



# Nanomaterials and molecular transporters to overcome the bacterial envelope barrier: Towards advanced delivery of antibiotics

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## ABSTRACT

With the dramatic consequences of bacterial resistance to antibiotics, nanomaterials and molecular transporters have started to be investigated as alternative antibacterials or anti-infective carrier systems to improve the internalization of bactericidal drugs. However, the capability of nanomaterials/molecular transporters to overcome the bacterial cell envelope is poorly understood. It is critical to consider the sophisticated architecture of bacterial envelopes and reflect how nanomaterials/molecular transporters can interact with these envelopes, being the major aim of this review. The first part of this manuscript overviews the permeability of bacterial envelopes and how it limits the internalization of common antibiotic and novel oligonucleotide drugs. Subsequently we critically discuss the mechanisms that allow nanomaterials/molecular transporters to overcome the bacterial envelopes, focusing on the most promising ones to this end – siderophores, cyclodextrins, metal nanoparticles, antimicrobial/cell-penetrating peptides and fusogenic liposomes. This review may stimulate drug delivery and microbiology scientists in designing effective nanomaterials/molecular transporters against bacterial infections.

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**Abbreviations:** OM, outer membrane; CM, cytoplasmic membrane; OMP, Outer membrane protein; LP, lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; WTA, wall teichoic acids; LTA, lipoteichoic acids; CD, cyclodextrin; TBDT, TonB-dependent transport; pmf, proton motive force; ABC, ATP-binding cassette; NAM, Nucleic acid mimic; PNA, peptide nucleic acids; PMO, phosphorodiamidate morpholino oligomers; LNA, locked nucleic acids; 2'OMe, 2'-OMethyl RNA; PO, phosphodiester linkages; PS, phosphorothioate linkages; MIC, minimum inhibitory concentration; NP, Nanoparticles; ROS, reactive oxygen species; LSPR, surface plasmon resonance; TEM, transmission electron microscope; AMP, Antimicrobial peptide; CPP, Cell-penetrating peptide; SNAPPs, structurally nanoengineered antimicrobial peptide polymers; PAMAM, Polyamidoamine; a.a., amino acid; X, 6-aminohexanoic acid; B, β-alanine; K, Lysine; F, Phenylalanine; R, Arginine; Q, Glutamine; I, Isoleucine; W, Tryptophan; N, Asparagine; CNS, Carbon nanostructures; CNT, carbon nanotubes; DOPE, dioleoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DOTAP, dioleoyltrimethylammoniumpropane; DMPC, dimyristoylphosphocholine; DHP, dihexadecylhydrogenphosphate; DPPS, dipalmitoylphosphatidylserine; Chol, cholesterol.

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## 1. Introduction

The ability to control infections with antibiotics has had a major impact on human and animal life over the last century [1]. However, the antibiotic era is threatened by resistance of bacteria to antibiotics and a very short pharmaceutical pipeline of new antibiotics [2]. Infections caused by resistant bacteria are already responsible for nearly 50,000 annual deaths in Europe and the United States [3,4].

The bacterial cell envelope is a sophisticated permeability barrier that can hinder the internalization/accumulation of antibiotics in bacterial cells, being a major cause of bacterial resistance to antibiotics [5]. Poor antibiotic internalization could be solved if antibiotics were "carried" into bacterial cells. Bacterial resistance to antibiotics can also arise from the expression of specific genes of resistance [6–8]. This could be solved by controlling gene expression in bacteria through anti-sense oligonucleotides [9]. However, oligonucleotides generally fail to be internalized in bacteria [10]. Nanomaterials and molecular transporters able to successfully interact with the bacterial envelopes are interesting to carry oligonucleotides and poorly internalized antibiotics across the bacterial envelope. Also, these carriers may prevent drug efflux from the bacterial cells, as intracellular delivery of a high dosage of drug into bacteria may overwhelm the efflux pumps [11,12]. The efflux of antibiotics will not be detailed here since it has already been extensively reviewed [13–16]. Instead of serving as carriers of bactericidal drugs into bacteria, certain nanomaterials and peptides interact with the bacterial envelope so extensively that they can disrupt it, thus serving as bactericidal per se to replace antibiotics. Clearly, the potential of nanomaterials and molecular transporters to overcome the bacterial envelope depends on the ability of the materials/transporters to efficiently interact with the different bacterial envelopes.

Nanomaterials and molecular transporters have been widely explored in mammalian cells which mostly take them up by endocytosis [17]. Differently, it is believed that bacteria do not endocytose (except a restricted group belonging to the phyla Planctomycetes [18]) and the use of nanomaterials/molecular transporters in bacteria is mostly based on a trial and error approach [1,11,12,19–21]. An in-depth discussion on how the different layers of bacterial envelopes may be potential barriers is pivotal to boost the rational development of more efficient nanomaterials/molecular transporters in the combat of bacterial infections. Although there are some interesting reports on the recent use of nanomaterials to manage infections [1,11,12,19–21], to the best of our knowledge the interaction of nanomaterials with bacteria has never been addressed.

In this review we first summarize the general structure of the bacterial envelope of Gram-negative bacteria and Gram-positive bacteria of the typical Firmicutes phylum (Actinobacteria with a different cell wall, such as that of the genus *Mycobacterium*, *Nocardia*, *Rhodococcus*

and *Corynebacterium*, are beyond the scope of this review). We then consider the permeability of these envelopes to external compounds and how it limits the internalization of common antibiotics and novel oligonucleotide drugs into bacteria. Subsequently, the nanomaterials and molecular transporters with potential to overcome the bacterial envelope, either by direct penetration or intracellular delivery of antibiotic/oligonucleotide drugs, are discussed. A special focus is given to the cell wall, i.e. the outermost layer of the bacterial envelope that determines the bacterial envelope permeability and the initial interaction with foreign compounds and materials. Seemingly much more research has been reported on the transport in Gram-negative bacteria compared to Gram-positive bacteria; nevertheless, this review aims for a global overview, including the relevant Gram-positive teichoic acids which are seldom considered. Finally, our conclusions and future perspectives are presented.

## 2. Bacterial cell envelope barrier

Bacteria, like mammalian cells, have their cytosol surrounded by a symmetric bilayer composed of amphiphilic phospholipids – the cytoplasmic membrane [22]. However, because they are unicellular organisms that often inhabit hostile environments, bacteria developed an extra cell wall that surrounds and protects the cytoplasmic membrane [23]. The cell wall provides protection against osmotic pressure and mechanical damage, while allowing permeation of key substrates for bacterial metabolism and communication with the environment [24,25]. Together, the cell wall and the cytoplasmic membrane compose the bacterial cell envelope. The cell wall, in turn, is subdivided into different layers. There is often lack of precision in the field on the reference to the different bacterial envelope layers. In this section, these envelope layers and their permeability will be described for Gram-negative and Gram-positive bacteria of the typical Firmicutes phylum, Figs. 1 and 2. The permeability of these envelopes is overviewed according to the current knowledge (which is still limited), with a focus on the transport through the cell wall – the critical intake barrier.

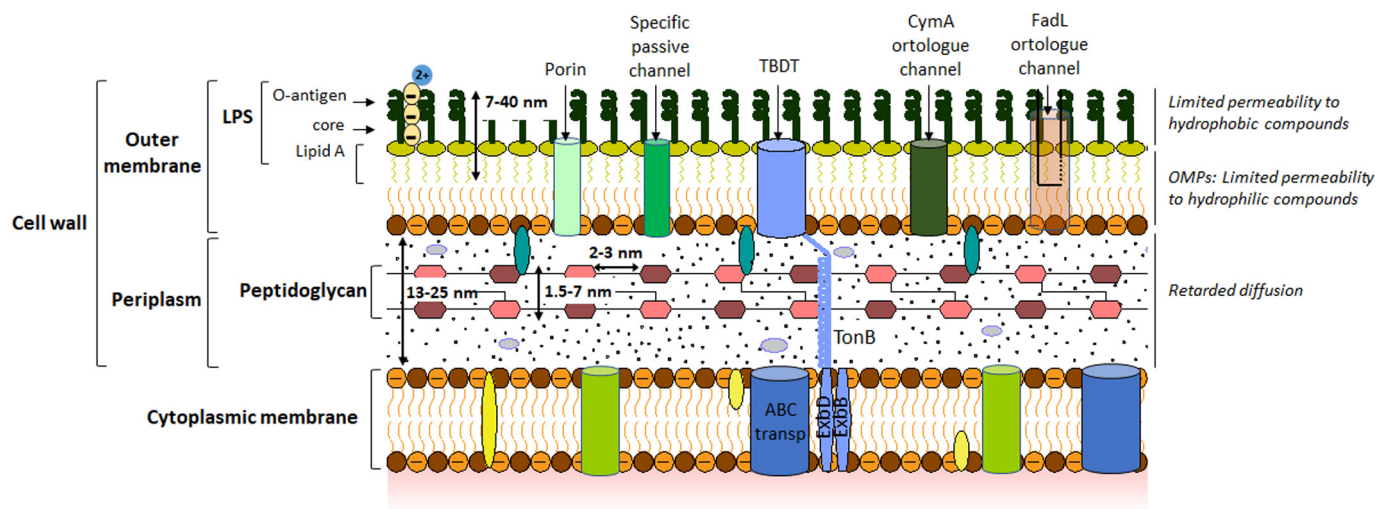
### 2.1. Gram-negative bacteria

The cell wall of Gram-negative bacteria comprises the outer membrane (OM) and the periplasmic space composed of a thin layer of peptidoglycan, Fig. 1 [26]. Since the OM is determined to define the permeability to foreign compounds, a particular focus is given below to the OM.

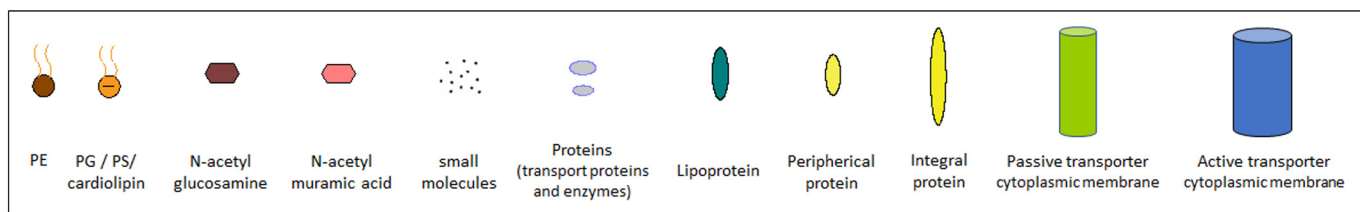
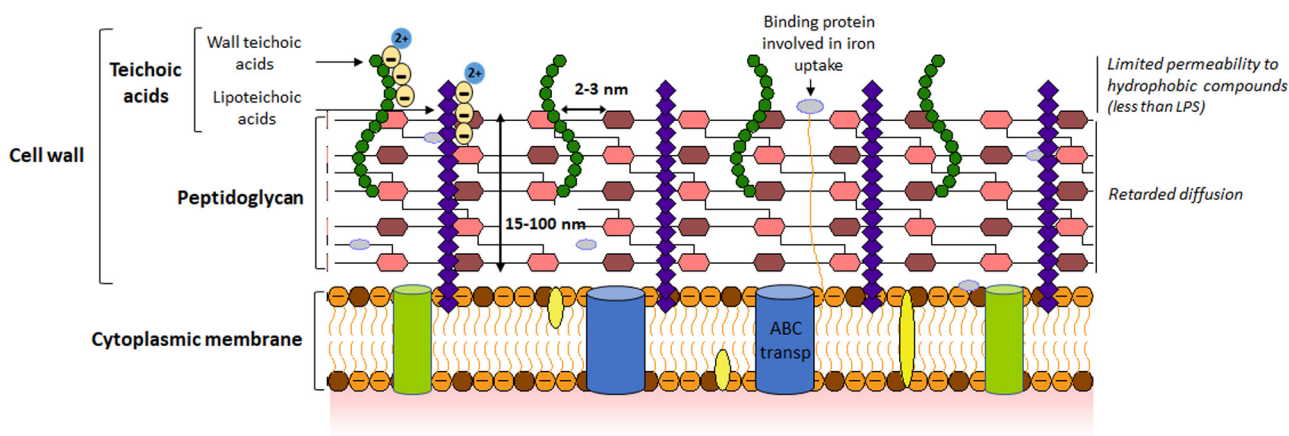
#### 2.1.1. Outer membrane

The OM is not a common phospholipid bilayer, but rather an asymmetric lipid bilayer – it is composed of phospholipids only in the inner

## Gram-negative bacteria



## Gram-positive bacteria







**Fig. 1.** Structure and permeability of the bacterial envelope of Gram-negative and Gram-positive bacteria of the typical Firmicutes phylum. Note that the TBDT transporter in the OM of Gram-negative bacteria is part of a complex system that spans the envelope, composed of (i) a specific TBDT at the OM that binds the substrate, (ii) the TonB complex (comprising the TonB protein at the periplasmic space and ExbB and ExbD proteins at the cytoplasmic membrane) that transduces the pmf of the cytoplasmic membrane for the conformational change of TBDT, (iii) a protein in the periplasm (light purple circle on top of the 'ABC transp') that captures the substrate that flows from the open TBDT channel and (iv) an ATP-binding cassette (ABC) transporter that transports the substrate across the cytoplasmic membrane using ATP [28,49]. Also, LPS in Gram-negative bacteria is represented with its three regions: the lipid A (a lipid with a large number of saturated fatty acids), the core polysaccharide (a complex anionic oligosaccharide) and the O-antigen (a specific O-polysaccharide that differs between bacteria and can be highly antigenic) [22,23,27,30]. LPS and teichoic acids are represented with their net negative charge (yellow circles) and the divalent cations attached (blue circles); the number of circles depicted is not representative and the divalent cations occur regularly along the LPS. Note that the peptidoglycan layer should be about 1.5–7 nm thick in Gram-negative bacteria [65,66] and about 15–100 nm thick in Gram-positive bacteria [23,66], while the peptidoglycan mesh is equally wide (2–3 nm) in both Gram types [54–56]. The thickness of LPS and periplasmic space in Gram-negative bacteria are believed to be 7–40 nm and 13–25 nm, respectively [27,30,67–69]. TBDT: TonB-dependent transporter; pmf: proton motive force; ABC transp: ATP-binding cassette transporter involved in the active iron uptake; LPS: lipopolysaccharide; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol.

leaflet and mostly lipopolysaccharide (LPS) in the outer leaflet [24,27,28]. In Gram-negative bacteria the major membrane phospholipid is phosphatidylethanolamine (PE), followed by phosphatidylglycerol (PG) and smaller amounts of cardiolipin [29,30] (Fig. 1). The LPS is a nonfluidic

amphiphilic structure composed of three covalently-linked regions (Fig. 1). LPS has a net negative charge, higher than the usual negatively-charged phospholipids, and it is held in position at the OM surface by divalent cations (Fig. 1) [23,24,27]. The presence of LPS in the OM decreases

## Gram-negative OMPs

Passive diffusion			Active transport
General Porins Do not bind substrates	Specific channels Bind scarce substrates with low affinity		TBDTs Bind very scarce substrates with high affinity
		Ligand-gated channels 	
$\leq 0.7\text{--}0.8\text{ nm}$ $\leq 600\text{ Da}$	$\leq 0.5\text{--}0.9\text{ nm}$ $\leq 850\text{ Da}$	<i>FadL</i> $0.8\text{ nm}$ $\leq 300\text{ Da}$	<i>CymA</i> $1.1\text{--}1.4\text{ nm}$ $\leq 980\text{ Da}$
<b>Substrates:</b> small hydrophilic compounds (e.g. small sugars, amino acids and ions)	<b>Substrates:</b> <i>LamB</i> : malto-oligosaccharides (linearized) <i>Tsx</i> : nucleosides and deoxynucleosides	<b>Substrates:</b> long fatty acids (e.g. oleic acid)	<b>Substrates:</b> $\alpha$ -cyclodextrin
			$\leq 2.5\text{ nm}$ $\leq 1360\text{ Da}$ <b>Substrates:</b> iron complexes, vitamin B12, colicins (unfolded)

**Fig. 2.** Channels formed by outer membrane proteins (OMPs) in Gram-negative bacteria. These can be passive diffusion channels, formed by general porins and specific channel, and active transporters, formed by TonB-dependent transporters (TBDTs). The diameter of the pores in the channels and the respective molecular weight exclusion (based on the substrates known to penetrate those channels) are presented. Specific channels are indicated in *italic*.

its permeability to hydrophobic compounds (Fig. 1) up to 50–100 times (as calculated from the oxidation's rate of a steroid probe in the cytosol of Gram-negative bacteria with respectively intact and LPS-deficient OM) [23,24,27,31,32]. Hydrophobic substrates that succeed in crossing the LPS are expected to become internalized into the cytosol by diffusion through the lipid bilayers of the outer and/or cytoplasmic membranes. In addition, the OM contains embedded proteins, mainly the outer membrane proteins (OMPs; which are integral proteins spanning the entire OM) and lipoproteins (LPs; which are mainly embedded in the cytoplasmic leaflet of the OM) (Fig. 1) [23]. The function of LPs is not yet completely resolved, they may act as enzymes and transporters [33]. Differently, the OMPs form aqueous channels crucial for bacterial intake of nutrients [23].

OMPs can serve either as passive diffusion channels (general porins and specific channels) or active transporters (Fig. 2).

General porins (or simply porins), the most abundant proteins of the OM, allow the internalization of small hydrophilic substrates (including sugars, amino acids and ions) that are available in high concentrations and can thus passively diffuse down the concentration gradient through porins (Fig. 2) [16,34–37]. Porins do not bind the transported compounds; rather, porins are relatively unspecific and mostly discriminate their substrates by size, although charge may also play a role [28]. According to the crystallography studies on the better known classical porins (OmpF, OmpC and PhoE) present in *Escherichia coli* (*E. coli*), OmpF is typically considered the largest porin – a pore size of 0.7 nm and an exclusion limit of about 600 Da [16,28,30]. OmpF allows the diffusion of both cationic and uncharged molecules, while OmpC is slightly cation selective, and PhoE is slightly anion selective [30,38–41]. Another exceptionally large porin named OmpG (with a pore diameter of 1.5 nm that can be constricted to 0.8 nm) was later found to exist in *E. coli* (and other bacteria), but only in rather small amounts [42]. The Gram-negative species investigated so far possess general porins with a permeability mostly similar to that of the general porins of *E. coli*, except *P. aeruginosa* which has a significantly lower permeability since its major OMP channel (OprF) exists as an open channel only at very low levels [22,28,35,43].

Hydrophilic substrates needed for bacterial growth are not always available in sufficiently high concentrations to diffuse fast through general porins [30]. Thus, specific passive OMP channels exist that can bind

substrates, with low affinities, in order to preferentially facilitate their passive diffusion (Fig. 2) [34,44]. They can also allow the unspecific flow of compounds, such as amino acids and carbohydrates, that are scarce in the environment [23,28,36]. Like in general porins, transport through specific passive OMP channels is still driven by the concentration gradient and is thus energy-independent [34]. The best well-known specific channels are LamB and Tsx from *E. coli* [16,36]. The LamB channel (which pore is 0.5–0.6 nm in diameter) is dedicated to the transport of maltose and larger malto-oligosaccharides, which need to be first converted in a linear form to fit the LamB channel [23,28,36,45]. In addition, ScrY, a channel homologous to LamB but larger ( $\approx 0.85\text{ nm}$  pore diameter) allows the specific transport of several sugars such as sucrose, in some *E. coli* and *Salmonella* strains [28,30,46]. The Tsx channel, on the other hand, is involved in the specific transport of nucleosides and deoxynucleosides; free bases or nucleoside monophosphates (nucleotides) are not internalized [30,46]. Tsx has several distinct binding sites in the channel; the part that binds the base moiety of nucleosides is only 0.3–0.5 nm wide, but the part that binds the sugar moiety is 0.7–0.8 nm wide [46].

Other noteworthy specific passive OMP channels were recently discovered that only open upon the presence of their substrate, without the need of energy, named ligand-gated channels (Fig. 2). This way the passive diffusion of a cyclic oligosaccharide, which is too big to pass through the classical OMP passive channels, was found possible without linearization (needed for malto-oligosaccharides to cross LamB) [45]. In particular,  $\alpha$ -cyclodextrin ( $\alpha$ -CD), with a cylindrical bulky structure of 973 Da and an outer diameter of 1.37 nm, was internalized via the CymA channel in *Klebsiella oxytoca* (a species closely related to *Klebsiella pneumoniae*) which, in the open state, has a diameter of around 1.1–1.4 nm [45,47]. Orthologues of CymA are present in the Enterobacteriaceae and Vibrionaceae, although the channel permeability has only been studied in *Klebsiella oxytoca* [45].

Another ligand-gated channel is dedicated to the transport of hydrophobic long fatty acids, therefore contradicting the general understanding that only molecules with a hydrophilic surface can diffuse through OMP channels [44,48]. In particular, oleic acid, 283 Da, could be internalized by FadL channels, which are widespread among Gram-negative bacteria, but better studied for *E. coli* [44,48]. Differently from hydrophilic compounds, the hydrophobic fatty acids are not transported across the



membrane via the polar central part of the channels, but rather through a lateral opening (of 0.8 nm in diameter) from where the fatty acids diffuse laterally in the OM (Fig. 2) [37,44].

Valuable hydrophilic substrates that are available only in extremely low amounts (such as micronutrients) need to be bound with high affinity by active (transport) OMPs to be internalized (Fig. 2). The transport by such OMPs occurs against the concentration gradient, thus requiring energy expense [34]. Active OMPs, named TonB-dependent transporters (TBDTs), form large channels generally used for the uptake of iron complexes (normally up to 1000 Da) and vitamin B12 (around 1355 Da) [34,37]. TBDTs are present in much lower amounts than passive channels and only open when triggered by substrate binding, similarly to ligand-gated passive channels [23,28,45]. However, differently from ligand-gated diffusion channels, energy from the proton motive force (pmf) of the cytoplasmic membrane (as the OM is not energized) has to be transmitted to the TBDT to open the channel [45], which explains that the TBDT in the OM is part of a complex system that spans the envelope (Fig. 1) [28,49]. For the active intake of iron, iron is typically in the form of complexes formed by iron chelating siderophores [49]. The siderophores are transported as a whole via the OM transporters into the periplasm where they bind to a periplasmic binding protein and only in the cytoplasm the iron is released from the complex [49,50]. Also vitamin B12, containing a  $\text{Co}^{2+}$  ion in a corrin ring, is transported by bacteria as a whole [49]. Recently, it was found that besides iron and vitamin B12, the TonB-dependent transport can also be used to take up other substrates such as nickel and carbohydrates, but with much lower affinity [49]. Also colicins (proteinaceous toxins) can be taken up by TBDTs, as studied for colicin M transported via the TBDT named FhuA in *E. coli* – the only example of a protein import by *E. coli* [51]. The FhuA pore diameter is around 2.5 nm in the fully open state, while colicin M is 3 to 4 nm of diameter in the folded state, so it first needs to unfold to become (fully or partially) imported [51,52].

### 2.1.2. Periplasm and cytoplasmic membrane

Compounds that cross the OM will encounter the periplasmic space located in between the outer and cytoplasmic membrane (CM) (Fig. 1) [27,30]. The periplasm is crucial for cell's structure maintenance, nutrition and protection against potentially harmful compounds [23,24,27,30]. As such, the periplasm is densely populated with transport proteins (involved in the transport of sugars, amino acids, vitamins and inorganic ions) and enzymes that degrade potentially harmful compounds and participate in envelope biogenesis (such as phosphatases, nucleases, proteases and  $\beta$ -lactamases) [23,24,27,30]. Besides proteins, the periplasmic space possesses a high concentration of small molecules (such as amino acids, mono- and oligosaccharides and biosynthetic precursors and degradation products of peptidoglycan) which results in a space of significantly higher viscosity than the bacterial cytosol [24]. This gel-like periplasm can, therefore, considerably retard diffusion; protein diffusion was slowed down up to 3.5 times when compared to diffusion in the cytosol, as measured for the periplasmic and cytoplasmic green fluorescent protein in *E. coli* by fluorescence recovery after photobleaching [53].

The periplasmic space comprises a thin layer of peptidoglycan (Fig. 1). Peptidoglycan is a rigid polymer that provides structure, mechanical protection and osmoregulation [23,24,27]. It is a disaccharide composed of alternating units of *N*-acetylglucosamine and *N*-acetyl-muramic acid cross-linked by short peptide chains with variable composition [23,27]. These form a coarse mesh that will normally offer little resistance to diffusion [22,30]. The peptidoglycan mesh pore size is similar in Gram-negative and Gram-positive bacteria and it is thought to be around 2–3 nm, as estimated for *E. coli*, *B. subtilis* and *B. megaterium* (Fig. 1) [54–56]. Using this pore size, it was calculated that peptidoglycan should be permeable to globular uncharged hydrophilic proteins up to 22–24 kDa and 50 kDa, respectively for unstretched peptidoglycan (isolated from bacteria) and stretched peptidoglycan (resembling growing bacterial cells) [55]. Nevertheless, doubts remain about the peptidoglycan

permeability; moreover it is known that it can depend on the bacteria, the bacterial growth rate and the degree of peptidoglycan's cross-linking [57,58]. For example, *Helicobacter pylori* was hypothesized to have a larger peptidoglycan's mesh than *E. coli* [59].

The periplasm covers the cytoplasmic membrane which, differently from the OM, is a symmetric phospholipid bilayer (Fig. 1) [30]. The phospholipid composition in the cytoplasmic membrane is similar to that of the inner OM's leaflet [23,29,30,54]. Besides phospholipids, integral and peripheral proteins are also present in the cytoplasmic membrane [2]. These are either structural proteins or transport proteins involved in the passive or active transport of hydrophilic substrates into the cytosol [2]. For instance, most sugars such as maltose are carried by periplasmic proteins to the ATP-binding cassette (ABC) transporters in the cytoplasmic membrane [46]. For nucleosides transport, no periplasmic binding proteins are known and they are transported across the cytoplasmic membrane via the transporters NupC and NupG in *E. coli* mainly energized by the pmf [46,60]. Differently, hydrophobic substrates will pass through the hydrophobic lipid bilayer of the cytoplasmic membrane into the cytosol by simple diffusion.

### 2.2. Gram-positive bacteria

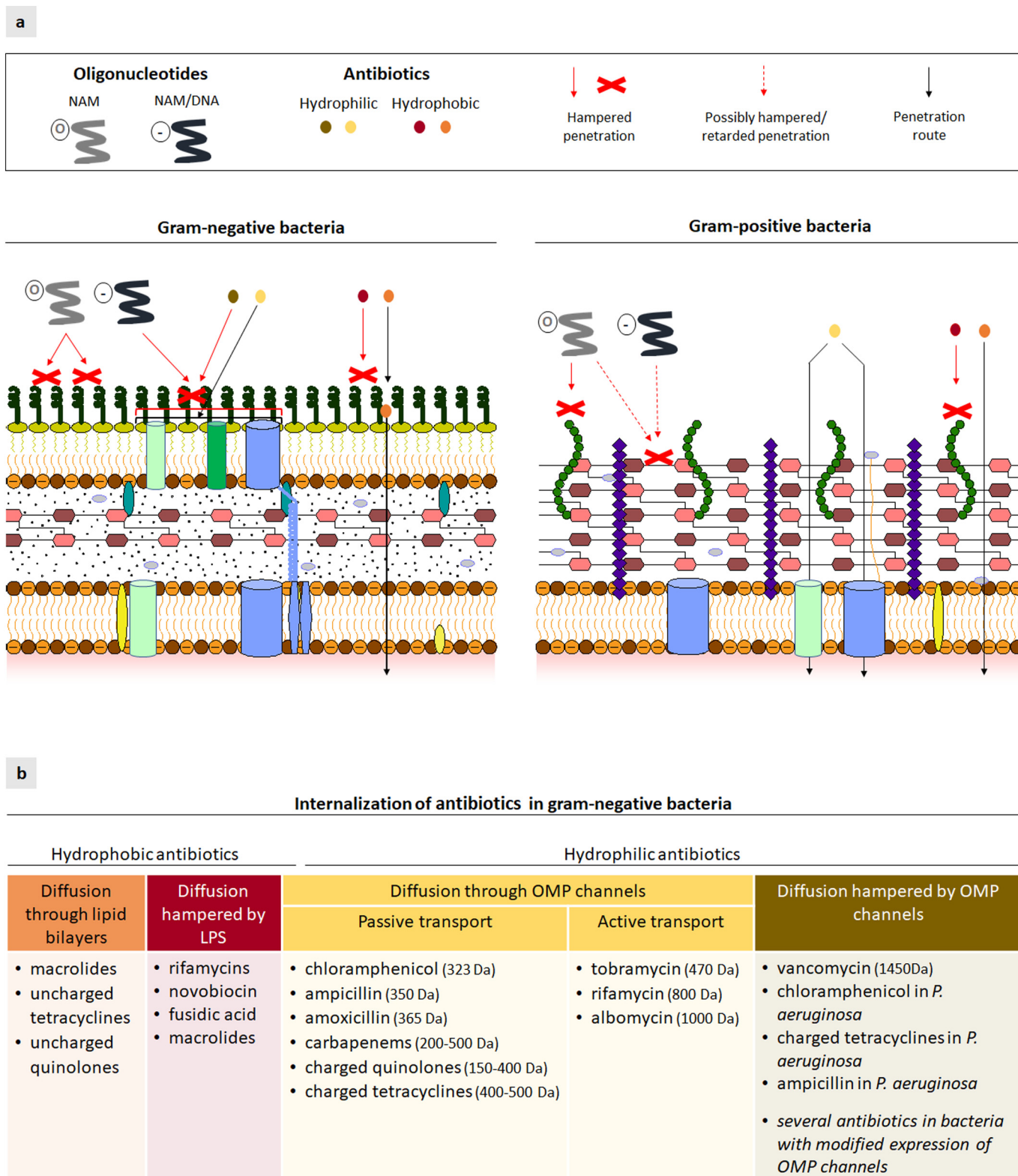
The cell wall that covers the cytoplasmic membrane in Gram-positive bacteria is markedly different from that of Gram-negative bacteria (Fig. 1). The Gram-positive cell wall is composed of teichoic acids and peptidoglycan, containing also proteins.

The cell wall is decorated with considerable amounts of anionic polysaccharides – the teichoic acids – that are in contact with the outer environment [23,30]. Teichoic acids in a certain way relate to LPS in Gram-negative bacteria. Due to their anionic charge they bind metal cations (mainly  $\text{Mg}^{2+}$ , but also  $\text{Ca}^{2+}$  and  $\text{K}^+$ ), regulating the envelope's rigidity and permeability [23,30]. Teichoic acids are divided in wall teichoic acids (WTA), covalently attached to the peptidoglycan, and lipoteichoic acids (LTA), anchored to the head groups of the cytoplasmic membrane (Fig. 1) [23,61,62]. Teichoic acids, in particular WTA, limit the permeation of hydrophobic compounds, in a similar way as LPS do but at a considerably lower extent [2,23,27,28,30,34,61].

The peptidoglycan in Gram-positive bacteria is similar in structure and porosity to that in Gram-negative bacteria [23,24,30]. However, to compensate the absence of an OM, in Gram-positive bacteria the peptidoglycan layer is significantly thicker and thus much more resistant to mechanical stress [23,24,30]. The thicker peptidoglycan layer can also retard the access of foreign compounds to the cytoplasmic membrane.

The Gram-positive's cell wall also comprises proteins, bound to the teichoic acids, the peptidoglycan, or the cytoplasmic membrane [23,30,63]. These proteins can be similar to the ones found in the periplasm of Gram-negative bacteria, being involved in defense, transport, synthesis and turnover of peptidoglycan, adhesion to other bacteria and to their host for infection [23,30,63].

The cytoplasmic membrane of Gram-positive bacteria is similar to that of Gram-negative bacteria, differing only in the relative phospholipid composition – Gram-positive bacteria generally possess a lower amount of PE and a higher amount of PG than Gram-negative bacteria (Fig. 1) [54,64]. Besides PE and PG, smaller amounts of cardiolipin and eventually phosphatidylserine are also present [54,64]. Like in Gram-negative bacteria, the cytoplasmic membrane contains peripheral and integral proteins, as well as passive and active protein channels for the internalization of hydrophilic compounds into the cytosol [2]. This includes the active transport of iron complexes, where a protein anchored on the cytoplasmic membrane (resembling the Gram-negative periplasmic binding protein) first binds the extracellular iron-siderophores and an ABC transporter brings the complex into the cytosol using ATP (Fig. 1) [50]. Hydrophobic substrates, on the other hand, diffuse passively through the lipids of cytoplasmic membrane bilayer into the bacterial cytosol.



**Fig. 3.** a) Limited permeability of the bacterial cell envelope of Gram-negative and -positive bacteria to oligonucleotides and common antibiotics. The depicted oligonucleotides include neutral oligonucleotides (NAMs such as PNA and PMO) and negatively charged oligonucleotides (DNA and NAMs such as LNA and 2'OMe). The antibiotics are represented as circles: yellow and orange circles – antibiotics that can be internalized; brown and red circles – antibiotics that cannot penetrate. The antibiotics identified as hydrophilic (brown and yellow circles) are too hydrophilic for diffusion through the lipid bilayers, contrary to the hydrophobic antibiotics (red and orange circles). On the Gram-positive bacteria, the cross on top of the wall teichoic acids should be considered as hampered penetration by teichoic acids in general (lipoteichoic acids and wall teichoic acids). b) Summary of the main internalization routes in Gram-negative bacteria that allow or hamper the influx of relevant antibiotics, based on the antibiotic's lipophilicity and size (as indicated by the approximate molecular weight of the exemplified antibiotics) [35,71,76,77]. The internalization of hydrophilic antibiotics can be further hampered if the expression of OMP channels in bacteria is modified or suppressed [35,82]. The envelope structures depicted are identified in Fig. 1.

### 3. The bacterial cell envelope limits internalization of antibiotics and antibacterial oligonucleotides

The bacterial cell envelope and in particular the cell wall are stringent barriers that can significantly restrict and even completely hinder the penetration of anti-infective drugs into bacterial cells. In the subsequent sections it is discussed how the bacterial envelopes of Gram-negative and -positive bacteria may pose a barrier to traditional antibiotics (Section 3.1) and to novel (antibacterial) oligonucleotides (Section 3.2).

#### 3.1. Internalization of antibiotics

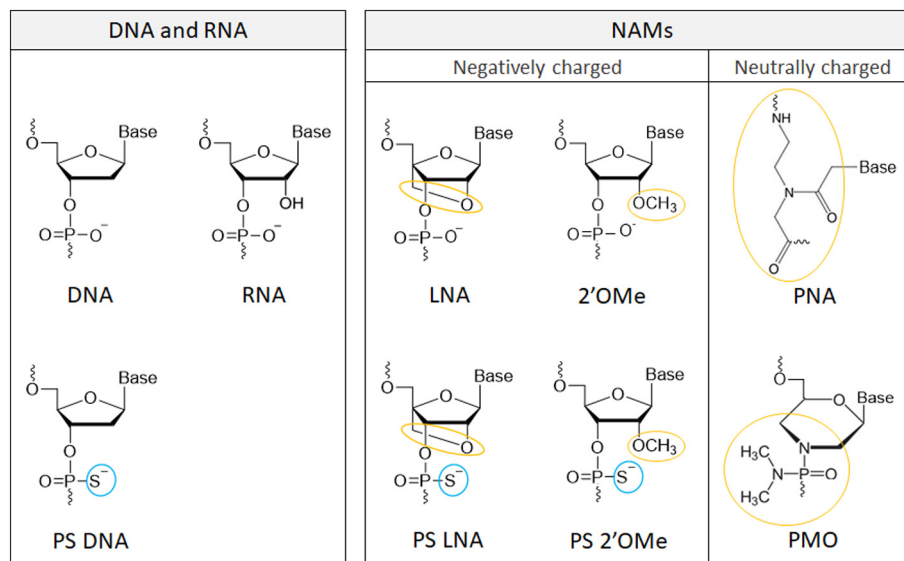
Antibiotics may act at the bacterial envelope or in the bacterial cytosol [70,71]. At the bacterial envelope, the peptidoglycan is a common target; the peptidoglycan's biosynthesis can be inhibited by  $\beta$ -lactam antibiotics (including penicillins, monobactams, cephalosporins and carbapenems) and glycopeptide antibiotics (vancomycin being the most common one) [72–74]. Intracellularly, antibiotics can target (i) DNA/RNA synthesis, as is the case of quinolones (e.g. ciprofloxacin), ansamycins (e.g. rifamycin), actinomycins, novobiocin and albicidin, (ii) protein synthesis, as is the case of tetracyclines, nitrofurans, macrolides (e.g. clarithromycin and erythromycin), aminoglycosides (e.g. tobramycin, streptomycin, kanamycin, gentamicin and amikacin), chloramphenicol, or fusidic acid and (iii) folate synthesis, being the case for sulphonamides and trimethoprim [70,75].

Teichoic acids in Gram-positive bacteria and LPS in Gram-negative bacteria restrict the permeation of hydrophobic antibiotics (Fig. 3a) [27,28,30,61]. In particular, LPS can hinder the penetration of macrolides, rifamycins, novobiocin, or fusidic acid, contributing to the resistance of Gram-negative bacteria to these antibiotics (Fig. 3b) [35,71,76,77]. The peptidoglycan is generally believed not to be a significant barrier for the permeation of antibiotics [22]. Some antibiotics, such as aminoglycosides, may also “force” their penetration into bacteria by disturbing the bacterial cell wall, a mechanism known as “self-promoted uptake” [28]; studies on Gram-negative bacteria indeed suggest that these antibiotics may compete with divalent cations for binding to LPS, thus destabilizing LPS and forcing their own penetration [28].

After crossing the teichoic acids/LPS, the majority of antibiotics that are able to reach the cytosol do so by passive diffusion [36]. Only a minority of antibiotics, such as tobramycin, may be actively transported

across the OM (Fig. 3a and b) [36,78]. Also 0.8 kDa rifamycin and 1 kDa albomycin (albomycin is comprised of an antibiotic moiety naturally covalently linked to  $\text{Fe}^{3+}$ -siderophores) are known to use the active TBDT channel named FhuA [36,79].

Passive diffusion of antibiotics can either occur (i) across the lipid bilayers of bacterial membranes – being the case for most antibiotics (e.g. novobiocin, macrolides, tetracyclines and quinolones), since they have some degree of hydrophobicity, or (ii) across the Gram-negative OM's general porins and specific passive channels – being the case for the small hydrophilic antibiotics (such as ampicillin, amoxicillin, chloramphenicol, carbapenems, tetracyclines and quinolones), Fig. 3b [16,28,35,36]. Quinolones and tetracyclines, depending on the pH, may exist in an uncharged or charged form, respectively crossing the OM via lipid mediated diffusion or via the porins (Fig. 3b) [28,35,80]. Penicillins, such as the zwitterionic ampicillin and amoxicillin, can be translocated via the general porin OmpF in *E. coli*; however, OmpF prevents anionic penicillins as carbenicillin to translocate, due to electrostatic repulsion between the anionic residues in the OmpF channel and the antibiotic [35,81]. The carbapenem imipenem can also use the general OmpF to penetrate *E. coli* and the specific OprD channel (which uptakes basic amino acids and peptides structurally similar to this carbapenem molecule) in *P. aeruginosa* [38]. Albicidin, a relatively high molecular weight (~850 Da) antibiotic, can passively diffuse through the Gram-negative Tsx specific channel [36,46]. Nevertheless, the size filtering effect of the OMP channels hinders or severely retards the diffusion of bigger hydrophilic antibiotics, as it is the case for glycopeptides like e.g. vancomycin (molecular weight of 1450–1500 Da) (Fig. 3b) [22,71,73]. These antibiotics, together with lipophilic antibiotics which influx is restricted by LPS (as described above), are thus only active against Gram-positive bacteria (Fig. 3b) [71]. Therefore, Gram-negative bacteria are intrinsically resistant to several antibiotics and among Gram-negative bacteria *P. aeruginosa* has a particularly low permeable OM [22,28]. In addition, functional change or loss/significant reduction in the number of expressed porins in Gram-negative bacteria can further decrease the OM's permeability to antibiotics [35,82]. Moreover, even antibiotics able to permeate the bacterial envelope in Gram-negative and -positive bacteria can become inactivated by bacteria expressing specific genes of resistance that code for enzymes that modify/degrade the antibiotic, or for a competitive inhibitor of the antibiotic or for altered forms of the antibiotic's substrate [6,71]. This antibiotic resistance crisis demands for novel antibacterial therapies.



**Fig. 4.** Chemical structure of the most studied nucleic acid mimics (NAMs). LNA: locked nucleic acids; 2'OMe: 2'-OMethyl RNA; PNA: peptide nucleic acid; PMO: phosphorodiamidate morpholino oligomer. The sugar modifications are circled in yellow and the internucleotide linkage modification (phosphorothioate instead of the natural phosphodiester linkage) is circled in blue.



### 3.2. Internalization of antibacterial oligonucleotides

To respond to the antibiotic crisis, antisense oligonucleotides could become a promising alternative as new antimicrobials. They act by specifically hybridizing in situ with complementary bacterial RNA and can, consequently, inhibit the expression of selected genes [10,83,84]. These can be essential bacterial genes, thus preventing bacterial growth, or genes involved in the resistance to antibiotics, hence restoring susceptibility of bacteria to antibiotics. This strategy could thus provide a potentially endless source of active antibacterials. Even if the bacterial target undergoes a point mutation that renders the oligonucleotide inactive, the oligonucleotide can be easily redesigned to become an effective drug again.

Among oligonucleotides, nucleic acid mimics (NAMs) are especially interesting, as, contrary to traditional DNA oligonucleotides, NAMs are composed of modified DNA or RNA sugars that make them resistant to endonuclease's degradation and improve their affinity towards RNA targets [85–88]. In particular, the charge neutral NAMs peptide nucleic acids (PNA) and phosphorodiamidate morpholino oligomers (PMO), as well as the negatively charged NAMs locked nucleic acids (LNA) and 2'-OMethyl RNA (2'OMe) are promising to target bacteria (Fig. 4) [89–97]. These can be further modified on the backbone by including phosphorothioate internucleotide linkages (PS), instead of the normal phosphodiester linkages (PO), further improving the stability and affinity (Fig. 4) [98,99].

The improved affinity of NAMs towards the target RNA, compared to traditional oligonucleotides, allows the design of shorter NAMs sequences [98,100] which in turn may lower their penetration's restraint into bacteria. Actually, decamers PS-LNA/2'OMe were seen to be able to target *H. pylori* cells adhered on a slide [96] and undecamers bactericidal PMO could reduce the growth of *E. coli* in pure culture and in an infected mice, although growth recovery was observed after 4 h in vitro and 12 h in vivo with multiple doses necessary to sustain the growth reduction [95]. Also, heptadecamers LNA/DNA were found to penetrate *E. coli*, but a very long incubation of 18 h was performed and still only 14% of the bacteria showed association with the LNA/DNA [91]. Clearly, internalization of NAMs in bacteria may happen, depending on the specific bacteria and on the NAM's structure, through currently unresolved mechanisms. However, internalization of oligonucleotides into bacteria is generally poor and insufficient to eradicate bacteria. Thus, although NAMs may solve the stability and affinity issues of natural oligonucleotides, penetration into bacteria remains a major bottleneck of antisense based antibacterial therapy [10,94,101,102].

Very few studies investigated so far the role of the individual bacterial envelope structures in the restricted internalization of nucleic acids in bacterial cells. Studies using the neutrally charged PNA in *E. coli* showed that the OM is the rate limiting layer in the kinetics of PNA penetration [89]. The LPS, in particular, was suggested to be a major barrier for PNA penetration into *E. coli* [83], probably due to the relatively high hydrophobicity of PNA, compared to charged oligonucleotides (Fig. 3a). After the OM, PNA needs to cross the peptidoglycan. Good et al. observed that antibiotics, which block peptidoglycan formation, did not improve PNA potency against *E. coli* and thus inferred that the peptidoglycan is not a barrier for PNA penetration [83]. However, this might not be the case for all bacteria. Studies using PNA to hybridize in different bacteria suggested that thick peptidoglycan layers from Gram-positive bacteria, such as *Bacillus cereus* (which has a peptidoglycan 6–7 times thicker than that of *E. coli* [66]), may significantly retard PNA penetration (Fig. 3a) [103,104].

For negatively charged NAMs (Fig. 4) to cross the OM, they need to overcome the presumable electrostatic repulsion to the negatively charged LPS and the size filtering effect of OMPs (Fig. 2). Most used NAMs have a molecular weight of about 2–4 kDa [10,90]. Therefore, passive diffusion of the NAMs through the OM is highly unlikely, considering the size exclusion of porins to be 0.7–0.8 nm in diameter and 600 Da in molecular weight (Fig. 3a, left panel) [105]. Even if

specific passive channels and active TBDT channels could accommodate the NAMs despite being structurally very different to their substrates, these channels would still be most of the times too narrow for NAMs translocation (Figs. 2 and 3a). Therefore, the potential of oligonucleotides in general, and NAMs in particular, as novel antibacterial drugs to solve the antibiotic crisis can only be fulfilled if oligonucleotides become “carried” across the bacterial envelope.

### 4. Behaviour of nanoparticles and molecular transporters at the bacterial cell envelope

There is a growing interest in nanoparticles (NPs) and molecular transporters to mediate the delivery of antibiotics/oligonucleotides in bacteria [20]. Also, certain NPs and peptides have bactericidal potential per se and have thus gained attention to replace antibiotics [12]. However, so far the exploration of NPs and molecular transporters (or carriers) to these ends has mostly relied on a trial and error approach. Clearly, to better understand the potential of NPs and carriers to overcome the bacterial envelope a ‘dialogue’ between nanotechnologists and microbiologists is highly needed.

#### 4.1. Siderophores as ‘trojan horses’ for antibacterial drugs

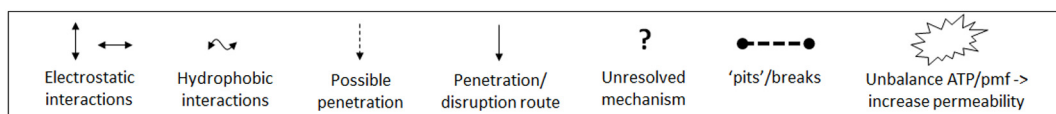
As mentioned in the Section 2, siderophores are low molecular weight compounds, secreted by bacteria under iron-limited conditions, which have a high affinity for Fe(III) ions and are actively transported as a complex into the bacterial cytosol [106,107]. Thus, conjugation of antibacterial drugs to siderophores holds the potential to allow active uptake by bacteria, so that siderophores can be used as ‘trojan horses’ for antibacterial drugs (Fig. 5). This idea arose from sideromycins, a group of antibiotics formed by analogues of bacterial siderophores linked to low molecular weight metabolic inhibitors, which are taken up by the iron transport system and release the antibacterial inhibitor upon internalization [106]. Therefore, several compounds containing a catechol/hydroxamate siderophore analogue linked via a stable linker to  $\beta$ -lactams or vancomycin or via a cleavable linker to fluoroquinolones or macrolides have been tested against Gram-negative and -positive bacteria [106,107]. Synthesized catecholate-type siderophores linked to  $\beta$ -lactams (ampicillin, amoxicillin, cephalixin and cefaclor) demonstrated high activity against the Gram-negative *P. aeruginosa*, *Stenotrophomonas maltophilia*, *E. coli*, *K. pneumoniae* and *Serratia marcescens* [108,109]. For instance, the siderophore conjugated ampicillin increased the potency of free ampicillin up to 2000-fold in *P. aeruginosa* and *Stenotrophomonas maltophilia*, 500-fold in *Serratia marcescens*, 62-fold in *K. pneumoniae* and 15-fold in *E. coli* [109]. BAL30072, a  $\beta$ -lactam with a siderophore mimicking moiety (for the structure the reader is referred to [110]), also showed interesting results against several Gram-negative bacteria, including multi-resistant and impermeable bacteria as *Burkholderia pseudomallei*, *P. aeruginosa* and *Acinetobacter baumannii* in vitro [111–114]. The in vitro potency of BAL30072 compared to free  $\beta$ -lactam antibiotic comparators was found to be up to 30–375 times, 47 times and 8 times higher respectively against strains of *B. pseudomallei*, *Acinetobacter baumannii* and *P. aeruginosa* [110–113]. These encouraging results led BAL30072 to enter clinical trials (Basilea Pharmaceutica Ltd.) [114,115].

Although to the best of our knowledge it has never been tested, siderophores may not be an efficient ‘trojan horse’ for the uptake of antibacterial oligonucleotides, considering that oligonucleotides are around 2–5 times bigger than sideromycins and also larger than the pore size of iron transporters in *E. coli* OM (as discussed in Section 3.2). Nevertheless, since our knowledge on the OM permeability is limited it may be worth to investigate.

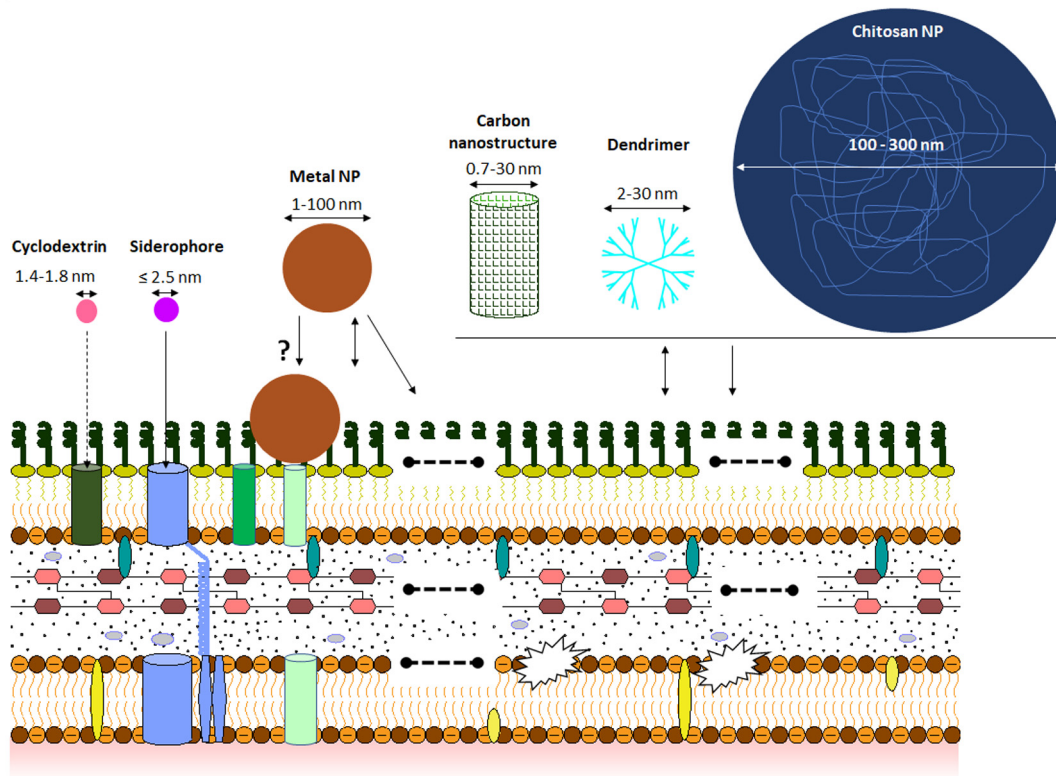
#### 4.2. Cyclodextrins as ‘trojan horses’ for antibacterial drugs

As described in Section 2, bulk cyclodextrins (CD) are able to diffuse through the CymA channel in *Klebsiella oxytoca*; orthologues of CymA

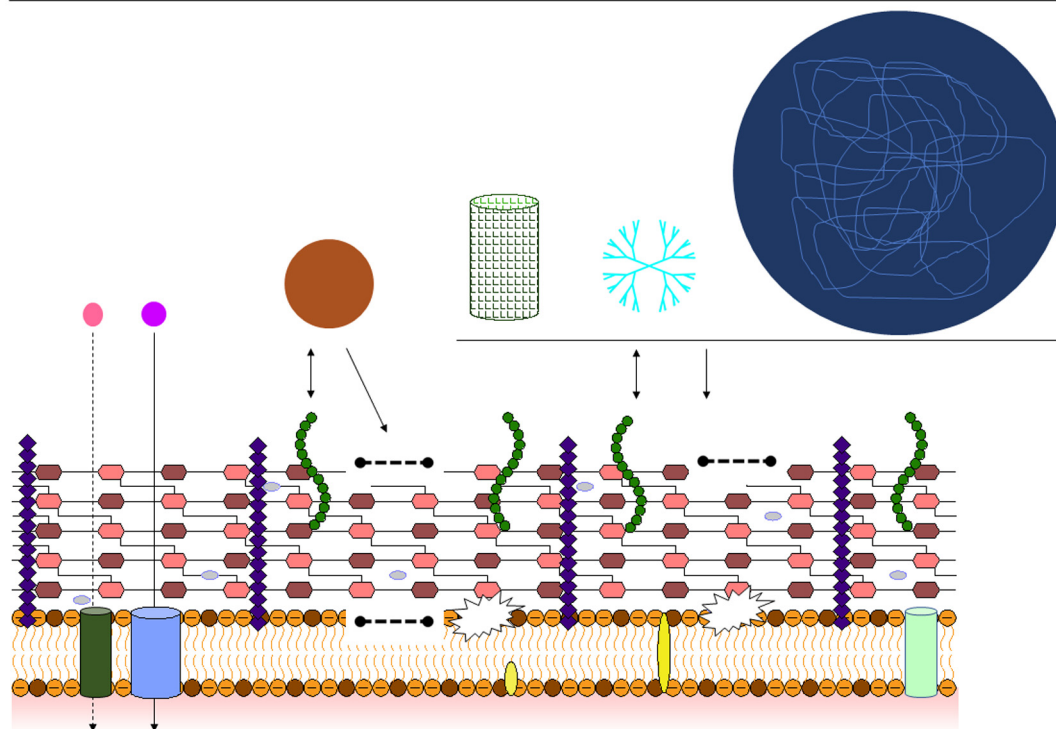




### Gram-negative bacteria



### Gram-positive bacteria



were also found in Enterobacteriaceae and Vibrionaceae [45]. Therefore, cyclodextrins may hold potential as ‘trojan horses’ for antibacterial drugs (Fig. 5). Cyclodextrins (typically 1.4 to 1.8 nm of outer diameter [116]) are investigated for nearly 70 years as drug carriers to improve the bioavailability, stability and solubility of drugs targeted to mammalian cells [116,117]. They form water soluble cyclic oligosaccharides with a hydrophobic cavity that can enclose hydrophobic antibiotics via noncovalent interactions [118].  $\beta$ -CD and its derivatives have been used for the inclusion or association of several antibiotics, such as macrolides, ryfamycins, quinolones,  $\beta$ -lactams, cephalosporins and tetracyclines, improving the antibiotic potency against Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp. and *A. baumannii*, and Gram-positive bacteria such as *S. aureus* [118–123]. For instance, when tested in *Staphylococcus* spp., *Klebsiella* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Citrobacter* spp.,  $\beta$ -CD carriers decreased the minimum inhibitory concentration (MIC) of ampicillin and amoxicillin up to 4 times and the MIC of cefadroxil up to 16 times [120]. Besides improving the antibiotic's stability and solubility,  $\beta$ -CD (and its derivatives) were considered to mediate enhanced permeation of the antibiotics [118–122]. In particular, it has been hypothesized that  $\beta$ -CD may drive internalization of the  $\beta$ -CD-antibiotic complex possibly via (i) CymA orthologue channels, (ii) enhanced adhesion to the bacterial surface (including pore channels) with potential local release of the antibiotic, and (iii) via destabilization of the bacterial envelope [119–122]. Also,  $\beta$ -CD capping silver NPs (AgNPs) improved their interaction at the bacterial envelope of *E. coli*, *P. aeruginosa* and *S. aureus* and enhanced the intracellular delivery of antibacterial silver ions [124].

The potential of CDs as drug carriers into bacterial cells can be restricted by poor colloidal stability of CDs which may result in the formation of aggregates of tens to hundreds of nanometers [118,121,122,125]. Despite being less colloiddally stable than  $\alpha$ -CD and  $\gamma$ -CD,  $\beta$ -CD is by far the most used CD carrier in drug delivery [126].  $\beta$ -CD's can be modified to become more stable, as seen for instance for (2-Hydroxypropyl)- $\beta$ -CD and for PEGylated CDs [25,126]. Nevertheless, theoretical studies on the CymA channel (Section 2.1.1) suggested that even colloiddally stable  $\beta$ -CD may be too large to be efficiently internalized in bacteria via CymA [47]. Differently, the smaller  $\alpha$ -CD can better flow through CymA [47]. Therefore,  $\alpha$ -CD would theoretically be a better ‘trojan horse’ for bacteria than  $\beta$ -CD. However, the narrow 0.47–0.53 nm cavity of  $\alpha$ -CD limits the enclosure of most antibiotics [116,118]. Further studies on the structure of CymA's orthologue channels and the transport of different CDs in various bacteria may help elucidate the chances of CDs to penetrate bacteria with associated antibiotics.

Concerning antibacterial oligonucleotides, they cannot be carried into the CD's cavity due to its hydrophobicity. CDs can instead be associated with cationic polymers to electrostatically complex the negatively charged oligonucleotides [116], but this would probably hamper CD's mediated uptake. Hence, similarly to siderophores, CDs may be promising to carry low molecular weight antibiotics, but not oligonucleotides.

#### 4.3. Metal NPs to disrupt and penetrate the bacterial envelope

Metals NPs (1–100 nm) like silver, gold, zinc oxide and titanium dioxide NP can have antibacterial properties against Gram-negative and -positive bacteria [20,25]. The antibacterial effect of metal NPs depends on their ability to interact with the bacterial envelopes. For most antibacterial metal NPs this interaction results in lethal disruption of the bacterial cell wall and/or the cytoplasmic membrane [6,12,20,25]. For some antibacterial metal NPs, this interaction allows the internalization of metal NPs, so that these are lethal by acting on the bacterial cytosol,

targeting RNA, DNA and proteins [6,12,20,25]. The antibacterial properties of metal NPs are long known, but how they interact with the bacterial envelope remains rather unclear [1,127,128]. Their action depends on the NP's size, concentration, shape, surface, charge and the capping agents used for stabilisation (which may affect the NP's surface charge), as well as on the type of bacteria [20,128,129].

##### 4.3.1. Interactions of metal nanoparticles with the bacterial cell envelope

The interaction of metal NPs with the bacterial cell wall has been mostly studied for AgNPs and it is frequently suggested to be driven by electrostatic attractions between the positively charged NPs and the negatively charged bacterial envelope surface – probably the LPS in Gram-negative bacteria and the teichoic acids in Gram-positive bacteria (Fig. 5) [25,130–132]. However, this does not seem to be a prerequisite since negatively charged AgNPs (diameter from 5 to 20 nm) were also shown to interact at the cell envelope of Gram-negative and -positive bacteria [133–135]. Negatively charged NPs were proposed to interact with metals bound to the LPS in the Gram-negative cell wall (see Fig. 1), causing metal depletion and thus perturbing the assembly of LPS and compromising the OM's permeability [134,136]. Likewise, it is conceivable that anionic metal NPs could also interact with metals bound to teichoic acids in the Gram-positive cell wall. Still, anionic AgNPs were considerably less toxic than cationic AgNPs against Gram-positive *Bacillus* species [135].

Besides electrostatic effects, binding of NPs to certain groups at the bacterial envelope surface has been suggested as well; for instance AgNP have a high affinity towards thiol groups and their antibacterial action was blocked by thiol containing agents [127,137].

Cell wall's disturbance may be further aided by the formation of pores, called ‘pits’, by metal NPs (Fig. 5), as proposed for AgNP [131,138,139]. Such ‘pits’ may be breaks in the OM, including LPS – as reported for 5 nm AgNPs against the Gram-negative *E. coli* [138] – or on the peptidoglycan bonds between *N*-acetylglucosamine and *N*-acetyl-muramic acid – as suggested for 18 nm AgNP against the Gram-positive *S. aureus* [139].

Some authors discuss that the extent of metal NP interaction at the cell wall may depend on the bacteria Gram type. Several studies report that the thicker peptidoglycan layer in Gram-positive bacteria may render them more resistant to metal NPs [134,139,140]. However, the opposite has also been reported [129,130,132,141]. Thus, the different Gram cell wall does not solely explain the interaction of metal NP on bacteria, but clearly the effect is strain dependent as well [141].

Overcoming the cell wall, metal NPs that reach the cytoplasmic membrane may disturb it directly by the formation of ‘pits’ similar to the ones formed in the cell wall (Fig. 5). Indeed, AuNPs (2–29 nm of diameter) and PtNPs (2–19 nm of diameter) caused substantial cell wall and cytoplasmic membrane disruption in *Salmonella enteritidis* and *Listeria monocytogenes* [142]. Catechin-CuNPs (5.3 nm of average diameter) were also seen to cause separation of the cytoplasmic membrane from the cell wall, in *S. aureus* [132].

In addition, metal NPs can disturb the CM by interfering with the ATP balance and pmf gradient, eventually causing cell leakage (Fig. 5) [6,12,131,132,143]. Vardanyan et al. verified that 3–15 nm anionic AgNPs increased ion flux across *E. coli* and *Enterococcus hirae* (*E. hirae*) [129]. Moreover, the CM's ATPase activity in *E. hirae* was decreased and the coupled  $H^+$  flux, as well as the associated  $H^+$ - $K^+$  exchange, were affected [129]. Upregulation of several OMPs (as OmpA, OmpC, OmpF) with accumulation of their precursors in the cytoplasm was also found as a consequence of pmf/ATP gradient dissipation in *E. coli* exposed to 9.3 nm AgNPs – without energy, OMP precursors cannot be translocated to the cytoplasmic membrane, therefore remaining in the cytoplasm [137].

**Fig. 5.** Most relevant mechanisms for the direct penetration/disruption of the bacterial envelope by nanosized materials like cyclodextrins, siderophores, metal nanoparticles, carbon nanostructures, chitosan nanoparticles and dendrimers in Gram-negative (top) and -positive (bottom) bacteria. The double arrow vectors represent electrostatic interactions with the LPS, in Gram-negative bacteria (top), and the teichoic acids (wall teichoic acids and lipoteichoic acids) in Gram-positive bacteria (bottom). The envelope structures depicted are identified in Fig. 1.

#### 4.3.2. Evidence for internalization of metal nanoparticles in bacteria

There are several reports on internalized AgNPs, CuNPs, PtNPs, ZnO NPs, TiO<sub>2</sub> NPs, MgO NPs [127,132,133,142,144–148]. It is frequently difficult to evaluate how NPs penetrated the bacterial envelope, because several reports use bactericidal concentrations [127,132,142] and so NP's penetration may be a consequence of their toxicity. Therefore, we only focus on published results obtained at a non-bactericidal concentration of metal NPs. It was found that 1–1.5 mM ZnO NP (14 nm of diameter) were able to be internalized in *E. coli* and even increase the number of *E. coli* colonies, probably because bacteria can metabolize Zn<sup>2+</sup> as an oligoelement [146]. Anionic ZnO and TiO<sub>2</sub> NPs up to 70 nm were also suggested to penetrate the Gram-negative *Salmonella Typhimurium* (as tested by flow cytometry and TEM) and be transmitted to daughter bacteria, while bigger NP agglomerates adhered to the bacterial envelope [147]. Similarly, AgNPs (normally anionic, as produced by citrate and borohydride reduction [145,149]) up to 80 nm were found to penetrate live Gram-negative *P. aeruginosa* with bigger NPs remaining mostly adhered on the bacterial surface [144,150]. In live Gram-positive *B. subtilis*, AgNPs from 8 to 33 nm [145] and even from 84 to 100 nm were reported to be internalized as well [151]. The majority of these NPs are clearly much larger than the reported pore sizes of the bacterial envelopes (as shown in Figs. 1, 2 and 5). The penetration of these NPs on live bacteria may be aided by non-specific mild envelope perturbation including transient 'pit' formation [146], while keeping bacteria viable (Fig. 5). Alternatively, passive diffusion has been considered for the influx of the ZnO and TiO<sub>2</sub> NPs up to 70 nm (in Gram-negative bacteria) [147] and of AgNPs up to 80 nm (in Gram-negative and -positive bacteria) [145,151]. However, it was reported that not all internalized AgNPs remained inside, but some were actively exported from *P. aeruginosa* and *B. subtilis* by active transporters (typically involved in antibiotics efflux); bigger AgNPs were more quickly exported while smaller AgNPs remained longer inside bacterial cells [144,145,151]. These studies on AgNPs transport were performed in live bacteria to assess real-time transport of AgNPs in and out of bacteria, using a method based on the size-dependent localized surface plasmon resonance (LSPR) spectral colors of single AgNPs by dark-field optical microscopy and spectroscopy [150]. The authors used this LSPR-based microscopy to distinguish internalized AgNPs from AgNPs extracellularly adhered to the bacterial envelope based on the scattering intensity of the AgNPs – intracellular NPs appear blurry and with lower scattered intensity, while extracellular NPs look sharper and brighter (due to the additive scattering of the bacterial membrane) [144,145,151]. Looking only at the microscopy images, it is not straightforward to visually discriminate envelope adhered and internalized AgNPs [145]. Nevertheless, the presented method was initially validated by transmission electron microscope (TEM) [144,150]. Still, it is intriguing that such NPs could diffuse through bacterial active and passive channels despite being up to 10–50 times larger than the respective channel's pores (Figs. 2 and 5). The authors argue that this should be due to an enormous ability of the channel's pores to adapt their permeability in live conditions, something that can be overlooked in the crystallography analysis of the protein channels normally used to establish the pore sizes of the bacterial channels [144]. Clearly this is an unresolved issue that needs to be further investigated; we anticipate that the development of microscopy techniques with improved resolution will help to directly visualize in real-time NPs internalization in live bacteria, in the future.

#### 4.4. Carbon nanostructures, dendrimers and chitosan nanoparticles to disrupt and penetrate the bacterial envelope

Although their interactions with the bacterial envelope have been less studied, NPs other than metal NPs like carbon nanostructures (as carbon nanotubes and graphene oxide nanoparticles), chitosan NPs and dendrimers may overcome the bacterial envelope as well [152]. However, their internalization in bacteria without disruption of the bacterial envelope has never been reported to our knowledge. Differently, they all have potential to overcome the bacterial envelope by disrupting

it [12,20] (Fig. 5). Therefore, they have been used against Gram-positive and -negative bacterial infections; while carbon nanostructures (CNS) have been mostly used as antimicrobials per se [153–156], dendrimers and chitosan NPs have been used both alone and as carriers of antibiotics [157–163].

When used in lower concentrations, dendrimers (mostly polyamidoamine (PAMAM) dendrimers), and chitosan NPs, may serve as non-bactericidal reservoirs which can improve antibiotic's half-life, bioavailability and biodistribution [157,158,160,161]. In addition, they may also perturb the bacterial envelope making it somewhat more permeable which eventually improves the delivery of the antibiotic once it has been released from the NPs [159,164].

At higher concentrations, the perturbation caused by dendrimers and chitosan NPs can be irreversible and lead to bacteria's disruption [162–164]. This bactericidal effect arises from the NPs highly positive surface charge which promotes electrostatic adsorption of the NPs to the negatively charged bacterial surface, with possible displacement of the metal cations bound to LPS/teichoic acids, and induces permeabilization of outer and cytoplasmic membrane, leakage of cytosolic contents and bacteria disruption (Fig. 5) [6,11,12,164].

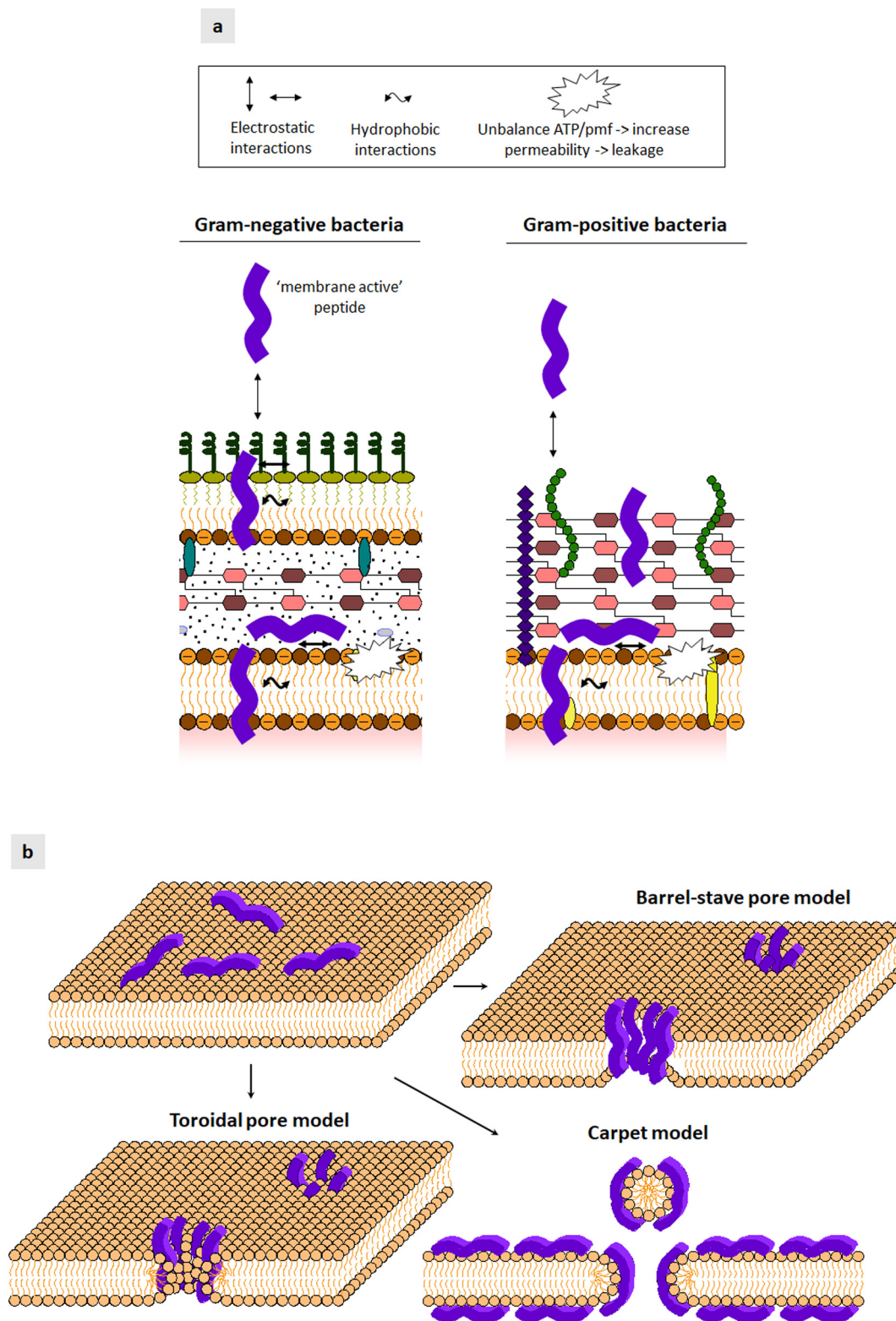
Carbon nanostructures (CNS), such as carbon nanotubes (CNTs) and graphene nanosheets, have recently gained increased attention as antimicrobials [1]. Their needle-like shape, small size and electronic properties offer interesting bactericidal potential against Gram-positive and negative bacteria [165–167]. The direct disruption of the bacterial envelope by CNS appears to mainly occur at the cytoplasmic membrane [153]. The sharp edges of graphene insert/partially penetrate into the cytoplasmic membrane, leading to the extraction of large amounts of phospholipids (as shown for graphene nanosheets on *E. coli*) and consequent degradation of the membranes with release of intracellular RNA, electrolytes and proteins [154–156] (Fig. 5). In addition, the oxidative nature of graphene may also induce lipid peroxidation [153,168]. These antimicrobial effects of CNS seem to be generally favored by their small size. A decrease in the area of graphene nanosheets from 0.65 to 0.01  $\mu\text{m}^2$  increased its bactericidal activity against *E. coli* up to 4-fold [153]; the decreased diameter of single-walled CNTs (0.9–1 nm) compared to multi-walled CNTs (15–30 nm) allowed improved penetration into *E. coli* [156] and a much higher bactericidal effect against Gram-positive and Gram-negative bacteria [156,165].

#### 4.5. 'Membrane active' peptides to disrupt and penetrate the bacterial envelope

'Membrane active' peptides refer to antimicrobial peptides (AMPs) and cell-penetrating peptides (CPPs), both acting on the cytoplasmic membrane [169]. This 'membrane activity' allows AMPs and CPPs to respectively kill bacteria and transport drugs across the bacterial envelope. There is some confusion in the literature on the denominations; while AMPs are used to kill bacteria per se, CPPs are molecular transporters used to carry attached drugs into the cytosol of mammalian or bacterial cells (without cell lysis) [170,171].

AMPs naturally exist to protect higher organisms against pathogenic microorganisms [172]. AMPs selectively act against bacteria and not mammalian cells, because the higher fluidity and anionic character of bacterial CM, compared to that of mammalian cells CM, favour the peptide's binding and insertion [26,171,173]. The higher fluidity of bacterial membranes derives from the absence of cholesterol and the presence of the fluid lipid PE. The higher anionic character, on the other hand, results from the presence of the anionic PG at the bacterial CM's surface (in mammalian cells the anionic lipids are sequestered in the inner leaflet of the membrane and the outer leaflet is mostly decorated with zwitterionic phosphatidylcholine (PC) and sphingomyelin) [26,54,171,173,174]. Most AMPs are bactericidal by disrupting the bacterial CM [26], but some AMPs exist, such as buforin II, indolicin and pleurocidin, that cross the CM without disruption and lethally inhibit





**Fig. 6.** a) Interactions of ‘membrane active’ peptides with the bacterial cell envelope of Gram-negative and -positive bacteria, mainly determined by i) electrostatic interactions between the charged residues of the peptide and the LPS (in Gram-negative bacteria), the teichoic acids (in Gram-positive bacteria) and the phospholipids (in both Gram types), and ii) hydrophobic interactions between the hydrophobic residues of the peptide and the lipid layers [54,182,195]. b) Different models for the peptide insertion into the bacterial cytoplasmic membrane, similar for Gram-negative and -positive bacteria (thus the represented membrane phospholipids are not differentiated) [26,174]. The envelope structures depicted are identified in Fig. 1.

the synthesis/activity of DNA, RNA or proteins in the bacterial cytosol [171,175].

CPPs, on the other hand, started to be earlier applied in mammalian cells and only more recently became also investigated to carry drugs into bacterial cells [170]. Hence, AMPs and CPPs have been mostly

discussed separately, although similar mechanisms of ‘membrane activity’ have been proposed in the literature for CPPs and AMPs [170,175]. Actually, despite the diversity of AMPs and CPPs (for a comprehensive description of different AMPs and CPPs the reader is referred to [175,176], respectively), most of them share general features: (i) small

size, with less than 50 (AMPs) and 30 (CPPs) amino acids, (ii) net positive charge, and (iii) a significant amount of hydrophobic amino acids [174,177,178]. Therefore, we will discuss the interactions of AMPs and CPPs with the bacterial envelope together, as ‘membrane active’ peptides.

#### 4.5.1. Interactions of ‘membrane active’ peptides with the bacterial envelope

As referred above, ‘membrane active’ peptides are active at the cytoplasmic membrane. Therefore, to reach the cytoplasmic membrane they first need to interact with the cell wall, through a rarely studied mechanism. Nevertheless, it is believed that the initial interaction is mediated by non-specific electrostatic interactions of the cationic peptides with the anionic LPS and teichoic acids, respectively in Gram-negative and -positive bacteria (Fig. 6a) [171,175]. Although a minority, anionic AMPs and CPPs also exist [175,179]; we can thus envision that similarly to metal NPs, anionic AMPs and CPPs may be electrostatically attracted to the divalent cations bound to LPS/teichoic acids. Electrostatic interactions together with hydrophobic interactions may help the peptides to further translocate the OM in Gram-negative bacteria (Fig. 6a) via a “self-promoted uptake” [89,180–182]. Alternatively, it has been suggested that specific interactions at the cell wall surface, for instance with LPS, PE, or lipid II (peptidoglycan precursor), can promote the initial interaction of peptides with the bacterial envelope [26,171,173].

Reaching the peptidoglycan, it has been assumed in the literature that the peptides (with a molecular weight typically below 5 kDa) may freely diffuse through the peptidoglycan mesh (considering the size exclusion for globular hydrophilic molecules of 50 kDa) [54,181]. However, the role of peptidoglycan in the interaction with the peptides is not clear [54]. Still, the peptides should pass the cell wall very fast, as dissipation of the electrochemical gradient across the CMs is seen within a few seconds of bacteria exposure to AMPs [173,183].

‘Membrane active’ peptides normally cross the cell wall without disrupting the OM in Gram-negative bacteria [182]. An exception was recently found for the newly developed 7.7 nm large “star-shaped nanoengineered AMP polymer” (SNAPP), composed of cationic lysine and hydrophobic valine monomers polymerised from a polyamidoamine (PAMAM) dendritic core [182]. Due to its particular architecture, SNAPPs cannot transverse the OM via a “self-promoted uptake” like general AMPs do [182]. Differently, after electrostatically binding to LPS, the SNAPPs (at high concentration) cause lethal OM’s destabilization and fragmentation [182]. SNAPPs can also further mildly perturb the CM and unbalance its ion movement [182]. This explains that the SNAPPs were not only active against Gram-negative bacteria, but also showed a moderate activity against Gram-positive bacteria [182].

Reaching the cytoplasmic membrane, where typical AMPs and CPPs are active, the peptides first bind to the membrane by electrostatic interactions (Fig. 6a) [26,78,175,184]. When the peptides reach a threshold concentration on the membrane, which for AMPs is about full membrane coverage, the peptides insert themselves into the membrane bilayer via hydrophobic interactions (Fig. 6a) [26,78,175,184].

The modes of membrane insertion/translocation depend on the peptide and the membrane [26]. For mammalian cells, CPPs have been proposed to translocate the mammalian membrane either by endocytosis or by direct penetration [169,170]. Endocytosis is not applicable in bacteria. Differently, the direct penetration of CPPs in mammalian cells is proposed to occur via three possible models which are the same exact models proposed for the insertion of AMPs into bacterial CMs [170,175]. Thus, carrier CPPs and antimicrobial AMPs should use the same mechanisms to force their translocation/insertion into the bacterial CM [185]. The three typically proposed models are: the barrel-stave pore, the toroidal pore and the carpet mechanism (Fig. 6b) [170,171,173,174]. In the barrel-stave pore the peptides insert perpendicularly to the membrane surface and pack together parallel to the hydrocarbon chains, forming an aqueous pore (Fig. 6b) [171,173,174]. The cytoplasmic diameter of the barrel-stave pore formed by the AMP alamethicin has been estimated

by structural studies to be around 1.8 nm [186]. In the toroidal pore model, the peptides also insert perpendicularly in the bilayer but they induce a toroid-like curvature in the membrane, so that lipid inner and outer leaflets are forced to bend towards one another establishing continuity [171,173–175]. The pore is thus formed by both the inserted peptides and the phospholipid head groups (Fig. 6b) [171,173–175]. The AMPs melittin and magainin form toroidal pores in lipid vesicles of 2.5–3 nm and 3.0–5 nm, respectively [187,188]. The (barrel-stave/toroidal) pores may allow the passage of molecules as big as 40 kDa, according to studies performed using the AMP maculatin that forms pores of 1.4 and 4.5 nm in diameter on lipid vesicles mimicking *S. aureus* membrane [189]. The formation of barrel-stave/toroidal pores requires that the peptide is long enough to span the hydrophobic core of the bilayer [189]. Differently, this is not needed in the carpet model, since the peptides absorb parallel to the bilayer surface and produce a detergent-like effect that eventually results in membrane disintegration into micelles (Fig. 6b) [171,173–175,177]. Therefore, smaller peptides, can act via the carpet model [190]; this is the case for the AMPs aurein and cecropin, the later shown to form pores on *E. coli* of 4.2 nm in diameter [191].

Instead of (or in addition to) self-assembling to form pores, adsorption of the peptides onto the membrane may dissipate the transmembrane potential, pH gradient and osmotic balance (Fig. 6a) [177,181,189]. Different modes of action can be related, depending on the peptide concentration. For instance, at low concentrations the AMP cecropin was bactericidal to *E. coli* by dissipation of transmembrane electrochemical ion gradients (as judged from ion gradients dissipation in lipid vesicles), while higher concentrations were needed to release cytoplasmic contents [192]. The extent and duration of the membrane action of the peptides will dictate the viability of the cytoplasmic membrane. It may depend, besides peptide’s concentration, on the peptide’s charge, hydrophobicity, sequence, structure and size [171,175,185,193].

Peptides able to remain inserted into the bacterial CM long enough to form irreversible pores or lesions will kill bacteria. This is the case for most AMPs. However, after insertion into the cytoplasmic membrane, CPPs (with its attached cargo) and the minority of AMPs that have their target in the bacterial cytosol (nucleic acids and proteins) still need to desorb from the CM to reach the cytosol. How exactly it happens is still a matter of debate. For the AMPs (with a cytosolic target) the peptides forming the pore might be randomly internalized by disintegration of the pore [185,194]. The same mechanism may be reasoned to occur for CPPs. In addition, we can reason that peptides free in solution (which are expected to be in a relative higher number for CPPs than AMPs, owing to the specifically high affinity of AMPs to bacterial CMs) might flow into the bacterial cytosol via the formed pore. This may be possible if the formed pore is as large as 2–5 nm with a size exclusion of 40 kDa, as reported for AMPs [186–188,195].

#### 4.5.2. CPPs as molecular transporters of antibacterial drugs

CPPs have been explored to carry covalently conjugated antibiotics and, most commonly, antisense oligonucleotides across the bacterial envelope into the cytosol.

The highly cationic 12 amino acids (a.a.) Pen peptide (RQKIKWFQNRW, where R is arginine, Q is glutamine, I is isoleucine, K is lysine, W is tryptophan, F is phenylalanine and N is asparagine) was designed based on the 16 a.a. penetratin (a well-known CPP for mammalian cells) and conjugated to the antibiotic tobramycin (lethal by ribosome inhibition), in order to increase the antibiotic’s uptake in persister *E. coli* and *S. aureus* [78]. Persister bacteria have decreased active transport and thus do not take up tobramycin. The Pen peptide alone caused extensive permeabilization which was per se bactericidal [78]. The conjugation of the antibiotic to the peptide, forming the conjugate Pentobra, decreased the permeabilization potential of the peptide [78]. Nevertheless, Pentobra killed more 4–6 logs of persister bacteria than the free tobramycin, showing the importance of the combined effect of tobramycin and the Pen peptide [78].

An earlier report prepared the cationic peptide (KFF)<sub>3</sub>K to permeabilize the Gram-negative OM to hydrophobic antibiotics (which penetrate intact OM very poorly, Fig. 3) [196]. Pre-treatment of bacteria with this peptide was found to sensitize enteric bacteria, such as *E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. typhimurium*, to the hydrophobic antibiotic rifampin [196]. The peptide (KFF)<sub>3</sub>K was also found to permeabilize the OM of *E. coli* to antisense PNA [83,89,92]. Since then, the peptide (KFF)<sub>3</sub>K has been widely covalently conjugated to antisense oligonucleotides to transport them into the bacterial cytosol. However, this has been nearly restricted to neutrally charged oligonucleotides (PNA and PMO) [89,90,197,198], since the covalent conjugation to negatively charged is technically difficult [100]. Besides *E. coli*, (KFF)<sub>3</sub>K was also shown to improve the potency of PNAs and PMOs into the Gram-negative *Salmonella typhimurium*, *Klebsiella pneumoniae* and Gram-positive *Staphylococcus aureus*, *Bacillus subtilis* and *Corynebacterium efficiens* [9,199,200]. This improved potency was ascribed to (KFF)<sub>3</sub>K mediated improved penetration [89]. Depending on the concentration, the (KFF)<sub>3</sub>K could also contribute to the killing efficacy of the antisense PNA by causing bactericidal cell leakage [89]. (KFF)<sub>3</sub>K could act not only against the cytoplasmic membrane, as expected, but it also disrupted the OM of *E. coli* [89,182].

Other CPPs have been tested as well, which – compared to the peptide (KFF)<sub>3</sub>K – had a lower toxicity towards bacteria and when conjugated to an antisense PNA still allowed improved bactericidal effects. This was the case for the CPPs Tat (which is derived from the transactivator of transcription (TAT) of HIV and has the sequence GRKKRRQRRRPQ) [184,201,202], (RXR)<sub>4</sub>XB and (RFR)<sub>4</sub>XB (G is glycine, P is proline and X is 6-aminohexanoic acid) [102,198]. The Tat conjugates were tested against the Gram-positive *Streptococcus pyogenes* [184]. The (RXR)<sub>4</sub>XB and (RFR)<sub>4</sub>XB conjugates were tested against the Gram-positive *Listeria monocytogenes* in vitro and in a *Caenorhabditis elegans* infection model [102]. Conjugates of (RXR)<sub>4</sub>XB were also tested in vitro against the Gram-negative *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Shigella flexneri* and also in vivo in a mice model infected with *E. coli* or *S. flexneri* [198].

Besides the peptide sequence, the extent of CPP permeabilization has been observed to depend on the bacteria. Vaara et al. showed that (KFF)<sub>3</sub>K was bactericidal per se against the Gram-positive *Micrococcus* tested, while it did not affect the viability of the tested Gram-negative (*E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. typhimurium*) [196]. Hatamoto et al. also found that the Gram-positive bacteria *Bacillus subtilis* and *Corynebacterium efficiens* exhibited increased susceptibility to (KFF)<sub>3</sub>K conjugated to antibacterial PNA than the Gram-negative *E. coli*; however, the Gram-negative bacterium *Ralstonia eutropha* was not affected by the conjugate [200]. Hence, it is not evident that a Gram-dependent susceptibility exists.

The cargo transported by the peptide can also affect the conjugate's translocation in different ways. For instance, in the aforementioned example of Pentobra, the conjugation of the cationic tobramycin to the Pen peptide decreased the permeabilization of *E. coli* and *S. aureus* compared to the Pen peptide alone [78]. In contrast, the conjugation of PNA to the (KFF)<sub>3</sub>K peptide made it more membrane-active towards *E. coli* than the (KFF)<sub>3</sub>K peptide alone [89]. The authors hypothesize that PNA, being neutrally charged, may increase the amphipathic character of the conjugate and thus benefit the conjugate's interaction with the bacterial membranes [89].

#### 4.6. Fusogenic liposomes to deliver drugs in bacteria

Liposomes started to be extensively investigated already in the 1970s for drug delivery into mammalian cells [203,204], but only recently liposomes attracted interest to carry antibacterial drugs. Liposomes, together with poly(lactic-co-glycolic acid) (PLGA) NPs, are the most popular NPs used in antimicrobial studies. However, they are mainly used to improve the pharmacokinetics and the tolerability of antibiotics; in particular they are used to protect antibiotics from degradation in the body and/or to

increase the local antibiotic concentration by sustained release [205–210]. Far less explored is the ability of some liposomes to overcome the bacterial envelope barrier by fusing with bacterial membranes, which may help to intracellularly deliver their antibacterial cargo [208]. Besides classic antibiotics, liposomes may offer an interesting solution for the delivery into bacteria of negatively charged oligonucleotides, as these can be easily complexed to cationic liposomes by electrostatic interactions. Fusion between liposomes and bacteria can only occur at the level of the Gram-negative OM lipids and the Gram-positive cytoplasmic membrane, which will be discussed in the sections below.

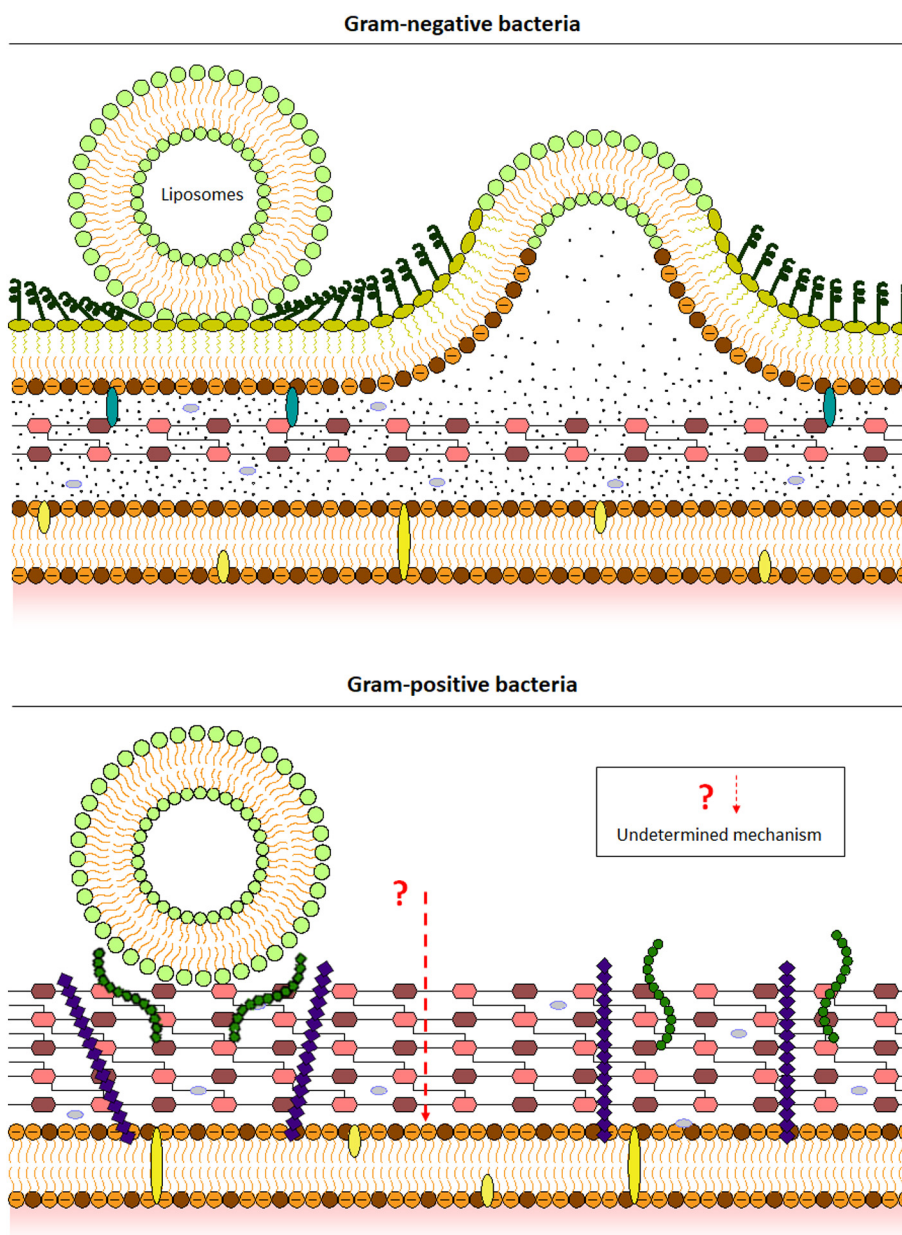
##### 4.6.1. Interactions of liposomes with the envelope of Gram-negative bacteria

For liposomes to be able to reach the OM lipids for fusion, they first need to overcome the LPS. Cationic liposomes will bind electrostatically to the negatively charged bacteria surface [211]; in particular, binding to the Gram-negative LPS may lead to LPS chains (O-antigen and core) flattening so that liposomes make contact with the negatively charged lipids of the OM [212] (top panel of Fig. 7). For negatively charged liposomes, such as dipalmitoylphosphatidylcholine (DPPC)/dimiristoylphosphatidylglycerol (DMPG) liposomes (Fig. 8), Ca<sup>2+</sup> is typically added to the liposome suspension which can limit the electrostatic repulsion between the anionic liposomes and the LPS; in addition, Ca<sup>2+</sup> may bridge the liposomes-OM interaction and contribute to dehydrate the OM's PE thereby improving fusion [212]. Apart from Ca<sup>2+</sup>, other divalent cations can also mediate fusion; nevertheless, cations with larger ionic radius seemed to be less efficient, as they probably increase the liposome-OM distance [212]. Electrostatic attraction of anionic liposomes to the metal cations bound to the LPS may also contribute to the initial interaction with the cell wall [134,136].

When in contact with the bacterial OM lipids, liposomes may then fuse with them. Fusion occurs when two bilayers, in this case the liposome bilayer and the OM lipid bilayer, merge into a single bilayer [213]. Fusion with the OM lipids is fast and occurs spontaneously by hydrophobic and van der Waals interactions [11]. Fusion critically depends on the lipid composition of the two bilayers involved [213]. The PE moiety is the most commonly referred fusogenic lipid [214,215]. PE has a low hydration of its polar head group, which may decrease the fluid spacing between bilayers and thereby facilitates energetically favorable interactions between lipid bilayers [214–216]. In addition, PE has a cone-shaped molecular shape (small head cross section and large chain cross section) and ability to promote bilayer-to-hexagonal phase transition which may trigger membrane destabilization [214,215]. Therefore, PE, in the form of the dioleoylphosphatidylethanolamine (DOPE) lipid (Fig. 8), has been incorporated into liposomes to produce fusogenic liposomes. DOPE containing liposomes have successfully improved antibiotics penetration into the Gram-negative *P. aeruginosa*, *E. coli*, *Klebsiella* spp. and *A. baumannii* [208,216,217] and have intracellularly delivered NAMs into the Gram-negative *H. pylori* [218]. In particular, the improved penetration of antibiotics in *P. aeruginosa* allowed a decrease of the minimum inhibitory concentration (MIC) of tobramycin of at least 640-fold [217], and of meropenem (a carbapenem) up to 4-fold [208]. Also, the delivery of vancomycin (too large to cross the bacterial envelope of Gram-negative bacteria (Fig. 3b)) reduced the MIC of vancomycin by up to 85-fold in *E. coli* and *A. baumannii*, up to 20-fold in *Klebsiella* spp. and up to 10-fold in *P. aeruginosa* [216].

Liposomes without DOPE have been also reported to be able to intimately interact and even fuse with bacteria, improving the permeation of antibiotics. A popular formulation is DPPC/DMPG negatively charged liposomes, frequently called “fluidosomes” [212,217,219–221]. This designation comes from the ability of DMPG to increase the fluidity of liposomes; indeed, DMPG is a phospholipid with short acyl chains and a high number of unsaturated bonds which results in a relatively low gel-liquid crystalline transition temperature (T<sub>c</sub>) [222,223]. Studies report efficient interaction of DPPC/DMPG liposomes with bacteria, improving the permeation of antibiotics [219–221] and even of one





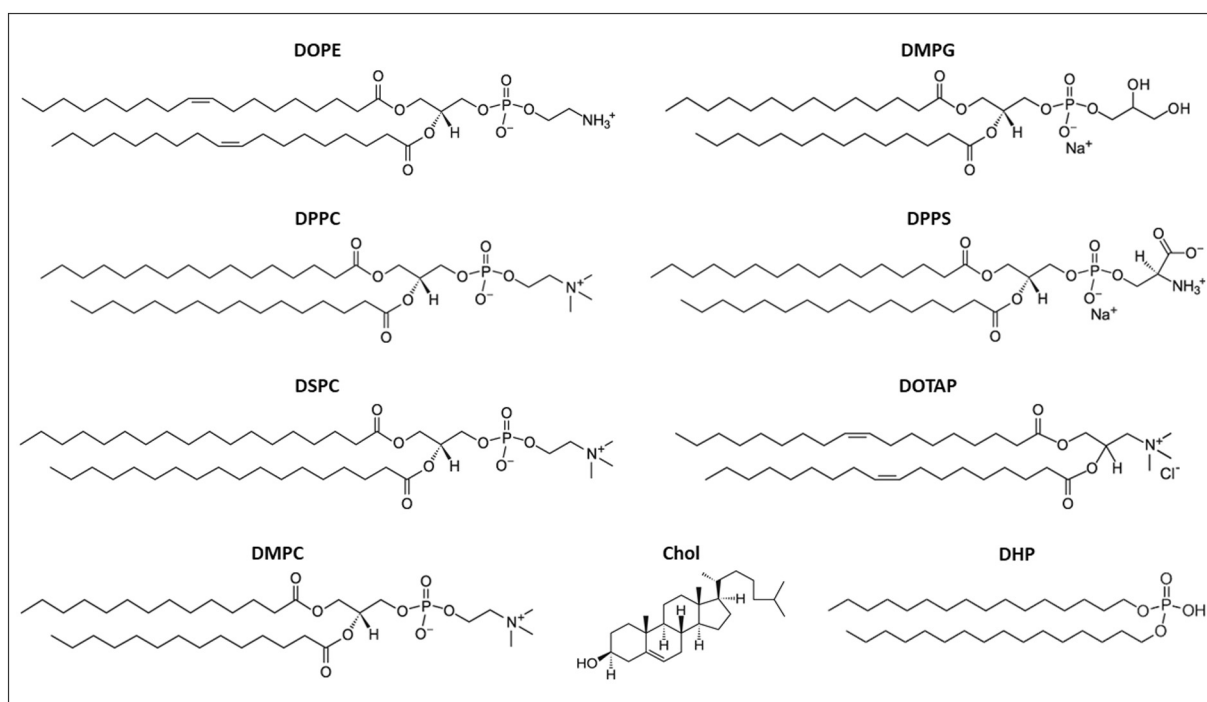
**Fig. 7.** Illustration of the intracellular delivery mechanism by liposomes able to fuse with bacterial membranes. In Gram-negative bacteria (top), liposomes fuse with the outer membrane (adapted from [212]). At the Gram-positive envelope (bottom), liposomes would have to cross the thick peptidoglycan layer to fuse with the cytoplasmic membrane, via an undetermined mechanism. The envelope structures depicted are identified in Fig. 1.

antisense PS DNA oligonucleotide [224]. However, it has also been reported that encapsulation of antibiotics in DPPC/DMPG liposomes even lowered the antimicrobial efficiency when compared to free antibiotic [208]. It was latter clarified that DPPC/DMPG are not fusogenic per se and that their improved antibiotic permeation depends on the presence of divalent cations as  $\text{Ca}^{2+}$  to reduce the electrostatic repulsion between the negatively charged liposomes and the bacteria surface [212,217].

In addition, intimate interactions were observed between the more rigid liposomal formulations, such as distearoylglycerophosphocholine (DSPC)/cholesterol (Chol), phosphatidylcholine (PC)/Chol/dioleoyltrimethylammoniumpropane (DOTAP) or DPPC/Chol (Fig. 8), and Gram-negative bacteria like *P. aeruginosa* and *Burkholderia cenocepacia* [211,225,226]. Despite the inclusion of lipids with increased Tc and Chol (which should increase liposomes rigidity/stability), fusion/adhesion of these liposomes with the bacterial OM was reported [211,225,226]. However, others have seen that the inclusion of only

10% Chol dramatically lowered DPPC/DMPG fusion in *P. aeruginosa* [212,217].

The studies using DPPC/DMPG and more rigid liposomes (containing Chol and DSPC) show that fusion with bacterial membranes may occur to some extent using non-DOPE liposomes. Although there is no PE in the liposomes, there is a high amount of PE in the Gram-negative OM that may mediate fusion [54,227]. Indeed DPPC/DMPG liposomes cannot fuse with the more rigid cytoplasmic membrane of human cells (which contain cholesterol and have PC as the major lipid on their membrane surface and a minor amount of PE sheltered in the inner CM's leaflet) [54,227]. However, DPPC/DMPG liposomes could fuse with Gram-negative bacteria and the higher the PE content of the bacterial OM, the better fusion occurred [212,227]. When PE (in the form of DOPE) is also included in liposomes, composed for instance of DPPC/DMPG liposomes [217], DPPC/Chol hemisuccinate liposomes [216] or PC/Octadecylamine [208], the tendency for fusion between bacteria



**Fig. 8.** Representative structure of common lipids used in liposomal formulations applied to bacteria. DOPE: dioleoylphosphatidylethanolamine; DMPG: dimyristoylphosphatidylglycerol; DPPC: dipalmitoylphosphatidylcholine; DSPC: distearoylphosphatidylcholine; DOTAP: dioleoyltrimethylammoniumpropane; DMPC: dimyristoylphosphocholine; DHP: dihexadecylhydrogenphosphate; DPPS: dipalmitoylphosphatidylserine; Chol: cholesterol.

and liposomes is further enhanced, as seen by the significant improvement of the antibiotics efficacy [211,216,217].

Although the composition of the liposomes affects their fusion ability, a clear effect of the size of liposomes has not been observed [217,224]. From the bacteria side, beside the PE content, the effect of other bacterial features on fusion is not well understood and may depend on fine details. For instance, various *P. aeruginosa* strains showed different degrees of fusion with PC/Chol/DOTAP liposomes, while the expression of one 18-kDa OMP was found to be positively correlated with fusion [211].

Upon fusion with the Gram-negative OM lipids the incorporated antibiotics/antibacterial oligonucleotides will be delivered into the periplasm (top panel of Fig. 7). From here, the molecules will have to cross the viscous periplasmic space and will make contact with the peptidoglycan, where antibiotics that disrupt the peptidoglycan synthesis have their site of action. Other antibiotics and oligonucleotides acting rather on the cytosol will have to cross the peptidoglycan and the cytoplasmic membrane as well, as discussed in Sections 3.1 and 3.2, respectively [36].

#### 4.6.2. Interactions of liposomes with the envelope of Gram-positive bacteria

Liposomes for the delivery of antibiotics and oligonucleotides into Gram-positive bacteria will first contact with the teichoic acids. It can be reasoned that liposomes will electrostatically interact with teichoic acids the same way that liposomes interact with LPS (described in the previous section), so that liposomes binding to and flattening of the teichoic acids layer may occur (bottom panel of Fig. 7). However, for fusion to occur in Gram-positive bacteria, liposomes do not only have to overcome the teichoic acids but also the thick peptidoglycan, in order to contact and fuse with the cytoplasmic membrane lipids.

It is currently unknown whether or not liposomes could overcome the peptidoglycan of Gram-positive bacteria (bottom panel of Fig. 7). The studies on fusion of liposomes with Gram-positive bacteria are few; they were performed with the bacteria *S. aureus*, *Enterococcus faecalis* and *Streptococcus agalactiae* and liposomes composed of

DPPC/DMPG (in the presence of  $\text{Ca}^{2+}$ ), egg PC/DMPG/DSPE-PEG, DPPC/DOTAP/DSPE-PEG (with and without wheat germ agglutinin as a targeting moiety), dimyristoylphosphocholine (DMPC)/Chol/DHP (dihexadecylhydrogenphosphate) and DMPC/Chol/DPPS (dipalmitoylphosphatidylserine), (Fig. 8) [212,228–230]. These studies suggest fusion to explain the improved efficiency of the encapsulated compared to the free antimicrobial drug [212,228–230]. Also, one study in *S. aureus*, resistant to  $\beta$ -lactam antibiotics, showed that the bacteria susceptibility could be improved by the use of anionic egg PC/DMPG/DSPE-PEG liposomes, carrying a PS DNA to downregulate a gene of resistance to  $\beta$ -lactams [228]. The PS DNA was first complexed with the cationic polymer polyethyleneimine (PEI), resulting in an 80 nm complex that was subsequently encapsulated into the liposomes [228]. The observed downregulation was interpreted as a result of liposomal fusion and intracellular delivery of the PS DNA [228], but the authors did not report the effect of the complex alone nor the interaction of liposomes with *S. aureus*. Actually only one of the studies on Gram-positive bacteria explicitly demonstrates interaction between liposomes and bacteria, using flow cytometry and fluorescence microscopy [231], and none of them show experimental evidence of direct intracellular delivery by liposomes. Therefore, it may be possible that liposomes only enable sustained local drug release close to the Gram-positive bacterial surface [229], resulting in an increased drug concentration gradient across the bacterial envelope. For drugs with limited (but not impossible) permeation of the bacterial envelope this may be sufficient to improve the drug diffusion across the peptidoglycan and cytoplasmic membrane [229]. Note that local drug release may also contribute to the delivery into Gram-negative bacteria, especially when non-fusogenic liposomes (liposomes without DOPE) are employed.

Even when interaction of liposomes and the OM of Gram-negative bacteria occurs, it remains very challenging to experimentally distinguish between adhesion of the liposomes on the OM with (only) local drug release and fusion of the liposomes with the OM followed by intracellular delivery. This is due to technological limitations. Electron microscopy typically shows generic interaction/adhesion at the bacterial

envelope. Immunohistochemistry combined with TEM can be useful to find intracellularly delivered drugs, but it depends on the availability of specific antibodies. Epi-fluorescence microscopy and flow cytometry based on fluorescent constructs (liposomes carrying fluorescent molecules) can hardly distinguish between surface adhered fluorescent constructs and internalized fluorescent molecules, since the optical resolution limit is rather close to the bacteria size (optical resolution being typically not higher than  $\sim 0.25\ \mu\text{m}$  while width of most bacteria  $\sim 0.5\text{--}1\ \mu\text{m}$ ). Fillion et al. proposed to distinguish in *E. coli* surface adhesion of DPPC/DPMG liposomes (carrying a fluorescently labelled PS DNA) from intracellular delivery of the fluorescently labelled PS DNA by incubating *E. coli* with the constructs respectively at  $4\ ^\circ\text{C}$  vs  $37\ ^\circ\text{C}$  and measuring the fluorescence by fluorescence-activated cell sorting (FACS) [224]. However, it is our belief that incubation at  $4\ ^\circ\text{C}$  is not a sufficiently good control, because adhesion at  $4\ ^\circ\text{C}$  may be lower than the adhesion that can occur at  $37\ ^\circ\text{C}$ . Differently, our group showed recently the use of fluorescence microscopy to visualize bacteria with fluorescence coming exclusively from intracellularly delivered fluorescent molecules; fluorescence coming from constructs remaining adhered on the OM could be removed by a mild triton wash [218]. Thus, the fraction of molecules actually delivered intracellularly by the liposomes could be quantified based on the fluorescence intensity [218].

## 5. Conclusions and future perspectives

Nanoparticles and molecular transporters may provide valuable tools to overcome the bacterial cell envelope which limits the internalization of bactericidal drugs. As discussed along this review, such NPs/molecules can be (i) bactericidal per se, if they directly penetrate the bacterial envelope causing envelope disruption (metal NPs, CNS, chitosan NPs, dendrimers and AMPs), (ii) carriers of bactericidal drugs that penetrate the bacterial envelope with the attached drugs without necessarily causing disruption (CPPs, siderophores and eventually cyclodextrins), or (iii) delivery vectors that do not penetrate bacteria but intracellularly deliver the drugs into bacteria (fusogenic liposomes). According to the current knowledge CPPs and liposomes are the best positioned candidates. These are especially interesting to intracellularly transport novel NAM drugs which suffer from very poor internalization, in particular CPPs to transport neutral NAMs and liposomes to transport anionic NAMs. NAMs are promising to be used as antibacterial drugs, by targeting essential bacterial genes, and as drug adjuvants, to restore bacteria susceptibility to antibiotics. NAMs can provide a virtual endless source of drugs, since even if the bacterial target undergoes a mutation the NAM can be easily redesigned to become effective again. Therefore, combining NAMs with a CPP/liposomal carrier holds promise to address the antibiotic crisis and redirect the fight against bacterial infections. Nevertheless, the design of efficient constructs would benefit from a better understanding of the dynamics at the bacterial envelope interface. X-ray crystallography has allowed very useful characterization of some protein channels and more still need to be studied. Still, it would be interesting that these studies could be complemented with investigating the real-time transport in living bacterial cells, so that the eventual adaptability of bacterial envelope's permeability could be evaluated and the chances of the NPs to overcome the bacterial envelopes could be fully understood. The possibility to test transport in live bacteria is challenged by the small size of bacterial cells which limits the direct appreciation of NPs internalization by common techniques as flow cytometry and fluorescence microscopy. Super-resolution microscopy is starting to be used to elucidate the dynamics of some bacterial physiological processes [232–234]. We envision that its continuous advance and availability will position super-resolution microscopy as a critical tool, in the future, to evaluate NPs interaction with bacterial cells and boost the application of nanomedicine towards bacterial cells.

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