the AUC obtained in orally dosed lymph-cannulated animal to the AUC from a sham operated animals after i.v. injection. Therefore, besides lymph duct cannulation, the models include carotid artery cannulation for blood collection. If necessary, jugular vein cannulation can be adopted for intravenous administration. In some cases, such as in unconscious animals, duodenum cannulation can be performed for administration of formulations and rehydration solution. Interested readers can refer to literatures for detailed surgical procedures (Edwards et al., 2001; Boyd et al., 2004; Hauss et al., 1998; Girardet and Benninghoff, 1973; Girardet, 1975).

Rats are the most commonly used animals for cannulation of lymph duct because they are robust and inexpensive, and the model is relatively simple to establish. Based on the status of the animals, the model can be further subdivided into unconscious, conscious restrained, and conscious unrestrained rat models. The surgical procedures are the same and performed under anaesthetized state, except that animals are fully recovered from anesthesia in the latter two models. The recovered rats are placed in a restraining cage in the conscious restrained rat model, or move freely with a harness containing changeable lymph collection bottles in the conscious unrestrained rat model. Although the anaesthetized animal

model excludes problems associated with animal movement, the lymph flow is significantly reduced to 0.1–0.6 mL/h from 1–3 mL/h in conscious rats. Accordingly, the conscious unrestrained rat model is more favorable. In this model, continuous lymph collection over 12 h and simultaneous systemic blood collection over 30 h can be achieved.

20.5.2 DERMAL DELIVERY

Dermal delivery is attractive for treatment of both systemic and local diseases. For systemic delivery, the dermal route provides sustained drug plasma levels, with reduced fluctuation and circumvents hepatic first-pass elimination. The topical treatment also reduces systemic side effects associated with parenteral or oral drug administration and increases local drug concentration. However, the *stratum corneum*, which separates the internal environment of the body from the external environment, presents as a barrier to exogenous substances. Only a limited number of drug molecules with proper physicochemical properties, such as molecular weight less than 500 Da and log *P* between 1 and 3, can penetrate the skin (Guy and Hadgraft, 1987). However, nanocarriers, especially LNs, are advantageous to improve drug permeation into the skin (Bolzinger et al., 2012; Gupta et al., 2012; Khan et al., 2015). LNs may adsorb onto the skin surface and prolong the residence time of drugs (Kuntsche et al., 2008; Chen et al., 2006; Puglia et al., 2008; Jensen et al., 2011), whereas the LNs film may reduce the barrier function of the *stratum corneum* due to the occlusion effects (Jensen et al., 2011). In addition, excipients used in LNs, such as surfactants, may interact with skin lipids and loosen the packing of corneocytes, facilitating drug penetration (Khurana et al., 2013; Lv et al., 2009). Moreover, LNs may fuse and mix with *stratum corneum* lipids, promoting drug partition into the skin (Kuchler et al., 2010). However, debates still exist concerning the penetration of integral nanoparticles, the transdermal routes and factors governing penetration. It is important to establish ex vivo and in vivo models to evaluate skin permeation and elucidate the penetration mechanisms.

20.5.2.1 Ex vivo permeation study

Permeation study is the most important ex vivo characterization for topical formulations. It is generally performed in Franz diffusion cells. Skin is mounted between the donor cell and the receptor cell with epidermis facing the donor side. The dermis is bathed from below with receiving media which is maintained at 32°C to mimic the skin temperature. A stirring bar can be put at the bottom of the receptor cell for electromagnetic stirring. Formulations are directly applied to the epidermis through the open cap of the donor cell, while receiving media can be withdrawn at predetermined intervals to analysis the permeated drug amount. The flux (permeation rate) can be obtained from the slope of the cumulative amount permeated per unit area versus time plot. It is worth noting that the skin model, temperature, receiving media, dose application and diffusion area can all

significantly influence the results. Human skin is the most favorable, but is usually hard to acquire. Due to the availability, the skin of rodents (mice, rats, and guinea pigs) is the most used *ex vivo* model in permeation studies.

20.5.2.2 *In vivo* evaluation

For systemic delivery, blood pharmacokinetics should be tested to evaluate the systemic bioavailability. The process is similar to the general procedures in pharmacokinetic study, except that the formulations are applied to the skin. After penetration, the skin sample can be extracted for quantitation of the retention and penetration of drugs into the skin. Skin slicing, vertical or horizontal, can also be utilized for qualitative evaluation. Micrographs, such as fluorescence microscopy, confocal laser scan microscope (CLSM), and confocal Raman microscopy or spectroscopy, can provide information on the penetration depth and paths of LNs into the skin (Zhang et al., 2014; Charoenputtakun et al., 2015; Guo et al., 2015).

However, for topical therapy, the efficiency depends on the concentration of the active agent at the target site. The general procedures used for pharmacokinetics via other routes, for example, oral and intravenous, are not applicable because only limited amount of drugs enter into systemic circulation for topical formulations. The dermatopharmacokinetic profile is essential to assess the rate and extent of topical drug bioavailability. Tape stripping and dermal microdialysis are the promising tools to study the dermatopharmacokinetics, which can also be applied to humans (Incecayir et al., 2011).

In the tape stripping experiment, pre-weighed adhesive tapes are successively pressed onto and removed from the treated skin area to remove the corneocytes layer by layer (usually 15–20 tapes). The first tape strip is discarded to prevent contamination from residual drug on the surface of the skin. Each tape strip was weighed again to determine the weight of the removed *stratum corneum*. The tapes are then extracted to quantify the drug content. Usually, the formulation is applied to the skin for a certain time and removed. The tape stripping experiment is performed in determined intervals post-removal of the formulation. The tape stripping is a noninvasive method, but it mainly focuses on the *stratum corneum* and cannot provide information on local bioavailability in the underlying skin layers, that is, epidermis and dermis, where many dermatological diseases manifest. The *stratum corneum* is the principle barrier for topical penetration. Results from tape stripping can be used to predict the drug levels in the underlying viable tissues.

On the contrary, dermal microdialysis can be used to assess drug concentrations directly in dermis. The basic principle of dermal microdialysis is to insert a tube with semipermeable dialysis membrane into the dermis, parallel to the skin surface, to mimic the small blood vessels. A tissue-compatible fluid was continuously pumped through the tube by a peristaltic pump. The unbounded drugs in extracellular spaces of dermis can be removed by diffusing through the dialysis membrane of the tube for content analysis. A real-time drug penetration into the

dermis can be continuously monitored by dermal microdialysis, providing the full pharmacokinetic profiles.

20.5.3 PULMONARY DELIVERY

Pulmonary delivery has attracted significant attention for treatment of both local and systemic diseases, because the lung offers huge surface area, extensive vasculatures as well as extremely thin epithelium for rapid absorption, and circumvents the first pass effect of the liver (Paranjpe and Muller-Goymann, 2014; Sakagami, 2006; Weber et al., 2014). Due to relatively less enzyme activity, degradation of drugs in the lung is comparatively slow. The pulmonary route may also provide targeted drug delivery, reducing potential systemic toxicity. Over past decades, LNs have become one of the most interesting nanocarriers for pulmonary application, due to biocompatibility, biodegradability, sufficient drug loading, and stability during nebulization. However, development of LNs for pulmonary applications is still in its infancy.

20.5.3.1 *In vitro* aerosol characterization

Pulmonary delivery of LNs can be achieved by inhalation of nebulized LNs suspension or dry powders (Weber et al., 2014). It is necessary to achieve good aerodynamic properties of the generated aerosols for sufficient deposition in the lung and respiratory tract. Since the alveolar is the most favorable site for pulmonary absorption, it is necessary to deliver as many as possible nanoparticles to this site. Usually, inhaled aerosols larger than 5–10 μm are mainly retained in the oropharyngeal airway and accordingly cannot reach the lung; instead, aerosols smaller than 5 μm can penetrate into the lung; whereas, the smallest aerosols $\leq 1 \mu\text{m}$ can be exhaled without deposition (Wolff and Dorato, 1993; Sakagami, 2006). Therefore, the aerodynamic particle size distribution is important to evaluate the quality of LNs (Jaafar-Maalej et al., 2011; Patlolla et al., 2010).

A cascade impactor can be used for *in vitro* aerosol characterization. The instrument consists of a series of stages, each made up of a plate with specific nozzle arrangement and collection surface. Sample laden air is drawn into the impactor, flowing sequentially through the stages with nozzle size and total nozzle area decreasing with stage number. During the experiment, LNs are nebulized into the cascade impactor under certain flow rate, for example 30 L/min. Particles that pass through the nozzles either remain entrained in the air stream or impact with the collection surface based on the differences in inertia. Consequently, particles with sufficient inertia are collected, the rest pass onto the next stage. As nozzle size decreases, velocity increases, allowing the collection of increasingly small particles, and residual material is captured in a final stage or filter. Therefore, each stage is associated with a cut-off diameter and the sample is separated into a series of size fractions. For example, the effective cutoff aerodynamic diameter for each stage in an eight-stage next generation cascade impactor is in the following order of 11.71 μm , 6.39 μm for, 3.98, 2.3, 1.36, 0.83, and 0.54 μm

from stage 1 to 8 (Marple et al., 2003). The deposited drug in each plate is then extracted and quantified to draw the curve of cumulative mass distribution versus aerodynamic diameter. The aerosolization behavior can be evaluated by mass median aerodynamic diameter and geometric standard deviation (Majoral et al., 2006). The fine particle fraction, defined as the cumulative drug mass fraction with an aerodynamic diameter less than 5 μm , can be directly obtained from the curve.

20.5.3.2 Systemic pharmacokinetics and in vivo lung deposition

Systemic pharmacokinetics and in vivo lung deposition is performed in whole animals, usually small rodents like mice, rats, and guinea pigs. The general procedures are similar to that used in other administration route, but pulmonary administration may be challenging. Intratracheal instillation is the first experimental method established for the conduct of in vivo studies assessing drug absorption and disposition in the lung. Following anesthesia, the trachea of the animal is exposed and an endotracheal tube is inserted between the tracheal rings. A typical volume of 10–200 μL suspensions or solution can be instilled into the airways through the tube (Sakagami, 2006). Instead of undertaking a tracheotomy, orotracheal intubation by direct injection or spraying of nanoparticle suspensions into the lung can be employed. Moreover, nose-only models are introduced, in which, the nose of the animal is fixed in a chamber and directly exposed to the aerosol for inhalation. Several sophisticated dosing devices have been available for rodents, either being custom-made or from scientific suppliers. After exposure for a specified time, the animal is sacrificed, and the bronchoalveolar lavage fluid (BAL) is analyzed for the desired components.

20.6 CELLULAR MODELS

It is essential for nanoparticles to deliver the payload to specific organs, tissues, and cells, which requires that the nanoparticles overcome a number of transport barriers to reach the targets. There are barriers for absorption from the administration sites to the systemic circulation, such as the pulmonary, gastrointestinal, and transdermal barriers. Conversely, nanoparticles may enter into the target cells and release the drug molecules effectively. Understanding the interaction between nanoparticles and cells is extremely important for translation into clinical application.

20.6.1 ENDOCYTOSIS

20.6.1.1 Endocytosis pathways

Endocytosis is the process by which a cell takes up extracellular molecules into the cells by engulfing them in an energy-consuming process. Particles in blood

are opsonized by opsonins such as IgG and C3b. The opsonized particles can be recognized by phagocytic cells and bind to the cell membrane. The bound particles are internalized inside the cells in the form of endosomes, which enters into lysosomes and release the loaded drugs.

Endocytosis pathways can be subdivided into three categories, namely phagocytosis, pinocytosis, and receptor-mediated endocytosis. Phagocytosis is the process by which cells bind and internalize particulates larger than 1 μm in diameter. In the progress, pseudopods are formed through depression of plasma membrane, surrounding and internalizing particles. Pinocytosis usually occurs from highly ruffled regions of the plasma membrane, in which small particles are brought into the cell, forming an invagination, and then suspended within small vesicles. The vesicle then travels into the cytosol and fuses with other vesicles, such as endosomes and lysosomes, to hydrolyze (break down) the particles. This process requires energy in the form of adenosine triphosphate. Pinocytosis is used primarily for the uptake of extracellular fluids, including all solutes present. Receptor-mediated endocytosis, also known as clathrin-mediated endocytosis, is a process by which cells absorb metabolites, hormones, other proteins, and in some cases viruses (endocytosis), by inward budding of plasma membrane vesicles containing proteins with receptor sites specific to the molecules being absorbed.

20.6.1.2 Cellular uptake

The selection of cell types is dependent on the experimental purposes. For example, Caco-2 cell lines, derived from human epithelial colorectal adenocarcinoma cells, can be used to evaluate the absorption of LNs in intestinal epithelia (Chaudhary et al., 2015; Shah et al., 2013, 2015). In some cases, the LNs are utilized to deliver anticancer drugs (Xu et al., 2016; Akanda et al., 2015; Singh et al., 2016; Arranja et al., 2016). The corresponding tumor cells can then be applied. During the experiment, cells are seeded in plates and cultured until cells attach to the culture plate. Lipid nanoparticles can be then coincubated with the cells for a time period. In predetermined intervals, LNs suspensions are removed from the plates, and the ingested drug concentration can be measured following lysis. Alternatively, the LNs can be labeled with fluorescence, and cellular uptake can be detected by flow cytometry.

Except for quantification of cellular uptake, it is important to study the endocytosis pathways for LNs. The general experimental procedure is similar to the cellular uptake, but prior to the addition of LNs, specific inhibitors for specific cellular uptake pathways are pre-incubated with the cells. For example (He et al., 2013), methyl- β -cyclodextran and nystatin inhibits lipid raft/caveolae dependent endocytosis by cholesterol depletion and sequestration effects, respectively; 5-(*N*-ethyl-*N*-isopropyl)-amiloride is Na^+/H^+ ion channel blocking agent and inhibits macropinocytosis; phenylarsine oxide inhibits clathrin-mediated endocytosis by interacting with vicinal dithiol containing molecules. Compared with the group without inhibitors, the endocytosis pathways for specific LNs can also be proposed. Furthermore, the intracellular trafficking of nanoparticles can be performed

by subcellular organelle labeling. LysoTracker probe, ERTracker probe and MitoTracker probe can be used to label lysosome, endoplasmic reticulum, and mitochondria, respectively. The intracellular fluorescence-labeled LNs can be localized by CLSM.

20.6.2 TRANSLOCATION

Cell culturing on Transwell inserts is the most commonly used in vitro barrier model (Fig. 20.3). The Transwell insert is composed of an apical and a basolateral compartment separated by a permeable membrane. Cells are seeded on the membrane and cultured to mimic the barriers. Transwells can be used to quantify the cellular uptake of nanoparticles from the apical compartment and penetration of nanoparticles across the barriers. Based on the cell types, different epithelial monolayers can be established to simulate various biomembranes, for example, gastrointestinal and pulmonary epithelia. However, the Transwell inserts are not suitable to mimic the dermal epithelia, because the *stratum corneum*, the principal barrier for skin absorption, is hard to simulate. Instead, ex vivo Franz diffusion model is preferable for this purpose.

20.6.2.1 Intestinal permeability models

Caco-2 cell lines are the principle cell type for studies of nanoparticle translocation. Caco-2 cells are not used as individual cells but form confluent monolayer on the insert membrane. Under specific culture conditions, Caco-2 cells are differentiated to form a polarized epithelial cell monolayer that resembles the enterocytes lining the small intestine. The characteristic of enterocytes, such as tight junctions, microvilli, enzymes (peptidases, esterases), and transporters (P-glycoprotein, amino acids transporters, bile acids transporters, etc.) can be expressed by Caco-2 cells. Before the permeation experiment, Caco-2 cells are seeded in the Transwell inserts and cultured to form monolayers. Trans-epithelial electrical resistance, expressed as Ω/cm^2 , can be measured to verify the cell monolayer integrity, both before and after the experiments. During the permeation experiment, formulation can be added either in the donor compartment to study the transportation from the apical side to the basolateral side ($A \rightarrow B$), or in the acceptor compartment for $B \rightarrow A$ transportation.

However, the conventional Caco-2 monolayers cannot secrete mucus layer, which consists of mucin glycoproteins and forms viscoelastic gels. Because the

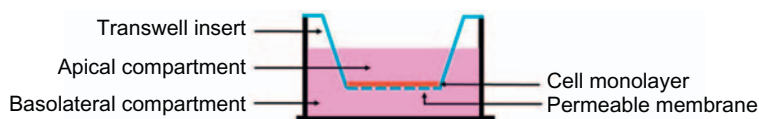


FIGURE 20.3

Structural illustration of Transwell.

mucus layer protects the intestinal epithelium from bio-relevant fluids in the lumen, it represents an actual barrier allowing selective passage of materials. Due to the entrapment in the mucus layer, the translocation of nanoparticles can be reduced (Braakhuis et al., 2015). Coculturing with HT29-MTX cells (human colon adenocarcinoma mucus-secreting cells) can be used to overcome this drawback of Caco-2 monolayers. In addition, Caco-2 monolayer is also lack of human intestine microfold (M) cells, which only account to less than 1% of intestinal epithelium, but are responsible for uptake and translocation of relatively larger particles. Caco-2/Raji coculture monolayer model can be used to mimic the function of M cell-associated phagocytosis (Martinez-Argudo and Jepson, 2008).

Recently, cellular uptake and trans-monolayer transport of ACQ-probe labeled SLN was studied in Caco-2 cells, Caco-2/HT29-MTX monolayers, and Caco-2/Raji coculture monolayer model, respectively (Hu et al., 2016b). The CLSM results from Caco-2 and Caco-2/HT29-MTX cell lines indicated bioadhesion of SLNs to cell membranes, instead of cellular uptake of intact nanoparticles. Due to the barrier of mucus layer, the bioadhesion of SLNs to Caco-2 cell lines is significantly higher than that in Caco-2/HT29-MTX cocultures. The trans-monolayer permeation studies on Caco-2 and Caco-2/HT29-MTX monolayers also confirmed no permeation of intact SLNs across both cell monolayers, as only faint fluorescence was measured in the receptor chamber. However, permeation of intact SLNs across Caco-2/Raji cell monolayers was observed, which is ascribed to the M cell pathway.

20.6.2.2 *In vitro lung barrier models*

Frequently used lung epithelial cell lines are A549, Calu-3, H441, and 16HBE14O, and the latter three can form tight junctions (Braakhuis et al., 2015). Rat alveolar cells can also be used to perform the *in vitro* translocation studies (Fazlollahi et al., 2011; Geys et al., 2009). Based on the culture method, the *in vitro* lung barrier models can be divided into the submerged and the air–liquid model. Although the submerged model is technically simple, the culture medium may affect the interaction of nanoparticles with the cells. Air–liquid models is more complex to maintain constant temperature and humidification, but mimic more realistically the inhalation exposure (Xie et al., 2012; Lenz et al., 2013).

20.7 CONCLUSION

LN combine the advantages of both o/w nanoemulsions and polymeric nanoparticles, while circumventing their drawbacks. Due to physical stability, protection of loaded drugs, controlled release, high biocompatibility, and ease of scaling-up, LNs show great potential in drug delivery and cosmetics. Several techniques have been developed to prepare LNs, improved to incorporate both hydrophobic and hydrophilic compounds. Routine issues of characterization for nanoparticles, for

example, particle size, zeta potential, morphology, drug loading, and drug release, are also applicable to control the quality of LNs, while polymorphism and degree of crystallinity and lipolysis are more specific for the lipidic features of LNs. Pharmacokinetics, biodistribution, and in vivo imaging provide important information on the in vivo performance of LNs. Specific models have also been established for in vivo characterization of LNs of different administration routes, such as lymphatic absorption, dermal, and pulmonary delivery. In addition, different cell models have been introduced to evaluate the acting mechanisms of LNs. With the improvement of preparation techniques, in vitro and in vivo characterization technologies, it is believed that more and more LNs preparations can be available in the near future.

ACKNOWLEDGMENTS

The authors thank for the financial support from National Natural Science Foundation of China (81573363, 21372063), Natural Science Foundation of Shanghai (16ZR1403500, 15ZR1403000), Shanghai Commission of Science and Technology (14JC1490300), and National Key Basic Research Program (2015CB931800).

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Index

Note: Page numbers followed by “f” and “t” refer to figures and tables, respectively.

A

- Abraxane, 27, 63, 342, 350–352
- Absorption, distribution, metabolism and excretion (ADME), 52–53
- Absorption window, 588–589, 595–596
- Aceclofenac, 604t
- Acinetobacter baumannii*, 274–275
- Acoustic streaming, 170
- Acrylic resins, 573
- Active targeting, 42, 123, 303–304, 343–344, 690–691, 692f
- Acyclovir, 604t
- Acylated β -CD, 720t
- Adeno-associated viral vector, 481t, 485
- Adeno-associated virus (AAV), 485, 526–528
 - AAV/P hybrid vector, 481t
- Adeno-associated virus-mediated gene therapy with radiation therapy, 501–502
- Adeno-associated virus/phage (AAVP), 487–488, 499, 503–504
 - clinical potential of, 492
 - for eukaryotic cell transduction, 491–492
 - studies to date, 489t
- Adeno-associated virus/phage-tumor necrosis factor with radiation therapy in metastatic melanoma model, 502–503
 - toxicity and preclinical safety of, 500–501
- Adenoviral vectors, 481t, 484–485
- Adenovirus, 484–485, 525–526
- Adhesion, mechanism of, 573–574
- Affibodies, 345
- Agglomeration, 48–49
- Aggregation caused quenching (ACQ) effect, 767, 767f
- Aggregation number, 296
- Aggressive variant prostate cancer (AVPC), 494–495
- Albumin-paclitaxel nanoparticles, 384f
- Alentuzumab (Campath), 20
- Alginate, 571
- Alginate-based hydrogels, 658
- Alkylated β -CD, 720t
- Alkylcyanoacrylates, 104
- α -glucosidase inhibitor (antidiabetic), 604t
- Altered density system, 580, 600
- Aminopropyltriethoxysilane (APTS), 284
- Amoxicillin, 604t, 609t
- Amphiphilic cyclodextrin nanoparticles, 724–730
- Amphiphilic cyclodextrins, 721–724
 - anionic, 723–724
 - cationic, 723
 - nonionic, 722–723, 722f
- Amphiphilic di-block copolymers, 319
- Amphiphilic star copolymers, 535
- Angiogenesis, 691
- Anionic liposomes, 531
- Anionic β -CDs, 720t
- Anthracycline-based drugs, 12–14
- Antiadherents, 586
- Anti androgens, 9t
- Antianginal, 609t
- Antiasthmatic, 609t
- Antibiotic resistance, 261–262, 286–287
- Antibiotics as antitumor drugs, 12–14
- Antibody-drug conjugates (ADC), 25–26
- Antibody mediated targeting, 692
- Anticancer, 604t, 609t
- Anticancer therapeutic agents, delivering via stimuli-responsive drug delivery systems, 468–470
- Antidiabetic, 604t, 609t
- Antifibrinolytic drug, 604t
- Antigen-responsive hydrogels (ARHs), 649t
- Antigens, 19, 121
- Antihyperlipidemic, 604t
- Antihypertensive, 604t, 609t
- Antimalarial, 604t
- Antimetabolites, 11–12, 12t
- Antimicrobial resistance (AMR), 261–262
- Anti-miRs, 521, 522f, 533, 545
- Antioxidant, 90–91, 609t
- Antiplatelet drug, 604t
- Antiviral, 604t
- Apoptotic vesicles, 532
- Aptamers, 347, 539, 692–693
- Aquasomes, 149
- Area under the curve (AUC), 763–764, 768
- Aromatase inhibitors, 9t
- Artesunate, 604t
- Asialoglycoprotein receptors (ASGPR), 125
- Aspergillus fumigatus*, 231
- Atom free radical polymerization (ATRP), 105, 636
- Atomic force microscopy (AFM), 58–61, 416, 758

Atom transfer radical polymerization (ATRP), 105, 308–311, 464
 Atopic dermatitis (AD), 395–396
 ATP binding cassette (ABC) transporter, 43–44
 ATP-sensitive potassium channels (K_{ATP})
 channels, 674*f*, 676–678
 Atrial natriuretic peptide (ANP)-modified NPs, 390
 Avastin (bevacizumab), 220
 Average quasi valence number (AQVN), 358
 Azobenzene, 317–319, 463–464

B

Bacillus subtilis, 90–91, 270–274
 Bacterial biofilms, 626
 Bacteriophage, 481*t*, 487
 Bacteriophage vector, 482–483
Basella alba, 100
 Bcl-2, 525
 β -blocker, 604*t*
 β -glycerolphosphate, 228
 β -lactum antibiotic, 604*t*
 Bevacizumab, 20, 220
 Bioadhesion of a polymer, 573–574
 Bioadhesive oral delivery, 591–592
 Bioavailability, defined, 763
 Biocompatibility, 47, 67–68, 445
 Biodegradability, 47
 Biodegradable polymers, 314, 392, 536
 Bioluminescence imaging (BLI), 493, 765–766
 Biopharmaceutical classification system (BCS), 566, 581–582
 Biopolymer-based nano-encapsulated substances, 54
 Biosensor and biolabels, 134–135
 BK virus, 484–485
 Bleomycin, 469*t*
 Blood–brain barrier (BBB), 86–87, 107, 269–270, 674–675
 versus blood–brain tumor barrier, 674*f*
 Blood–brain tumor barrier (BTB), 675–676
 assessment of, 678–681
 biochemical modulation of, 676
 functional ion channels on, 677–678
 nanotechnology for improved brain tumor
 detection and treatment, 682–683
 permeability, 675–676
 versus blood–brain barrier, 674*f*
 Blood–retina barrier (BRB), 204
 Bortezomib, 16
 Bouquet-like CDs, 723
 Bowel conditions, treatment of, 604*t*
 Bowman's membrane, 203

Bradykinin (BK), 676
 Brain, nanoparticles targeting, 392–394
 Brain capillary endothelial cells (BCEC), 392
 Branched β -CD, 720*t*
 Breast cancer, 12–14, 495
 Brownian motion, 47–48
 Buckminsterfullerene, 89–90
 Buckyballs, 89
 Budesonide, 604*t*
 Bulk powder properties, 583

C

Caco-2 cell lines, 774–775
 Caco-2 cell model, 592–593
Caenorhabditis elegans, 513
 Calcium channel blocker, 609*t*
 Calcium-dependent potassium channels
 Camptothecin, 174–175, 731
 CaNanoLab, 366
 Cancer, treatment strategies in, 1, 3*f*
 chemotherapy, 9–17
 antibiotics as antitumor drugs, 12–14
 antimetabolitic agents, 11–12
 cell cycle inhibitors, 10–11
 different types of drug delivery, 14
 main logic of chemotherapeutic application, 10
 proteasome inhibitor drugs, unexpectedly
 good results from, 16–17, 17*f*
 topoisomerase inhibitors, 14–16
 conventional surgery, types of, 4–5
 curative or primary surgery, 4
 diagnostic, 4
 palliative surgery, 4–5
 preventive (prophylactic) surgery, 5
 staging, 4
 surgery to debulk cancer, 4
 hormone therapy, 8
 immunotherapy, 18–20
 importance of diagnosis in cancer therapy, 1–2
 nanotechnology, smart role of, 26–31
 radiotherapy, 6–8
 main logic of radiotherapy application, 8
 types of, 7–8
 reconstruction, 5
 surgical application, history of, 3
 types of surgery, 5–6
 well known immunotherapeutic agents, 20–26
 adoptive cell therapies by using lymphocytes, 23–24, 24*t*
 antibody-drug conjugates, 25–26
 cellular immunity, 20–23
 genetic engineering in T cell, 24–25

- Candida albicans*, 90–91, 169, 231
- Candidate diseases, 149
- Captopril, 604*t*
- Carbohydrate-specific (lectin) receptors, 124
- Carbomer, 224–225, 574
- Carbon dioxide, 266–267
- Carbon disulfide, 90
- Carbon nanomaterials, 98
- Carbon nanotubes (CNTs), 136, 159
 - CNT-doped TiO₂ thin films, 275
- Carbopol, 569–570
- Carbopol 934, 574
- Carbosilane dendrimers (CBS), 703
- Carboxyfluorescein succinimidyl diacetate ester (CFSE), 423–424
- Cardiac cells, nanoparticles targeting, 390
- Carrageenans, 211
- Carrier systems, types of, 127*f*
- Carrier target identification, 140, 142
- Carvedilol, 604*t*, 609*t*
- Cascade molecules, 97
- Catalase, 428
- Catechol-conjugated poly(vinylpyrrolidone) sulfobetaines (C-PVPS), 285–286
- Cationic lipid compatible systems, 148–149
- Cationic lipids, 148
- Cationic liposomes, 227–228, 530–531
- Cationic polyplexes, 537–538
- Caveolin-dependent endocytosis, 421
- CD4 + T helper cells, 23
- CD8 + cytotoxic T cells, 23
- CD44, 175, 400, 542
- CD47, 167–168
- CD polymeric nanoparticle technology (CDP), 739–740
- Celecoxib, 223–224
- Cell and organ targeting, using nanoparticles, 379–380
 - characteristics of nanoparticles affecting drug targeting, 381–382
 - drug release, 382
 - particle size, 381
 - surface properties of nanoparticles, 381–382
- intracellular targeting, 380–381
- modifying nanoparticles for cell, organ specific targeting, 382–383
- nanoparticles targeting cells, 383–390
 - cardiac cells, 390
 - lymphocytes, 387–388
 - neutrophils, 388–390
 - tumor cells, 383–387
- nanoparticles targeting organs, 390–399
 - brain, 392–394
 - inner ear, 399
 - intestinal epithelium, 398–399
 - lungs, 390–391
 - nanoparticles in diagnosis, 400–401
 - protein, 399–400
 - skin, 395–398
 - vascular endothelium, 394–395
- Cell cycle inhibitors, 10–11
- “Cell-free layer” (CFL), 395
- Cell-level targeting, 122
- Cell membranes
 - lipid components of, 121
 - receptors on, 121
- Cell-penetrating peptides (CPPs), 349–350
- Cell surface antigens, 120–121
- Cellular immunity, 20–23
- Cellular uptake, 137
- Cellulose ethers, 572
- Cellulosine, 716
- Central nervous system (CNS), 86–87
- Cephalexin, 604*t*
- Cephalosporin, 604*t*
- Ceramide, 410
- Cerium dioxide (CeO₂), 389
- Cetuximab (Erbix), 20
- cGMP, 676
- Chemical cross-linked hydrogels, 627–628
- Chemical cross-linking, 648–654
 - by chemically reacting complimentary groups, 654–656
 - cross-linking by addition reaction, 655–656
 - cross-linking by condensation reactions, 656
 - cross-linking with aldehydes, 654–655
 - by high energy irradiation, 656–657
 - by radical polymerization, 654
 - using enzymes, 657
- Chemical exchange saturation transfer (CEST), 328
- Chemical stimuli, 648
- Chemical stimuli-responsive delivery, 171–176
 - enzymes stimulation, 175–176
 - pH stimulation, 171–174
 - redox activity stimulation, 174–175
- Chemistry, manufacturing, and controls (CMC), 594–595
- Chemotherapy, 9–17
 - antibiotics as antitumor drugs, 12–14
 - antimetabolite agents, 11–12
 - cell cycle inhibitors, 10–11
 - different types of drug delivery, 14
 - main logic of chemotherapeutic application, 10
 - topoisomerase inhibitors, 14–16
 - unexpectedly good results from the proteasome inhibitor drugs, 16–17, 17*f*
- Chimeric antigen receptors (CAR), 24–25

- Chitosan, 223–224, 228, 239–241, 391, 538, 571, 694–695
- Chitosan-graft-poly(*N*-vinyl caprolactam) nanoparticles, 164
- Chitosan-modified gold nanoparticles, 282–283
- Chitosan-modified PLGA nanoparticles, 698
- Chitosan nanoparticles (CNPs), 106, 175
- Chitosan/ γ -PGA nanoparticles, 272
- Chloroform, 99, 453–455
- Chloroquine, 504–505
- Cholesterol-PEG solid lipid nanoparticles, 173–174
- Chronic kidney disease (CKD), detection of, 401
- Cinnamon bark extract (CBE), 272
- Cinnarizine, 173
- Ciprofloxacin, 223–225, 227
- Circadian rhythm, 569
- Cisplatin, 469*t*
- Clathrin-mediated endocytosis, 421, 773
- Coacervation/ionic gelation method, 100
- Co-enzyme A (CoA), 640–641
- Cold homogenization technique (CHT), 754–755
- Colloidal carrier systems, 123
- Colloidal drug delivery system, nanoparticles as, 224–225
- Colony-forming units (CFUs), 390–391
- Composite hydrogels, 641–642
- Computed tomography (CT), 235, 400–401
- Computer modeling, 592–593
- Cone cells, 204
- Confocal laser scan microscope (CLSM), 770
- Continuous solvent evaporation method (CSE), 451
- Controlled drug release, 164
- Controlled/living radical polymerization, 105
- Controlled release dosage forms, 564–565
- extended release, 565
- prolonged release system, 565
- sustained release, 564
- Conventional eye drops, 201–202
- Conventional hydrophobic polymers, 314
- Copolymeric hydrogels, 208, 646
- Corneal regeneration capacity
- effect of hydrogels on, 237
- Corticosteroids, 8
- COX-2 inhibitor, 223–224
- Coxsackie-adenovirus receptor, 484–485
- Critical gel concentration (CGC), 631–632
- Critical micelle concentration (CMC), 296–297, 534
- Critical quality attributes (CQAs), 594–595
- CRLX101, 739–740
- Cross-linking, defined, 642
- Cross-linking methods, 660
- advantages and disadvantages of, 655*t*
- Cryosurgery, 5
- Cryptosporidium parvum*, 135
- Cup and ball CDs, 722
- Curcumin, 383–384, 418–419, 427, 460–461
- Cyberknife, 7
- Cyclic oligosaccharides, 638–639, 700
- Cycloamyloses. *See* Cyclodextrins (CDs)
- Cyclodextrin-based polyrotaxanes (CDPRs), 733–736
- Cyclodextrins (CDs), 638–639, 641, 700–701, 715–724
- amphiphilic CDs, 721–724
- anionic, 723–724
- cationic, 723
- nonionic, 722–723, 722*f*
- amphiphilic cyclodextrin nanoparticles, 724–730
- cyclodextrin-based polymeric nanomedicines, 733–735
- derivatives, 717–721, 720*t*
- natural cyclodextrins, 717–721
- polymer-drug conjugates, 735–736
- polymeric nanoparticles incorporating, 730–733
- polymer-modified drug carriers, 738–740
- supramolecular hydrogels, 737
- Cyclomaltoses. *See* Cyclodextrins (CDs)
- Cyclopamine, 175
- Cyclosporine, 226
- CYP3A4, 582
- CytImmune, 27
- Cytomegalovirus (CMV), 484–485
- Cytoplasmic delivery, 86
- Cytosine deaminase, 428
- Cytotoxic CD8⁺ T cells, 387
- D**
- D-Ala2- D-Leu5-enkephalin, 682
- DaunoXome, 342
- Degree of deacetylation (DDA), 694
- Delayed release, 565, 590–591
- Dendrimers, 57, 97, 129–130, 159, 538–540, 701–703
- carbosilane dendrimers (CBS), 703
- glycodendrimers, 703
- poly(amido amine) dendrimers, 702
- polypropylenimine dendrimers, 702–703
- as thermo-responsive substances, 466–467
- Dendritic cell-derived exosomes, 432
- Dendritic cells, 19
- Density gradient centrifugation, 414
- 2-Deoxy-D-glucose, 166
- Depocyt, 342

Derjaguin, Landau, Vervy and Overbeek (DLVO) theory, 47–48

Dermal delivery, of lipid nanoparticles, 769–771
 ex vivo permeation study, 769–770
 in vivo evaluation, 770–771

Desolvation, 105–106

Detonation nanodiamonds, 388

Dextran, 638, 658

2-Diazo-1,2-naphthoquinone (DNQ), 319–320, 321f

o-Dichlorobenzene, 90

Dichloromethane, 99

Diethylenetriamine pentaacetic acid (DTPA), 327

Diferuloylmethane, 460–461

Differential scanning calorimetry (DSC), 760

Diffusion-controlled products, 577

Diffusion-controlled release pattern, 633

Diffusion matrix devices, 598–599

Diffusion reservoir devices, 598

Diffusion systems, 598–599

N,N-(3-Dimethylaminopropyl)-*N*-ethyl-carbodiimide (EDC), 656

2,3-Dimethylmaleic anhydride, 173

1,2-Dioleoyl-3-dimethylammonium propane (DODAP), 531–532

1,2-Dioleoyloxy-*N,N*-dimethyl-3-aminopropane (DODMA), 531

Dipyridamole, 604t

Dissolution-controlled products, 577–578

Dissolution controlled system, 597–598

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine, 173

Disulfide bonds, 174–175

Disulfide bridge. *See* Disulfide bonds

Dithienylethene, 317–319

Docetaxel, 10–11, 728, 732

Dose dumping, 587, 595–596

Dose/solubility ratio, 581

Dosimetry, 65–66

Double emulsion (DE) method, 101, 453

Double-network hydrogels, 640–641

Double targeting, 124

Doxil, 27, 342

Doxorubicin (DOX), 12–14, 175, 180, 307, 425–426, 459–460, 469t, 736, 738–739
 DOX-loaded PLGA microspheres, 166

Drug delivery to targets, 41–44

Drug entrapment efficiency and drug loading, 758

Drug loading and release properties,
 pharmaceutical systems for the
 modification of, 221–232

Drug-loading techniques, 130

Drug penetration and promotion, studies of, 239–241

Drug-resin complexes, 578–579

Drug transport, mechanism for, 131

Drug uptake mechanisms, 138
 applications, 139

Dual-loaded anticancer drug nanoparticles, 175

Dual responsive hydrogels, 648

Dual stimuli-responsive nanosystems, 177–182

Dual targeting, 123–124

Dua's layer, 202–203

D value, 567

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), 678–680

Dynamic light scattering (DLS), 58–61, 217, 417, 756

E

Econazole nitrate, 302–303, 719

Ectosomes, 409, 414–415

Egalet, 590–591

Electrical responsive systems, mechanism of
 action of, 462

Electric-field responsive hydrogels
 applications of, 462–463
 preparation method, 462

Electrochemical double layers (EDL), 264–265

Electromagnetic radiation, 169

Electron–ion interaction potential
 (EIIP), 358

Electron microscopy, 58–61, 415–416

Electron spin resonance (ESR), 761

Electrophoretic light scattering (ELS), 757

Electroporation, 144

Electro-responsive drug delivery systems,
 461–463

Electrosensitive hydrogels (ESHs), 649t

Electro-sensitive hydrogels, 644–645

Emulsifying agents, 455–456

Encapsulated dissolution systems, 597

Endocytosis, 86, 392, 772–774
 cellular uptake, 773–774
 pathways, 772–773

Endogenous lectins, 124

Endohedral metallofullerenes, 91

Endosomal sorting complex required for the
 sorting/transport (ESCRT), 410

Endothelium, 203

Enhanced permeability and retention (EPR) effect,
 303, 341–342, 468–470, 691, 763–764

Enhanced permeation, 42

ENOS-pVAX plasmid, 706

Enterococcus faecalis, 272–274, 278–279

Enzyme-linked immunosorbent assay (ELISA),
 496–497

- Enzyme-responsive strategy, 176
 - Epidermal growth factor (EGF), 431
 - EGF receptor, 347–348
 - Epstein-Barr virus (EBV), 484–485
 - Erodible molded multilayer tablet, 590–591
 - Erosion controlled release, 577–578
 - ERTracker probe, 773–774
 - Erythrocytes, 138–139
 - Escherichia coli*, 90–91, 135, 270–271, 281–282, 385–386
 - Ethyl cellulose (EC), 572
 - Eudragit, 173, 573
 - Eukaryotic AAVs, 485
 - Eukaryotic cell transduction
 - targeted adeno-associated virus/phage vectors for, 491–492
 - Evaporation methods, 101
 - Exocarta, 410–411, 429
 - Exogenous stimuli-responsive systems, 463–467
 - light-responsive systems, 463–464
 - thermo-responsive systems, 464–467
 - hydrogels as external temperature-responsive material, 465–466
 - novel dendrimers as thermo-responsive substances, 466–467
 - Exogenous thermo-responsive drug delivery systems, 465
 - Exosome/ectosome isolation, 413–415
 - affinity, 414
 - density gradient centrifugation, 414
 - general extracellular vesicle isolation problems, 414–415
 - polymer-based precipitation, 414
 - size exclusion, 414
 - ultracentrifugation, 413–414
 - Exosome-mimics (EMs), 430
 - Exosomes, as drug delivery platforms
 - for proteins, 428
 - for RNAs, 429–431
 - miRNA, 430–431
 - short interfering RNA, 429–430
 - for small molecules, 425–428
 - External dual stimuli-responsive systems, 467–468
 - External stimuli, 155–157, 156f
 - External-stimuli-responsive systems, 445–463, 445f
 - electro-responsive drug delivery systems, 461–463
 - magnetically responsive systems, 445–456
 - magnetic liposomes (MLs), 452–455
 - magnetic microspheres, 450–452
 - magnetic nanoparticles, 447–450
 - magnetic-responsive emulsions, 455–456
 - ultrasound-responsive systems, 456–461
 - biomedical applications of, 461
 - mechanism of action of, 456–457
 - types of, 457–461
 - Extracellular signal-regulated kinase (ERK), 409
 - Extracellular vesicle characterization, 415–417
 - atomic force microscopy (AFM), 416
 - dynamic light scattering, 417
 - electron microscopy, 415–416
 - flow cytometry, 417
 - fluorescence microscopy, 416
 - nanoparticle tracking analysis (NTA), 416
 - scanning force microscopy (SFM), 416
 - Western blotting, 416–417
 - Extracellular vesicles (EVs), 532–534
 - assessing EV uptake into recipient cells, 422–424
 - intravesicle probe, 424
 - labeling exosome cargoes, 423–424
 - labeling membrane, 422–423
 - biochemical and biological characterization, 413–417
 - exosome/ectosome isolation, 413–415
 - extracellular vesicle characterization, 415–417
 - biogenesis and composition, 408–411
 - calcium, pH, and hypoxia in biogenesis of as drug delivery platforms for therapeutic benefit in clinical trials, 431–432
 - exosomes as drug delivery platforms
 - for proteins, 428
 - for RNAs, 429–431
 - for small molecules, 425–428
 - loading of cells after EV isolation, 418–419
 - loading of cells before EV isolation, 418
 - mimetics, 419–420
 - recipient cells, EVs uptake into, 420–422
 - caveolin-dependent endocytosis, 421
 - clathrin-mediated endocytosis, 421
 - macropinocytosis, 421
 - participation of lipid rafts, 420
 - phagocytosis, 421–422
 - Eye, anatomical characteristics of, 202–207, 205f
 - ophthalmic administration, classic
 - routes of, 205–207
 - periocular drug delivery, 206–207
 - topical administration, 205–206
 - topical ocular drug delivery, barriers to, 204–205
- F**
- Fc receptors, 125
 - Femotidine, 604f

Fetal bovine serum (FBS), 533
 Fictionalization methods, 683
 Field-responsive polymers, 576
 Film characterization, techniques used on,
 218–219
 First-order targeting, 122
 Flow cytometry, 417
 Fluconazole-loaded niosomes, 229–230
 Fluorescein iso-thio cyanate (FITC), 635
 Fluorescence-based imaging, 329–330
 Fluorescence microscopy, 416
 Fluorescent imaging, 766
 Fluorine-containing anionic β -CDs, 723–724
 5-Fluorocytosine (5-FC), 428
 5-Fluorouracil, 165, 428, 721
 Folate receptors, 126, 385–386
 Food labeling nanosensors, 55
 Förster resonance energy transfer (FRET) imaging,
 766–767
 Fourth level of targeting, 122
 Franz diffusion cells, 224–225, 231
 Fulgides, 317–319
 Fuller, Richard Buckminster, 90
 Fullerenes, 89–91, 136
 Functional ion channels, on blood–brain tumor
 barrier, 677–678
 Future trends for nanomaterials (or nanoparticles)
 in drug delivery, 107–108

G

Gamma irradiation, 643–644, 727–728
 Gamma Knife, 7
 Ganciclovir (GCV), 493
 Gastrointestinal (GI) tract, 269–270
 GastroPlus, 592–593
Gaussia luciferase, 423
 Gelatin nanoparticles, 383–384
 Gellan gum, 210–211, 226
 Gemtuzumab (Myolotarg), 20
 GEN-1 (IL-12 plasmid), 706
 Gene delivery, 141–142
 approaches for gene therapy, 142
 prerequisites, 142
 vectors, 141–142
 Gene modification, 143
 replacement therapy, 143
 corrective gene therapy, 143
 Gene therapy, 30, 141, 143f, 689
 advantages and disadvantages of, 481t
 candidate diseases for, 150t
 clinical applications of, 480–481
 clinical trials of polymeric nanocarriers for,
 705–706
 delivery systems in, 148f
 early foundations of, 480
 future perspectives, 707
 somatic, 146–147
 Gene transfer techniques, 144–146
 biological (viral vectors), 146
 chemical (nonviral gene delivery), 146
 electroporation, 144
 molecular vibration, 144–145
 particle bombardment, 144
 photochemical transfection, 145–146
 ultrasound mediated transfection, 144
 Gene transfer to specific cell lines, 146–149
 candidate diseases, 149
 delivery systems in gene therapy, 147–149
 aquasomes, 149
 cationic lipid compatible systems, 148–149
 lipoplexes, 148
 liposomes, 147–148
 polymers, 147
 polyplexes, 149
 problems associated with gene therapy, 149
 somatic gene therapy, 146–147
 germline gene therapy, 126
 Genexol-PM, 342
 Germline gene therapy, 126
Giardia lamblia, 135
 Gliadel, 675–676
 Glioblastoma multiforme (GBM), 675–676, 680
 Gliomas, 675–676, 678, 680
 Glucocorticoids, 8
 Glucose regulated protein 78 (Grp78), 488, 495
 Glucose-responsive hydrogels (GRHs), 649t
 Glucose-responsive polymers, 576
 Glutaraldehyde-conjugated PVA hydrogels, 630
 Glycodendrimers, 703
 Glycolytic inhibitors, 386–387
 Glycotargeting, 44
 Gold (Au) nanoparticles, 739
 Gold nanocages, 167–168
 Gold nanoparticles, 67–68, 382–383, 543
 Gold nanorods, 167–168
 Gold nanoshells, 162–163
 Gonadotrophin releasing hormone blocker, 9t
 G-protein coupled receptors (GPCRs), 348–349,
 361–362
 Graphene oxide (GO), 544
 Gregoriadis, 127
 Growth factor receptors (GFRs), 348–349

H

Helicobacter pylori, 163–164, 271, 282–283
 Hemodynamic forces, 46

- Hepatocellular carcinoma (HCC), 400–401
- Heptakis (2- ω -amino-*O*-oligo-(ethyleneoxide)-6-hexylthio)- β -CD, 723
- Herpes simplex virus, 528–529
- High density lipoproteins (HDL), 30
- High pressure homogenization (HPH), 529–530, 754–755
- High speed homogenization (HSH), 536
- Hollow mesoporous silica spheres, 386–387
- Homopolymeric hydrogel, 646
- Hormone therapy, 8
- Hot homogenization technique (HHT), 754
- “Hot spot” effect, 446–447
- Human immunodeficiency virus (HIV)-1 protease, 90
- Human serum albumin (HSA), 384
- Hyaluronic acid (HA), 175, 538
- Hyaluronidases, 175–176
- Hybrid adeno-associated virus/phage vector, 487–491
- Hybrid viral phage nanoparticles
ligand-directed tumor targeting with, 479
- Hydrodynamic pressure controlled release system, 579–580
- Hydrodynamic therapy, 145
- Hydrogel as drug delivery systems, 207–221
in situ hydrogels, properties of, 212–219
rheometry, 212–213
shape and size change monitoring, 214–216
spectroscopic analysis, 216–218
techniques used on film characterization, 218–219
test tube inversion, 213–214
stimuli-responsive hydrogels as ophthalmic drug delivery systems, 219–221
stimuli-sensitive hydrogels, 208–212
ion-sensitive hydrogel, 210–211
multistimuli hydrogels, 211–212
pH-sensitive hydrogels, 209
temperature-sensitive systems, 209–210
- Hydrogel nanoparticles (HNPs), 465–466
- Hydrogels, 576, 625
biomedical applications of, 466*t*
classification, 645–648, 647*t*
based on properties, 646–647
stimuli-responsive hydrogels, 648
determining ocular residence time and ocular biodistribution of, 232–235
in drug delivery, 659, 661*t*
effect on corneal regeneration capacity, 237
as external temperature-responsive material, 465–466
first generation hydrogels, 629–631
based on cellulose, 631
based on cross-linking of water-soluble synthetic polymers, 630–631
prepared by polymerization of water-soluble monomers, 629–630
fourth generation, 640–642
composite hydrogels, 641–642
double-network hydrogels, 640–641
physiochemical properties of, 646*t*
properties of, 642–645
cytotoxicity and in vivo toxicity, 643
cytotoxicity and in vivo toxicity, 643–644
dynamics of swelling, 642–643
factors affecting swelling of hydrogels, 642
mechanical properties, 643
second generation hydrogels, 631–636
in situ-forming hydrogels, 635
temperature-sensitive hydrogels, 631–635
thermo-responsive systems, 635–636
synthesis of, 648–659
chemically cross-linked hydrogels, 648–657
physically cross-linked hydrogels, 657–659
third generation hydrogels, 636–640
hydrogels cross-linked by other physical interactions, 638–640
stereo-complexed hydrogels, 636–638
- Hydrolysis-activated micellar systems, 315–316
- Hydrophilic CDs, 719, 721
- Hydrophilic drugs, 91–92, 127, 755
- Hydrophilic-lipophilic balances (HLBs), 95
- Hydrophilic matrix systems, 570
- Hydrophilic poly(tert-butylacrylate-co-acrylate), 319
- Hydrophobic and hydrophilic drugs, 96
- Hydrophobic poly(methacrylate), 319
- Hydrophobic protoporphyrin (PpIX), 307
- Hydrosol process, 49–50
- Hydroxyalkylated β -CD, 720*t*
- Hydroxy-alkyl methacrylate (HEMA), 634–635
- Hydroxyl ethyl cellulose (HEC), 572
- Hydroxypropyl cellulose (HPC), 572
- N*-(2-Hydroxypropyl) methyl acrylamide (HPMA), 634–635
- Hydroxypropyl methylcellulose (HPMC), 571–572
- Hydroxypropyl methylcellulose phthalate (HPMCP), 172
- Hydroxypropyl- β -cyclodextrin (HPBCD), 231
- Hyperthermia, 56, 165, 457
- Hypoxia inducible factors (HIFs), 386–387
- I**
- Ibritumomab (Zevalin), 20
- Ibuprofen, 388–389

- ¹²⁵I-labeled norbiotinamide, 423
- Image-guided radiotherapy (IGRT), 7, 502–503
- Immature dendritic cells (imDCs), 425–426
- Immunoglobulin G (IgG), 637–638
- Immunotherapeutic agents, 20–26
- adoptive cell therapies by using lymphocytes, 23–24, 24*t*
 - antibody-drug conjugates, 25–26
 - cellular immunity, 20–23
 - genetic engineering in T cell, 24–25
- Immunotherapy, 17–20, 22*f*
- Indomethacin, 469*t*, 640–641, 737
- “Induced fit” hypothesis, 356
- Industrial applications, nanotechnology evaluation for, 54–68
- clinical trials, 58
 - developmental questions, 58–61
 - marketed products and technologies, assessment of, 61–63
 - nanodrug delivery, techniques for further development of, 63–64
 - nanotoxicology, 64–68
 - research pipeline, 55–58
 - dendrimers, 57
 - metal and inorganic nanoparticles, 56
 - polymeric micelles and polymerosomes, 57–58
 - polymeric nanoparticles and nanocapsules, 55–56
 - protein vaults, 57
 - self-nanoemulsifying drug delivery systems (SNEDDS), 56
 - solid lipid nanoparticles and nanostructured lipid carriers (SLN and NLC), 57
- Infectious diseases, nanomedicine for, 352–353
- Inflammatory breast cancer (IBC), 495
- Influenza virus
- targeting peptides for treatment of, 365
- Informational spectrum method (ISM), 358–365
- design of peptide mimetics of nanobodies, 361–363
 - design of peptides mimetics of vasoactive intestinal peptide, 363–365
 - targeting peptides, identification of, 365
- Inhibitors of apoptosis (IAPs) proteins, 506–507
- Injection molding (IM), 590–591
- Inner ear, nanoparticles targeting, 399
- Inorganic metal, 266–267
- Inorganic nanoparticles, 159, 542–544
- Inorganic nano-sized additives, 54
- In situ-forming hydrogels, 635
- In situ hydrogels, properties of, 212–219
- film characterization, techniques used on, 218–219
 - rheometry, 212–213
 - shape and size change monitoring, 214–216
 - spectroscopic analysis, 216–218
 - test tube inversion, 213–214
- Insulin, 172, 398, 604*t*, 640–641
- Intensity modulated radiotherapy (IMRT), 7
- Interfacial polymerization, 104–105
- Interleukin receptors, 125
- Internal stimuli, 155–157, 156*f*
- Inter penetrating networks (IPNs), 646–647
- Interpenetrating polymeric network hydrogels, 208
- Intestinal epithelium, nanoparticles targeting, 398–399
- Intracellular targeting, 86, 122
- using nanoparticles, 380–381
- Intravitreal drug delivery, 207
- In vitro-in vivo correlation (IVIVC), 592–593
- In vitro lipolysis, 761–762
- In vitro lung barrier models, 775
- In vivo distribution of nanoparticles, 764–765
- In vivo studies in animals and effectiveness of drugs, 238–239
- Ion-exchange resin-drug complexes, 599
- Ion exchange resins, 578–579
- Ion-exchangers, 599
- Ionic gelation method, 100
- Ion-sensitive hydrogel, 210–211
- Ion-sensitive polymers, 238*t*
- Irinotecan, 342
- Iris, 203–204
- Iron oxide nanoparticle (IONP), 284, 328, 543
- Isobutylcyanoacrylate, 104
- N*-Isopropylacrylamide, 209
- N*-Isopropylacrylamide thermosensitive hydrogels, 217
- Isotretinoin, 133
- Isradipine, 609*t*
- ## K
- Kaposi’s sarcoma, 12–14
- κ -carrageenan, 211, 211*f*
- Knowledge management, 594
- Kupffer cells (KC), 390–391
- ## L
- Lacrimal glands, 203
- Laparoscopic surgery, 5
- Large unilamellar vesicles (LUV), 529–530
- Laser diffraction (LD), 756
- Laser pulses, 383
- Laser surgery, 5
- Lentiviral vector, 481*t*, 485–486
- Lentivirus, 524–525

- Leukotriene (LTC₄), 676
- Levofloxacin, 212
- Ligand-directed nanoparticles, 486
- Ligand-directed tumor targeting, 479
 - adeno-associated virus/phage (AAVP), 487–488
 - clinical potential of, 492
 - for eukaryotic cell transduction, 491–492
 - studies to date, 489*t*
 - adeno-associated virus/phage-tumor necrosis factor
 - with radiation therapy in metastatic melanoma model, 502–503
 - adeno-associated virus/phage-tumor necrosis factor- α
 - toxicity and preclinical safety of, 500–501
 - bacteriophage, 487
 - combination adeno-associated virus-mediated gene therapy with radiation therapy, 501–502
 - future directions, 503–507
 - gene therapy
 - clinical applications of, 480–481
 - early foundations of, 480
 - hybrid adeno-associated virus/phage vector, 487–491
 - ligand-directed nanoparticles, 486
 - molecular-genetic pet imaging using RGD-AAVP-HSVTK, 493–495
 - Oct-adeno-associated virus/phage-tumor necrosis factor- α
 - preclinical trial in small animal model with, 499–500
 - octreotide-targeted adeno-associated virus/phage vector-mediated tumor necrosis- α in vitro assays, 499
 - RGD-targeted adeno-associated virus/phage vector-mediated tumor necrosis factor- α in vitro assay, 496–497
 - preclinical trial in large animal model with, 498
 - preclinical trial in small animal model with, 497–498
 - targeting tumor vasculature with, 495–496
 - targeted gene therapy, 486
 - vectors, 481–486
 - nonviral vectors, 481–482
 - viral vectors, 482–486
- Ligands, 140, 691–693
 - targeting ligands. *See* Targeting ligands
 - types of, 140*t*
- Light-responsive systems (photo-sensitive), 463–464
- Light-sensitive hydrogels (LSHs), 649*t*
- Light-sensitive polymers, 576
- Limitations/toxicology of nanomedicines, 106–107
- Lipid components of cell membranes, 121
- Lipid nanoparticles (LNs), 541–542, 751. *See also* Solid lipid nanoparticles (SLNs)
 - cellular models, 772–775
 - endocytosis, 772–774
 - translocation, 774–775
 - dermal drug delivery with, 133
 - in vitro characterization, 756–762
 - assessment of coexisting structures, 761
 - in vitro lipolysis, 761–762
 - polymorphism and degree of crystallinity, 759–760
 - routine characterization, 756–759
 - in vivo characterization, 762–767
 - biodistribution, 763–764
 - in vivo imaging, 764–767
 - pharmacokinetics and bioavailability, 762–763
 - models for specific administration route, 768–772
 - dermal delivery, 769–771
 - evaluation of lymphatic absorption, 768–769
 - pulmonary delivery, 771–772
 - preparation of, 754–755
 - high-pressure homogenization, 754–755
 - microemulsion technique, 755
 - solvent emulsification evaporation technique, 755
- Lipid rafts, participation of, 420
- Lipofectin, 530
- Lipophilic compounds, 602–603
- Lipophilic diisocyanate, 104
- Lipophilic drugs, 536, 759
- Lipophilic dyes, 422
- Lipoplexes, 148, 529–531
- Lipopolysaccharides (LPS), 427
- Lipoprotein receptors, 125
- Liposomal transportation, 129
- Liposomes, 54, 94–95, 127–128, 128*f*, 147–148, 158, 171–173, 444–445, 529–531
 - applications of, 129
 - drug entrapment in, 129
 - preparation of, 128*f*
- Liquid crystals (LCs), 397–398
- Listeria monocytogenes*, 135
- Liver disease
 - detection of, 400–401
 - treatment of, 604*t*
- Living imaging, 765
- LMWP-PR-DOX conjugate, 736
- “Lock-and-key” hypothesis, 356
- Lollipop CDs, 722

Lovastatin, 604*t*
 Low density lipoprotein (LDL), 693
 LDL receptors, 392
 Lower critical solution temperature (LCST), 209,
 311–313, 312*f*, 464–465, 634, 636
 Luciferin, 765–766
 Lungs, nanoparticles targeting, 390–391
 Luteinizing hormone blockers, 9*t*
 Lymphatic drug transportation, 768
 Lymphocyte-homing receptors, 125
 Lymphocytes
 adoptive cell therapies by using, 23–24, 24*t*
 nanoparticles targeting, 387–388
 LysoTracker probe, 773–774
 Lysozyme, 638

M

Macrophage cell membrane vesicles, 167–168
 Macrophages, 19
 Macropinocytosis, 421
 Magnetic emulsions, 444–445
 Magnetic ionic liquid surfactants (MILSs), 456
 Magnetic iron oxide nanoparticles, 285–286, 543
 Magnetic liposomes (MLs), 163, 452–455
 advantages of, 454
 biomedical applications of, 454–455
 disadvantages of, 454
 mechanism of action of, 454
 method of preparation of, 453
 Magnetic microspheres, 450–452
 marketed products of, 470*t*
 mechanism of action, 451–452
 method of preparation of magnetic
 microparticles, 450
 Magnetic nanoparticles, 98–99, 161–162,
 386–387, 444–445, 447–450
 applications of, 449
 effect on antimicrobial nanoparticle, 283–286
 magnetic nanomaterials, mechanism of action
 of, 448–449
 nanosized material, advantages of, 449
 preparation, 447
 by microemulsions method, 448
 by thermal decomposition method, 448
 Magnetic resonance imaging (MRI) contrast
 enhancers, 261–262, 328–329, 400–401
 Magnetic-responsive emulsions, 455–456
 advantages, 455–456
 applications, 456
 disadvantages, 456
 mechanism of action, 455
 preparation of, 455
 Magnetoliposomes. *See* Magnetic liposomes (MLs)

Mannitol, 676
 Mannose receptors, 125
 Mass spectrometry, 763
 Matrix diffusion-control devices, 599
 Matrix dissolution systems, 588
 Medusa-like CDs, 722
 Melanoma-associated antigen A3 (MAGE-A3),
 432
 Melanoma M21 cells, 496–497
 Mesenchymal stem cells (MSCs), 426–427
 Mesoporous magnetic nanoparticles (MNPs),
 386–387
 Mesoporous silica nanoparticles (MSNs), 174, 176,
 739
 Metal and inorganic nanoparticles, 56
 Metallic nanoparticles, 162–163, 165–166
 Metallofullerenes, 91
 Metallophthalocyanines (MPCs), 279
 Metalloproteases (MMP), 175
 Metal oxide-derived nanoparticles, 266–267
 Metastasis-associated protein 1 (MTA1), 483–484
 Metformin hydrochloride, 609*t*
 Methacrylic acid (MAA), 272
 Methicillin-resistant *S. aureus* (MRSA), 281–282
 Methotrexate (MTX), 398, 739
 2-(2-Methoxyethoxy) ethyl methacrylate, 636
 Methylated CDs, 720*t*
 Methylene blue (MB), 232–233, 272–274
 Methyl ether poly(ethylene glycol) (MPEG)-poly
 (beta-amino ester) (PAE) block copolymer
 (MPEG-PAE), 311
 Methyl methacrylate (MMA), 272, 704
 Metoprolol, 588–589, 609*t*
 Micelles, 159, 738–739
 Micelles and micelle-forming amphiphiles,
 299–301
 amphiphilic block copolymers used in
 fabrication of micelles, 300–301
 micelle shape and morphology, 299
 polymeric micelles, 57–58
 Microbubbles, 170–171, 457, 459
 Microemulsions, 95–96, 103
 Microemulsion technique, 448, 754–755
 MicroRNA (miRNA) modulators for cancer
 treatment, 513
 clinical trials and future perspectives, 544–545
 nonviral vectors, 529–544
 dendrimers, 538–540
 extracellular vesicles, 532–534
 inorganic nanoparticles, 542–544
 lipid nanoparticles, 541–542
 liposomes and lipoplexes, 529–531
 nanofibers, 540–541
 polymeric micelles, 534–535

- MicroRNA (miRNA) modulators for cancer treatment (*Continued*)
 polymeric nanoparticles and polyplexes, 536–538
 stable nucleic acid lipid particles, 531–532
 principal barriers, 522–523
 therapeutic potential of, 521–522
 viral vectors, 523–529
 adeno-associated virus, 526–528
 adenovirus, 525–526
 herpes simplex virus, 528–529
 lentivirus, 524–525
- Microspheres, 444–445
- Microtubule stabilizing taxane agent, 10–11
- Miglitol, 604*t*
- Miniemulsion, 102–103
- Minoxidil sulfate (MS), 680
- Mitogen-activated protein kinases (MAPK), 389–390
- Mitotic catastrophe, 10–11
- MitoTracker probe, 773–774
- Mohs micrographic surgery (MMS), 5–6
- Molecular diagnostics, 134–135
- Molecular-genetic pet imaging using RGD-AAVP-HSVTK, 493–495
- Molecular imaging, 678–679, 682–683
- Molecular-level targeting, 122
- Molecular vibration-mediated transfection, 144–145
- Monoclonal antibodies (mAbs), 121, 344–345, 383
- Mononuclear phagocytic system (MPS), 297–298
- Mucoadhesive system, 580
- Multidrug resistant (MDR) cancer, 427
- Multilamellar liposomes, 227
- Multilamellar vesicles (MLV), 94, 458–459, 529–530
- Multilayer matrix tablet, 598
- Multimeric molecular imaging, 679
- Multipolymer interpenetrating polymeric network (IPN) hydrogels, 208
- Multistimuli hydrogels, 211–212
- Multistimuli responsive nanosystems, 183
- Multivesicular body (MVB), 409–410
- Multi-walled carbon nanotubes (MWNTs), 98, 278
- Mycobacterium avium*, 90–91
- Myeloid cell leukemia 1 (MCL-1), 530
- Myocet, 342
- N**
- N-Ac-poly (L-histidine)-grafted poly-L-lysine nanocarriers (PLH-g-PLL), 699
- Naked DNA, 145–146
- Nanobiomagnetism, 446
- Nanodroplets, 459
- Nanodrug delivery, techniques for development of, 63–64
- Nanoemulsions, 56, 96
- Nanofibers, 540–541
- Nanofiltration, 54
- Nanogels, 159
- Nanohorns, 98
- Nanoinformatics, databases and targeting information, 366
- Nanomaterial Registry, 366
- Nanomedicine, 40, 678, 715–716
 active research areas in, 41*t*
 for infectious diseases, 352–353
 and nanoimaging, 678
 for targeted delivery of natural compounds, 350–352
 paclitaxel nanodelivery systems, 350–351
 peptide-targeted paclitaxel-loaded nanoparticles, 351–352
- Nanoparticle drug delivery systems (NDDSs/NPDDS), 64, 83–84
- Nanoparticles (NPs), 41–42, 83, 131–132, 380
 advantages of, 87–88, 87*f*
 affecting drug targeting, characteristics of, 381–382
 drug release, 382
 particle size, 381
 surface properties of nanoparticles, 381–382
 as a colloidal drug delivery system, 224–225
 disadvantages of, 88*f*
 drug release from, 88–89
 preparation methods, 99–106
 coacervation/ionic gelation method, 100
 controlled/living radical polymerization, 105
 desolvation, 105–106
 double emulsion and evaporation method, 101
 interfacial polymerization, 104–105
 microemulsion, 103
 miniemulsion, 102–103
 polymerization method, 100
 precipitation method, 102
 salting out method, 101
 solvent evaporation method, 99
 spontaneous emulsification or solvent diffusion method, 100–101
 spray-drying, 106
 supercritical fluid technology, 106
 preparation methods of, 133*f*
 release mechanism from, 132
 targeting cells, 383–390
 cardiac cells, 390
 lymphocytes, 387–388

- neutrophils, 388–390
 - tumor cells, 383–387
 - targeting organs, 390–399
 - brain, 392–394
 - inner ear, 399
 - intestinal epithelium, 398–399
 - lungs, 390–391
 - protein, 399–400
 - skin, 395–398
 - vascular endothelium, 394–395
 - Nanoparticle tracking analysis (NTA), 416
 - Nanoparticulate carrier systems, 715–716
 - Nanoparticulate drug delivery systems, 83*f*
 - Nanoscale-based sensors, 63–64
 - Nano-sized agrochemicals, 55
 - Nanosized material, advantages of, 449
 - Nanostructured lipid carriers (NLC), 398, 541–542, 752, 753*f*
 - Nano-targeted drug delivery, 44–53
 - regulation and guidelines, 53
 - shape and size, 45–53
 - agglomeration consequence, 48–49
 - approaches, 49–50
 - charge, 47–48
 - concerns, 45–46
 - delivery to the target of nanotherapeutics, 51–52
 - explicitness of nanodrug carriers, 50–51
 - in vitro–in vivo correlation, pharmacokinetics with, 52–53
 - matter, 47
 - performance based on, 46–47
 - surface characteristics of nanomaterial, 48
 - Nanotechnology, defined, 39–40
 - Nanotechnology-based drug delivery systems, 89–99
 - carbon nanomaterials, 98
 - dendrimers, 97
 - fullerenes, 89–91
 - liposomes, 94–95
 - magnetic nanoparticles, 98–99
 - microemulsions, 95–96
 - nanoemulsions, 96
 - solid lipid nanoparticles (SLNs), 91–93
 - routes of administration of, 93
 - Nanotechnology characterization laboratory (NCL), 61
 - Nanotechnology evaluation for industrial applications. *See* Industrial applications, nanotechnology evaluation for
 - Nanotechnology in drug delivery, 84–86
 - Nanotechnology research fields, 40
 - Nanotechnology Working Group (Nano WG), 366
 - Nano-textured food products, 54
 - Nanotherapeutics, 51–52, 59*t*, 62*t*
 - Nanotoxicology, 53, 64–68
 - Nanotrain, 30
 - Natural cyclodextrins, 717–721, 719*t*
 - Nb peptide mimetics (NPMs), 363
 - Near-infrared (NIR) light exposure, 285–286
 - Nerve growth factor (NGF) intravenous delivery, 393
 - Neurotrophins, 399
 - Neutrophil extracellular traps (NETs), 389–390
 - Neutrophils, nanoparticles targeting, 388–390
 - N*-hydroxysuccinimide (NHS), 656
 - Niosome-based hydrogels, 229
 - Nitroxide-mediated polymerization (NMP), 105
 - Nonbiodegradable polymers, 314
 - Nonporphyrins, 168–169
 - Nonsmall cell lung cancer (NSCLC) Stages IIIB/IV, 432
 - Nonsteroidal anti-inflammatory drug, 604*t*
 - Nonviral gene delivery, 146
 - Nonviral vectors, 529–544
 - dendrimers, 538–540
 - extracellular vesicles, 532–534
 - inorganic nanoparticles, 542–544
 - lipid nanoparticles, 541–542
 - liposomes and lipoplexes, 529–531
 - nanofibers, 540–541
 - polymeric micelles, 534–535
 - polymeric nanoparticles and polyplexes, 536–538
 - stable nucleic acid lipid particles, 531–532
 - Nuclear imaging, 327
 - Nuclear magnetic resonance (NMR), 761
 - Nucleic acids, 530, 693, 700–701
 - N*-vinyl-pyrrolidone (NVP), 704
- ## O
- Oct-adenovirus-associated virus/phage-tumor necrosis factor- α
 - preclinical trial in small animal model with, 499–500
 - Octreotide-targeted adenovirus-associated virus/phage vector-mediated tumor necrosis- α in vitro assays, 499
 - Ocular biodistribution, 232–235
 - Ocular drug delivery systems. *See* Stimuli sensitive ocular drug delivery systems
 - Ocular residence time, 232–235
 - Ocular tolerance, 236
 - Oil-in-water emulsion, 455–456
 - Oligo(ethylene glycol) methacrylate, 218, 636
 - Oligoethylenimine (OEI), 701
 - Oncaspar, 342

- Oncolytic adenoviruses, 526
- Oncolytic herpes simplex viruses (oHSVs), 528–529
- OncomiRNAs, 515, 516*t*
- One-bead-one-compound (OBOC) library, 355
- Ophthalmic administration, classic routes of, 205–207
 - periocular drug delivery, 206–207
 - intravitreal drug delivery, 207
 - subconjunctival drug delivery, 206
 - subtenon drug delivery, 206
 - topical administration, 205–206
 - semisolid ophthalmic preparations, 206
 - solutions, 205
 - suspensions, 206
- Opioid analgesic, 609*t*
- Opsonins, 297–298, 381–382
- Opsonization, 297–298, 381–382
- Oral controlled and sustained drug delivery systems, 563, 565–566
 - bioadhesive controlled release systems, 573–574
 - carbomers, 574
 - polyethylene oxide, 574
 - biological properties, 568–569
 - absorption, 568
 - circadian rhythm, 569
 - disease state, 569
 - elimination/biological half-life, 568
 - metabolism, 568
 - protein binding, 569
 - safety considerations and side-effects, 569
 - biopharmaceutical factors, 581–582
 - colonic stability, 582
 - gastrointestinal metabolism and elimination, 582
 - permeability, 581–582
 - solubility, 581
 - challenges and new technologies in, 586–593
 - limiting factors for, 586–589
 - new formulation designs for achieving desired release profiles, 590–593
 - oral controlled delivery for water-insoluble drugs, 589–590
 - classification, 564–566
 - controlled release dosage forms, 564–565
 - delayed release, 565
 - coating systems for, 584–586
 - excipients used in coating, 585–586
 - materials used in coating processes, 584–585
 - design and concepts, 577–580
 - altered density system, 580
 - diffusion-controlled products, 577
 - dissolution-controlled products, 577–578
 - hydrodynamic pressure controlled release system, 579–580
 - ion exchange resins, 578–579
 - mucoadhesive system, 580
 - osmotic pump systems, 578
 - pH-independent formulation, 580
 - swelling controlled drug release system, 579
- oral controlled release dosage forms, 577–596
- oral matrix systems, 570–572
 - alginate, 571
 - cellulose ethers, 572
 - chitosan, 571
 - hydroxypropyl methylcellulose (HPMC), 571–572
 - pectin, 571
- oral membrane controlled systems, 572–573
 - cellulose acetate, 572
 - ethyl cellulose, 572
 - polymethacrylates, 573
- oral sustained release dosage forms, 596–612
 - advantages, 601
 - biological factors, 601–602
 - coating systems for, 608–612
 - design and fabrication of, 597–600
 - disadvantages, 601
 - physicochemical factors, 602–607
- physicochemical properties, 566–568
 - aqueous solubility, 566
 - drug pKa and ionization of the drug, 568
 - molecular size and diffusivity, 567
 - partition-coefficient, 566–567
 - size of the dose, 567
 - stability of the drug, 567
- polymers for fabrication of oral modified drug delivery systems, 569–576
- preclinical and clinical status of, 612
- preformulation parameters, 583–584
 - active ingredient solubility, 584
 - particulate and mechanical properties of drug substances, 583
 - partition coefficient, 584
 - stability and compatibility, 583
- quality by design (QbD) approach in, 593–595
 - advantages and disadvantages of, 595–596
 - elements of, 594–595
 - enablers of, 593–594
- stimuli-responsive polymers, 574–576
 - bioresponsive polymers, 576
 - pH-responsive polymers, 575
 - temperature-responsive polymers, 575
- Organic nanoparticles, 159
- Organic nano-sized additives for food applications, 54
- Organ level targeting, 122

- Organs, nanoparticles targeting, 390–399
 OROS tablet, 588–589
 Oscillatory rheometers, 212
 Osmogen, 578
 Osmosis, 578, 600
 Osmotic pressure controlled systems, 600
 Osmotic pump systems, 578
 Oswald ripening, 95–96
 Ovarian cancer, 12–14
 Oxycodone, 609*t*
- P**
- p53 pathway, 515
 Paclitaxel (PTX), 385, 418, 425–426, 541–542
 Paclitaxel nanodelivery systems, 350–351
 S-Palmitoylation, 422
 P-amino benzoic acid (PABA), 397–398
 Pancreatic lipases, 761
 Pancreatic neuroendocrine tumors (PNETs), 499
 Panitumumab (Vectibix), 20
 Panning, 354
 Panoprogen, 224–225
 Paramagnetic substances, 328
 Parkinson's disease, 428
 Particle bombardment, 144
 Particle-fluid flow interactions, 264
 Particle–particle potential interactions, 264–265
 Particle size analysis, 583
 Partition coefficient, 566–567, 584, 597
 Passive targeting, 42, 122–123, 157–158, 341–342, 690–691, 692*f*
 PCL–PEG–PCL hydrogels, 633
 Pectin, 571
 PEG–PLA based systems, 632–633
 PEG–polyester block copolymers, 631–634
 PEGylated chitosan nanoparticles, 695
 Peptide-based targeting ligands, identification of, 353–355
 Peptide ligands, computational design of, 355–365
 long-range intermolecular interactions in
 biological systems, 356–365
 informational spectrum method, 358–365
 Peptides, 348–350
 drug delivery system for, 132–133
 Perfusion magnetic resonance imaging, 680–681
 Periocular drug delivery, 206–207
 intravitreal drug delivery, 207
 subconjunctival drug delivery, 206
 subtenon drug delivery, 206
 Peripheral benzodiazepine receptor (PBR)-targeted probe, 679
 Permanent cross-linked hydrogels, 627–628
 P-glycoprotein, 43–44, 343
 Phagocytosis, 138, 389, 421–422
 Phase separation emulsion polymerization method (PSEP), 450–451
 Phenothiazine, 267–268
 pH-independent formulation, 580, 599–600
 Photochemical internalization (PCI), 145–146
 Photochromophore, 316–317
 Photodynamic therapy (PDT), 145–146, 168–169, 267–268, 272–274
 Photon correlation spectroscopy (PCS), 756
 Photoreceptor cells, 204
 Photoresponsive micelles, 316–322
 photo-induced cleavage, 319
 photo-induced cross-linkable polymeric micelles, 320–321
 photo-induced energy conversion, 321–322
 photo-induced rearrangement type micelles, 319–320
 photoisomerization, 317–319
 Photo-responsive systems
 biomedical applications of, 464
 mechanism of action, 463–464
 preparation of, 464
 Photosensitizers, 97, 267–268
 for antimicrobial activity, 281*f*
 pH-responsive hydrogel (pRHs), 172, 649*t*
 pH-responsive micelles, 305–311, 309*t*, 328–329
 pH-sensitive hydrogels, 209
 pH-sensitive polymers, 238*t*, 575
 Phthalocyanines (PC), 267–268
 PC-based nanoparticle as photosensitizer, 280*f*
 Physical cross-linking, 657–658
 by crystallization of homopolymer systems, 658–659
 by hydrogen bonds, 659
 by ionic interactions, 658
 Physically cross-linked hydrogels, 627
 Physical–physical dual stimuli-responsive materials, 467–468
 Physical stimuli, 648
 Physical stimuli-responsive nanosystems, 160–171
 magnetic field stimulation, 160–164
 photostimulation, 167–169
 radiation stimulation, 169–170
 temperature stimulation, 164–167
 ultrasound stimulation, 170–171
 Physical targeting, 123
 Physiologically based pharmacokinetic (PBPK) modeling, 52–53
 Pigments, 586
 Pioglitazone, 604*t*
 “Planning for quality”, 593
 Plasmid DNA (pDNA), 698
 Plasticizers, 572, 585

- Plastic polymers containing nanomaterials, 54
- Pluronic-based hydrogels, 631–632
- Pluronic F-127, 210–212, 223–224
- Pluronic micelles, 459–460
- P(MMA-co-MAA) hydrogel, 272
- (P(NIPAAm-co-DMAAm)-*b*-PLC), 179–180
- Polo-like kinase-1 (PLK-1), 430, 730
- Poloxamer 188, 210
- Poloxamer 407, 210
- Poloxamers, 210, 569–570, 631–632, 698
- Poly [2-(dimethylamino)ethylmethacrylate] p (DMAEMA), 703–705
- Poly(2-hydroxyethylmethacrylate) (*p*-HEMA) hydrogel, 226
- Poly(3,4-ethylenedioxythiophene) (PEDOT), 285–286
- Poly(3-methylglycolide), 633–634
- Poly(4-vinylphenol) (PVP) monolayers, 218–219
- Poly(4-vinylpyridine), 307
- Poly(acrylic acid), 569–570
- Poly(alkylcyanoacrylate), 100
- Poly(amidoamine) (PAMAM), 129–130, 538–539, 641
- dendrimers, 702
- Poly(β -amino ester) (PAE), 307
- Poly (butylcyanoacrylate) (PBCA), 392–393
- Poly(ϵ -caprolactone) (PCL), 99
- Poly(δ -valerolactone), 633–634
- Poly-[di-(carboxylatophenoxy)phosphazene] (PCPP) polymer, 658
- Poly(ϵ -caprolactone) (PCL), 536, 732
- Poly(ϵ -caprolactone) (PCL), 282
- Poly(ϵ -caprolactone-co-lactide)-*b*-poly(ethylene glycol)-*b*-poly (ϵ -caprolactone-co-lactide) (PCLA_PEG_PCLA) tri-block copolymers, 633–634
- Poly(ethylene glycol) (PEG), 164, 300, 731–732
- PEG-*b*-carboxylated polycarbonate (PEG-PCCOOH), 308
- PEG-coated biodegradable nanoparticles, 380
- Poly(ethylene oxide) (PEO), 300, 569–570, 733
- Poly(ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide) (PEO–PPO–PEO) copolymer, 216
- Poly (histidine), 307
- Poly(hydroxy-alkyl methacrylate) (pHEMA), 630, 654
- Poly(lactic acid) (PLA) nanoparticles, 397, 632
- Poly (lactic-co-glycolic) acid (PLGA), 99, 163–164, 224–225, 270, 536–537, 697–699
- PLGA-PEG nanoparticles, 388–389
- Poly(L-glutamic acid) (PGA), 308
- Poly(L-lactic acid) (PLLA), 632, 636, 638
- Poly(L-lactic acid) (PLLA)-*b*-PEG-*b*-pHis, 308
- Poly(L-lactide)-based nanoparticles (PLLA-NPs), 283
- Poly (*N,N*-dimethylaminoethyl-methacrylate), 219, 575
- Poly(*N,N'*-diethylaminoethyl-methacrylate), 575
- Poly(*N,N'*-dimethyl aminoethyl methacrylate) (PDMAEMA), 307
- Poly(*N*-isopropyl acrylamide) (pNIPAAm), 272, 312–315, 631–632, 634
- Poly(*N*-isopropylacrylamide)-chitosan (pNIPAAm–CS), 221
- Poly(*N*-isopropylacrylamide-co-*N,N*-dimethylacrylamide-*b*- ϵ -caprolactone), 179–180
- Poly(*N*-isopropylacrylamide-co-*N,N*-dimethylacrylamide-*b*-lactide), 179–180
- Poly(propylene fumarate), 633–634
- Poly(propyleneimine), 538–539
- Poly(propylene oxide) (PPO), 733
- Poly-(tert-butyl acrylate)PtBA-Br initiators, 464
- Poly(trimethylene carbonate), 633–634
- Poly(vinyl alcohol), 227, 569–570
- Poly(vinylpyrrolidone) (PVP), 285–286
- Polyacrylamide (PAM), 579, 629–630
- Polyacrylic acid polymers (carbomers), 573–574
- Polyacrylonitrile (PAN), 627
- Poly-alkyl-cyanoacrylates (PACA), 536
- Polyarginine, 382–383
- Polybutylcyanoacrylate, 100
- Polybutylmethacrylate (PBMA), 315
- Polycaprolactone (PCL), 315, 633, 731–732
- Polydimethylaminoethyl methacrylate (PDMAEMA), 307, 535
- Poly dispersity index (PDI), 694–695, 756
- Polyethylene glycol (PEG), 44, 157–158, 270, 468–470, 630–631, 659, 695
- Polyethylene oxides (Polyox), 573–574
- Polyethylenimine (PEI), 534, 537–538, 696–697, 699, 706
- Poly(lactic acid) (PLA), 535
- Poly(lactide) (PLA), 99, 315
- Poly-L-histidine, 270
- Poly-L-lysine, 699–700
- Polymer-based precipitation, 414
- Polymer-drug conjugates, 735–736
- Polymeric micelles, 57–58, 230–231, 534–535
- in drug delivery applications, 301–303
- properties of, 296
- size, 297
- in targeted drug delivery, 303–304
- Polymeric nanoparticles, 176, 693–705
- chitosan, 694–695
- clinical trials of, for gene therapy, 705–706

- cyclodextrins, 700–701
 - dendrimers, 701–703
 - carbosilane dendrimers (CBS), 703
 - glycodendrimers, 703
 - poly(amido amine) dendrimers, 702
 - polypropylenimine dendrimers, 702–703
 - incorporating cyclodextrins, 730–733
 - and nanocapsules, 55–56
 - poly (lactic-co-glycolic) acid, 697–699
 - polyethylenimine, 696–697
 - poly-L-lysine, 699–700
 - polymethacrylate, 703–705
 - and polyplexes, 536–538
 - Polymerization method, 100
 - Polymer-modified drug carriers, 738–740
 - Polymerosomes, 57–58
 - Polymers, in gene therapy, 147
 - Polymer solvents, 101
 - Polymersomes, 58
 - Polymethacrylate (PMMA), 315, 569–570, 573, 703–705
 - Polymethacrylic acid, 215, 227
 - Polymorphonuclear neutrophil cells (PMNs), 389–390
 - Polyoxyethylene-polyoxypropylene block copolymers, 323
 - Polyplexes, 149
 - Polypropylenimine dendrimers, 702–703
 - Polypseudorotaxanes, 733, 735*f*
 - cyclodextrin-based, 734–735
 - Polyrotaxanes, 701, 733–734, 735*f*, 739
 - Polysaccharide alginate, 211
 - Polyvinyl alcohol (PVA), 44, 630, 643–644, 658–659, 698
 - Polyvinyl pyrrolidone (PVP), 630
 - Porphyrin dyes, 267–268
 - Porphyrins, 168–169, 272–274
 - Positional scanning synthetic peptide
 - combinatorial libraries (PS-SPCLs), 355
 - Positive surface potential IONP (p-IONP), 284
 - Positron emission tomography (PET), 234, 327, 493, 765
 - Precipitation method, 102
 - Prednisone, 388–389
 - Pressure-sensitive hydrogels (PSHs), 649*t*
 - Presystemic clearance, 588–589
 - Processed nano-structured food products, 54
 - Prodigious zeal, 635
 - Propanolol, 604*t*
 - Proteases, 175–176, 323
 - Proteasome inhibitor drugs, in cancer treatment, 16–17, 17*f*
 - Proteasome inhibitors, 16–17
 - Protein-based hydrogels (PHs), 649*t*
 - Proteins, 347–348
 - exosomes as drug delivery platforms for, 428
 - nanoparticles targeting, 399–400
 - Proteins, drug delivery system for, 132–133
 - Protein stability, 132–133
 - Protein vaults, 57
 - Proton sponge effect (PSE), 537–538, 696
 - Protopanaxadiol, 604*t*
 - Protoporphyrin IX (PPIX), 168–169, 278
 - Pseudomonas aeruginosa*, 268–269, 282–283
 - Pseudomonas elodea*, 210
 - (Pseudo)polyrotaxanes of CDs, 737
 - Pulmonary delivery, of lipid nanoparticles, 771–772
 - in vitro aerosol characterization, 771–772
 - systemic pharmacokinetics and in vivo lung deposition, 772
- ## Q
- QAR (quantitative autoradiography), 680
 - Quality by design (QbD), 593–595
 - advantages and disadvantages of, 595–596
 - elements of, 594–595
 - enablers of, 593–594
 - Quality risk management (QRM), 593–594
 - Quality target product profile (QTPP), 594–595
 - Quantum dots (QDs), 41–42, 135–136, 159–160, 382–383, 543–544
- ## R
- Radical polymerization, 654
 - Radioimmunoconjugates (RICs), 169–170
 - Radio-nuclear imaging, 765
 - Radiopharmaceuticals, 232–233
 - Radiotherapy, 6–8
 - main logic of radiotherapy application, 8
 - types of, 7–8
 - Raman spectroscopy, 760
 - Rapid expansion of supercritical solution (RESS), 106
 - Reactive nitrogen species (RNS), 266–267
 - Reactive oxygen species (ROS), 167, 262–263
 - Receptors on cell membranes, 121
 - Recombinant adeno-associated virus (rAAV), 527
 - Recombinant DNA technology, 690
 - Redox-sensitive carriers, 175
 - Repaglinide, 604*t*
 - Respiratory syncytial virus (RSV), 135
 - Retention effect, 42, 50
 - Reticuloendothelial framework (RES), 122–123
 - Reticuloendothelial system (RES), 157–158, 382–383, 530

Retina, 204
 Retinal pigmented epithelium (RPE), 204
 Retroviral vector, 481*t*, 483–484
 Retrovirus-based gene therapies, 141
 Reverse targeting, 123
 Reversible addition and fragmentation transfer
 chain polymerization (RAFT), 105
 Reversible cross-linked hydrogels, 627
 RGD (arginine glycine aspartic acid) short
 peptides, 384
 RGD-targeted adeno-associated virus/phage
 vector-mediated tumor necrosis factor- α
 in vitro assay, 496–497
 preclinical trial in large animal model with, 498
 preclinical trial in small animal model with,
 497–498
 targeting tumor vasculature with, 495–496
 Rheometry, 212–213
 Rhodamine-loaded poly(lactic-co-glycolic acid),
 732–733
 Rituximab (Rituxan), 20
 RNA-induced silencing complex (RISC), 430–431
 RNA interference (RNAi), 387, 521
 RNAs, exosomes as drug delivery platforms for,
 429–431
 miRNA, 430–431
 short interfering RNA, 429–430
 ROS/RNS, 267–268

S

Salmonella typhi, 135
 Salting out method, 101
 Scanning electron microscopy (SEM), 415–416
 Scanning force microscopy (SFM), 416
 Scanning ion occlusion sensing (SIOS), 756–757
 Scavenger receptors, 126
 Schardinger dextrans. *See* Cyclodextrins (CDs)
 Schizophrenic micelles, 316
 Scintigraphy image, 234*f*
 Sesamol, 609*t*
 Second order targeting, 122
 Secreted protein, acidic and rich in cysteine
 (SPARC), 384
 Self-nanoemulsifying drug delivery systems
 (SNEDDS), 56
 Self-triggered targeting, 43
 Semi-interpenetrating network (semi-IPN), 646
 Sericin nanoparticles, 173–174
 Severe combined immunodeficiencies (SCID), 690
 Short interfering RNAs (siRNAs), 51–52,
 429–430
 Silk-based nanoparticles, 383–384
 Silymarin, 604*t*

Single photon emission computed tomography
 (SPECT), 327, 682–683
 Single walled carbon nanotubes (SWCNTs), 98,
 641
 Site-specific drug delivery, 124–140
 carriers, 126–139
 carbon nanotubes, 136
 cellular uptake, 137
 dendrimers, 129–131
 drug-loading techniques, 130
 drug transport, mechanism for, 131
 drug uptake mechanisms, 138
 liposomal transportation, 129
 liposomes, 127–129
 molecular diagnostics, 134–135
 nanoparticles, 131–132
 nanoparticles, release mechanism from, 132
 quantum dots, 135–136
 resealed erythrocytes, 138
 transfection, 131
 ligands, 140
 receptors, 124–126
 asialoglycoprotein receptors (ASGPR), 125
 carbohydrate-specific (lectin) receptors, 124
 Fc receptors, 125
 folate receptors, 126
 interleukin receptors, 125
 lipoprotein receptors, 125
 lymphocyte-homing receptors, 125
 mannose receptors, 125
 scavenger receptors, 126
 transferrin receptors, 126
 Site-specific gene targeting, 691–693
 antibody mediated targeting, 692
 aptamers, 692–693
 ligands, 693
 Skin, nanoparticles targeting, 395–398
 Skirt-shaped CDs, 722
 Small interfering RNA (siRNA), 387, 765–766
 Small unilamellar vesicles (SUV), 529–530
 Smart hydrogels, 212, 219, 640–642
 Smart nanoparticle, 443–444
 Smart “ondemand” antibiotic delivery, 282–283
 Sodium alginate, 229, 390–391
 Sodium carboxymethylcellulose, 229
 Solid lipid nanoparticles (SLNs), 91–93,
 389–390, 541–542, 752, 753*f*, 767
 drug release from, 92*f*
 and nanostructured lipid carriers, 57
 routes of administration of, 93
 Soluplus, 230–231
 Solvent diffusion method, 100–101
 Solvent emulsification evaporation technique, 755
 Solvent evaporation method, 99

- Somatic gene therapy, 146–147
 - germline gene therapy, 126
- Sonazoid, 457
- Spacetabs, 597
- Spectroscopic analysis, 216–218
- Spherical fullerenes, 89
- Spiropyran, 317–319
- Spontaneous emulsification/solvent diffusion
 - method, 100–101
- Spray atomization technique, 584–585
- Spray-drying, 106
- S-S bond. *See* Disulfide bonds
- Stable nucleic acid lipid particles (SNALPs), 531–532
- Staphylococcus aureus*, 270–271, 279, 282
 - effect of cadmium oxide nanoparticles on, 273f
- Staphylococcus epidermidis*, 270–271
- Stealthing, 297–298
- Stereo-complexed hydrogels, 636–638
- Stereotactic ablative radiotherapy (SABR), 7
- Sterically stabilized mixed phospholipid nanomicelles (SSMM), 351
- Stilbene, 317–319
- Stimuli gel system
 - toxicity and safety of polymers used in, 236–237
- Stimuli-responsive hydrogels, 648, 654f
- Stimuli-responsive liposomes, 94
- Stimuli-responsive micelles, 304–323
 - ability to avoid uptake by mononuclear phagocytic system, 297–298
 - core-shell structure, 298–299
 - critical micelle concentration and micelle stability, 297
 - diagnostic applications, 324–330
 - fluorescence-based imaging, 329–330
 - magnetic resonance imaging, 328–329
 - nuclear imaging, 327
 - X-ray computed tomography, 329
 - micelles and micelle-forming amphiphiles, structural aspects of, 299–301
 - amphiphilic block copolymers used in fabrication of micelles, 300–301
 - micelle shape and morphology, 299
 - micelles in drug delivery applications, 301–303
 - micelles in targeted drug delivery, 303–304
 - photoresponsive micelles, 316–322
 - photo-induced cleavage, 319
 - photo-induced cross-linkable polymeric micelles, 320–321
 - photo-induced energy conversion, 321–322
 - photo-induced rearrangement type micelles, 319–320
 - photoisomerization, 317–319
 - pH-responsive micelles, 305–311
 - properties of polymeric micelles, 296
 - size, 297
 - thermoreponsive micelles, 311–316
 - as hydrophilic block of amphiphile, 313–316
 - as hydrophobic block of amphiphile, 312–313
- Stimuli-responsive nanomaterials, 443–444
- Stimuli-responsive nanosystems, 155, 160–183
 - chemical stimuli-responsive, 171–176
 - enzymes stimulation, 175–176
 - pH stimulation, 171–174
 - redox activity stimulation, 174–175
 - dual stimuli-responsive nanosystems, 177–182
 - future perspectives, 183–184
 - multistimuli responsive nanosystems, 183
 - physical stimuli-responsive nanosystems, 160–171
 - magnetic field stimulation, 160–164
 - photostimulation, 167–169
 - radiation stimulation, 169–170
 - temperature stimulation, 164–167
 - ultrasound stimulation, 170–171
- Stimuli-sensitive delivery systems, 443
 - delivering anticancer therapeutic agents via, 468–470
 - exogenous stimuli-responsive systems, 463–467
 - light-responsive systems, 463–464
 - thermo-responsive systems, 464–467
 - external multistimuli-responsive drug delivery systems, 467–468
 - external-stimuli-responsive systems, 445–463, 445f
 - electro-responsive drug delivery systems, 461–463
 - magnetically responsive systems, 445–456
 - ultrasound-responsive systems, 456–461
 - future directions, 471
- Stimuli-sensitive hydrogels, 208–212
 - ion-sensitive hydrogel, 210–211
 - multistimuli hydrogels, 211–212
 - pH-sensitive hydrogels, 209
 - temperature-sensitive systems, 209–210
- Stimuli-sensitive nanomaterials for antimicrobial drug delivery, 261
 - effect of stimuli on drug delivery, 269–286
 - effect of light on antimicrobial nanoparticle, 272–282
 - effect of magnet on antimicrobial nanoparticle, 283–286
 - effect of temperature and pH on antimicrobial nanoparticles, 270–272
 - effect of the enzyme on the antimicrobial nanoparticle, 282–283

- Stimuli-sensitive nanomaterials for antimicrobial drug delivery (*Continued*)
- future prospects, 286–287
 - mechanism of action of antimicrobial nanoparticles, 265–268
 - physicochemical behavior of nanoparticles on drug property improvement, 263–265
 - type of antimicrobial nanoparticles, 268–269
- Stimuli sensitive ocular drug delivery systems, 201
- drug loading and release properties, pharmaceutical systems for the modification of, 221–232
 - drug penetration and promotion, studies of, 239–241
 - eye, anatomical characteristics of, 202–207, 205*f*
 - periocular drug delivery, 206–207
 - topical administration, 205–206
 - topical ocular drug delivery, barriers to, 204–205
 - hydrogel as drug delivery systems, 207–221
 - in situ hydrogels, properties of, 212–219
 - stimuli-responsive hydrogels, 219–221
 - stimuli-sensitive hydrogels, 208–212
 - hydrogels' effect on corneal regeneration capacity, 237
 - in vivo studies in animals and effectiveness of drugs, 238–239
 - new technologies for determining ocular residence time and ocular biodistribution of hydrogels, 232–235
 - toxicity and safety of polymers used in stimuli gel system, 236–237
- Streptococcus pyogenes*, 274–275, 398, 769–770
- Streptomyces* spp., 12–14
- Subconjunctival drug delivery, 206
- Subtenon drug delivery, 206
- Sugar coating, 584–585
- Sulfobutylether- β -cyclodextrin (SBECD), 231
- Supercritical antisolvent (SAS), 106
- Supercritical fluid technology, 106, 536
- Superparamagnetic iron oxide (SPIO) nanoparticles, 162, 165–166, 328
- Supramolecular hydrogels, 737
- Supramolecular polymer micelles (SMPMs), 738–739
- Surface Gibb's free energy, 48–49
- Surface-modified nanoparticles, 132*f*
- Surface-to-volume ratio of nanoparticles, 50
- Surfactants, 586
- Sustained release matrix tablets, 600
- Swelling controlled drug release system, 579
- Synthetic gene delivery vectors, 145–146
- Synthetic polymers, 207, 448–449, 569–570
- ## T
- Tamoxifen, 9*t*
- Taniguchi, Norio, 39–40
- Targeted drug delivery system (TDDS), 119, 150–151
- Targeted gene therapy, 486
- Targeting, 118–124, 340–344
 - active targeting, 343–344
 - background, 118–119
 - concept and rationale, 120
 - levels of, 122–124, 122*f*
 - active targeting, 123
 - double targeting, 124
 - dual targeting, 123–124
 - passive targeting, 122–123
 - reverse targeting, 123
 - passive targeting, 341–342
 - target identification, 120–121
 - drug targets in the body, 121
 - genetic approach, 121
 - genomic approach, 121
 - types, 692*f*
- Targeting ligands, 344–350
 - antibody fragments, 345
 - aptamers, 347
 - monoclonal antibodies, 344–345
 - nanobodies as targeting therapeutics and targeting ligands, 345–346
 - peptides, 348–350
 - proteins, 347–348
- Targeting peptides from biological and chemical libraries, 353–355
 - bacterial display libraries, 354–355
 - biological library methods, 353–354
 - chemical libraries, 355
 - phage display, 354
- Target product profile (TPP), 594–595
- Targets for drug delivery, 86–87
- Target-specific delivery, 117
 - candidate diseases, 149
 - challenges and obstacles, 140–141
 - delivery systems in gene therapy, 147–149
 - aquasomes, 149
 - cationic lipid compatible systems, 148–149
 - lipoplexes, 148
 - liposomes, 147–148
 - polymers, 147
 - polyplexes, 149

- gene delivery, 141–142
 - approaches for gene therapy, 142
 - prerequisites, 142
 - vectors, 141–142
- gene modification, 143
 - replacement therapy, 143
- gene transfer techniques, 144–146
 - biological (viral vectors), 146
 - chemical (nonviral gene delivery), 146
 - electroporation, 144
 - molecular vibration, 144–145
 - particle bombardment, 144
 - photochemical transfection, 145–146
 - ultrasound mediated transfection, 144
- problems associated with gene therapy, 149
- site-specific drug delivery. *See* Site-specific drug delivery
- somatic gene therapy, 146–147
 - germline gene therapy, 126
- Taxol (TXA), 350–351, 469*t*
- T cell, genetic engineering in, 24–25
- Tear film, 203
- Temperature-responsive hydrogels, 649*t*
- Temperature-sensitive hydrogels
 - based on PEG–polyester block copolymer, 631–634
 - based on poly(*N*-isopropyl acrylamide), 634–635
- Temperature-sensitive polymers, 238*t*
- Temperature-sensitive systems, 209–210
- Terbinafine hydrochloride, 226
- Terbinafine-loaded nanoemulsions, 226
- Test tube inversion, 213–214, 214*f*
- 1,4,7,10-Tetraazacyclodecane-1,4,7,10-tetracetic acid (DOTA), 327, 682–683
- Tetraethoxysilane (TEOS), 284
- Tetrandrine, 604*t*
- Theophylline, 388–389, 609*t*
- Theranostics, 137–138, 165–166, 303, 324
- Therapeutic index (TI), 124, 569
- Thermal decomposition method, magnetic nanoparticles preparation by, 448
- Thermoresponsive polymer
 - as hydrophilic block of amphiphile, 313–316
 - as hydrophobic block of amphiphile, 312–313
- Thermo-responsive systems, 464–467, 635–636
 - hydrogels as external temperature-responsive material, 465–466
 - nonthermal effects, 456–457
 - novel dendrimers as thermo-responsive substances, 466–467
 - thermal effects, 457
- Thermo-reversible gels, 644
- Thermosensitive hydrogels, 212–213, 220–221
- Thermosensitive materials, 165
- Thin film hydration (TFH) method, 453
- Third order targeting, 122
- 3D conformal radiotherapy, 7
- Three-dimensional (3D) polymeric networks, 626
- Tight junction proteins (TJP), 674–675
- Tight junctions, 43–44
- Timolol maleate, 221, 227
- Titanium dioxide, 268, 385–386, 389
- T lymphocytes, 19
- Toluene, 90
- Toluidine blue O–tiopronin gold (TBO), 276
- Topical drug delivery, 205–206
 - semisolid ophthalmic preparations, 206
 - solutions, 205
 - suspensions, 206
- Topical glucocorticoids (TGs), 395–396
- Topoisomerase enzymes, 15–16
- Topoisomerase inhibitors, 14–16
- Tositumomab (Bexxar), 20
- Tramadol, 609*t*
- Tranexamic acid, 604*t*
- Transfection, 131, 144–145, 701, 705
- Transferrin receptors, 126, 347–348, 393
- Transforming growth factor- β (TFG- β), 517–520
- Transglutaminase, 657
- Translocation, 774–775
- Transmission electron microscopy (TEM), 415–416
- Transtuzumab (Herceptin), 20
- Transwell inserts, 774
- 1,4,7-Triazacyclononane-*N,N',N''*-triacetic acid (NOTA), 327, 682–683
- Trimetazidine, 609*t*
- Triple-layered nanogel (TLN), 282
- N*-Trymethylchitosan, 227–228
- Tumor angiogenesis, 495–496
- Tumor cells, nanoparticles targeting, 383–387
- TumorHoPe public database, 356, 366
- Tumor-infiltrating lymphocytes (TIL), 480
- Tumor-Suppressor miRNAs downregulated in cancer, 518*t*

U

- Ulex europaeus agglutinin 1, 604*t*
- Ultracentrifugation, 413–414, 758
- Ultrasonic responsive nanopreparations, marketed products of, 471*t*
- Ultrasound, 323
- Ultrasound enhanced gene transfer (UEGT), 144
- Ultrasound mediated transfection, 144
- Ultrasound-responsive systems, 456–461
 - biomedical applications of, 461

Ultrasound-responsive systems (*Continued*)
 mechanism of action, 456–457
 types of, 457–461
 ultrasonic-mediated polymeric micelles,
 459–460
 ultrasonic microbubbles, 457
 ultrasonic nanobubbles (nanodroplets), 459
 ultrasonic responsive liposomes, 458–459
 ultrasonic responsive microemulsions,
 460–461
 3' Untranslated region (3' UTR), 544
 Upper critical solution temperature
 (UCST), 311–312, 312*f*, 465
 Uracil phosphoribosyl-transferase
 (UPRT), 428

V

Vaccines, 352, 604*t*
 Van der Waals interactions, 46–47
 Vascular endothelial growth factor
 (VEGF), 303, 680
 Vascular endothelium, nanoparticles
 targeting, 394–395
 Vasoactive intestinal peptide (VIP)
 design of peptides mimetics of, 363–365
 Vectors, 481–486
 nonviral vectors, 481–482
 viral vectors, 482–486
 adeno-associated viral vector, 485
 adenoviral vector, 484–485
 bacteriophage vector, 482–483
 lentiviral vector, 485–486
 retroviral vector, 483–484
 Vessels that encapsulate the tumor cluster
 (VETC), 517–520
 Vinpocetine, 609*t*
 Viral vectors, 146, 481–486, 523–529
 adeno-associated viral vector, 485

adeno-associated virus, 526–528
 adenoviral vector, 484–485
 adenovirus, 525–526
 bacteriophage vector, 482–483
 herpes simplex virus, 528–529
 lentiviral vector, 485–486
 lentivirus, 524–525
 retroviral vector, 483–484
 Viscosifying agents, 205
 Vitamin A palmitate, 227–228
 Vitamin E, 302
 Vitamin E-d-alpha-tocopheryl PEG succinate, 44
 Vitreous humor, 204
 Volatile organic compounds (VOCs), 401
 Volumetric-modulated arc radiotherapy
 (VMAT), 7
 Voriconazole, 231
 Vögtle, Fritz, 97

W

Warburg effect, 386–387
 Water-in-oil emulsion, 455–456
 Water-insoluble polymers, 577, 608–612
 Western blotting, 416–417, 431

X

X-ray computed tomography, 329
 X-ray diffractometry (XRD), 760
 Xylene, 90
 Xyloglucan, 210

Z

Zeta potential, 47–48, 265, 266*f*, 757–758
 Zinc oxide (ZnO), 389
 Zn-phthalocyanine (ZnPC)- ϵ -polylysine (ϵ -PL)
 conjugates, 279

DRUG TARGETING AND STIMULI SENSITIVE DRUG DELIVERY SYSTEMS

Edited by
Alexandru Mihai Grumezescu

A global panel of experts shows how nanotechnology helps create more effective drug-targeting systems.

This book presents recent advances in the area of stimuli-sensitive drug delivery systems. The book presents an up-to-date overview of physical, chemical, biological, and multistimuli-responsive nanosystems. In addition, the book presents an analysis of clinical status for different types of nanoplateforms. Furthermore, the book:

- Shows how the use of nanomaterials can help target a drug to specific tissues and cells.
- Explores the development of stimuli-responsive drug delivery systems.
- Includes case studies to showcase how stimuli responsive nanosystems are used in a variety of therapies, including camptothecin delivery, diabetes, and cancer therapy.

Written by an internationally diverse group of researchers, the book is an important reference resource for biomaterials scientists and for those working in the medical and pharmaceutical industries who want to learn more about how nanostructures can be used to create more effective drug delivery systems.

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Pharmacology

ISBN 978-0-12-813689-8



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