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Chapter

TUMOR-SELECTIVE AND SELF-ACTIVATING CHEMILUMINESCENT PHOTOSENSITIZERS FOR PHOTODYNAMIC THERAPY

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ABSTRACT

Photodynamic therapy (PDT) is a minimally-invasive anti-tumor therapeutic modality, known for its selectivity and limited side-effects. PDT has three fundamental requirements: a nontoxic photosensitizer (PS), light of a specific wavelength, and molecular oxygen. The anti-tumor therapeutic effect is triggered by the photo-activation of the PS via irradiation of the target tumor site with light of a specific wavelength. This leads to a photochemical reaction in which the PS generates cytotoxic reactive oxygen species (ROS), which lead to tumor-selective cell destruction. Despite significant advantages, the necessity of an external light source impairs the use of PDT for most tumors, due to the poor penetration of light into deeply localized tissues. Given this, researchers have focused on developing strategies to improve the efficiency of this therapy and expand it for deeper tumors and metastatic cancer. One strategy is to use either chemi- (CL) or bioluminescent (BL) reactions as intracellular excitation sources of PSs for PDT. Namely, CL/BL are processes in which light is generated due to a chemical/biochemical reaction, without any light source, and therefore, can be used to excite the PS intracellularly. More importantly, an extra step can be taken and instead of using CL reactions as excitation sources, they can be used as the basis for tumor-selective and self-activating single-molecule drugs that are both the excitation source and the PS itself. In this chapter, we will provide a critical

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review about the evolving use of CL/BL reactions in PDT toward solving its light-related problems, focusing on the most recent and exciting development: tumor-selective and self-activating single-molecule PSs.

Keywords: photodynamic therapy; chemiluminescence; bioluminescence, photosensitizers; cancer.

INTRODUCTION TO PHOTODYNAMIC THERAPY

Cancer is one of the biggest scourges of our time, responsible for nearly 10 million deaths in 2020. [1] Moreover, the incidence of cancer in humans has increased significantly as a result of the increasing life expectancy worldwide, with 19.3 million cancer cases being diagnosed in 2020. [1] Considering the prominence of cancer in human society, it is not surprising that several therapeutics have been developed to treat the different cancer types known to science. Therapeutic modalities such as surgical removal, [2, 3] radiotherapy, [4, 5] chemotherapy, [6-8] immunotherapy, [9, 10] and hormonal therapy [11, 12] have been extensively studied by the scientific community, albeit not always with the intended efficiency. One of the most promising strategies to increase efficiency is combining different anticancer drugs or therapy modalities to treat a single disease.[8, 13] This can increase the efficacy, counter drug resistance, and diminish the required dosage for the treatment to be effective.[14]

Although the aforementioned strategies have been commonly employed to treat cancer patients and have led to significant increases in the survivability of cancer patients, they also have some drawbacks: they can be invasive, they can lack selectivity towards cancer cells and be toxic to healthy tissues, they present several side effects for the patient, and the tumor might develop resistance towards the therapy. As such, there is an increasing demand for the development of more efficient anticancer therapies.

Photodynamic therapy (PDT) is a new and minimally invasive therapeutic modality to treat various diseases (*e. g.* bacterial and fungal infections, skin diseases) as well as some types of cancer (*e. g.* esophageal cancer, non-small cell lung cancer, skin cancer), which is already in clinical use.[15-18] Over the past decade, the advances in the fields of nanotechnology and materials science and the improvements in the different components of PDT systems have promoted the rapid development of this treatment.[19-23] When compared to more traditional approaches, PDT offers significant advantages: it is minimally invasive, results in fewer side effects, has a high spatiotemporal precision (it can only be activated after irradiation with light of a specific wavelength), and allows for fast healing of the healthy tissues.[15-17, 24-26]

The working mechanism of PDT requires three fundamental parts: a non-toxic photosensitizer (PS), light of a specific wavelength, and the presence of molecular oxygen.[15-17] PDT is based on the production of reactive oxygen species (ROS), mainly singlet oxygen. This is achieved by photo-activation of a PS via irradiation of the targeted tissue with light.[27] Upon photoexcitation, the PS is transiently excited from its singlet ground state to a singlet excited state. Subsequently, the population of the singlet excited state can cross to comparatively long-lived triplet

states, resulting in the production of ROS through one of two different pathways (Figure 1).[26, 28] In type I, the PS, while in the triplet excited state, can transfer an electron to molecular oxygen (or biological substrates), prompting the formation of radical species such as the superoxide anion, which can then originate the highly reactive hydroxyl radical, starting a cascade of cytotoxic free radicals. In an alternative type I pathway, we have the transference of a hydrogen atom to the excited state PS. This originates free radicals that may react with molecular oxygen and create a mixture of ROS, causing oxidative damage. In the second pathway, type II, which is thought to be the most important for PDT, the triplet excited state of the PS can transfer its energy to molecular oxygen, resulting in highly reactive singlet oxygen.

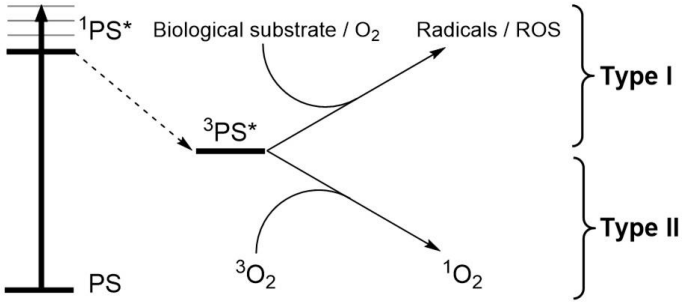


Figure 1. Schematic representation of PDT and its respective mechanisms of action.

Despite its advantages when compared to more conventional therapeutics, PDT has some limitations and drawbacks. PSs typically absorb strongly at around 400 nm and weakly between 600 and 800 nm. Therefore, PSs are generally excitable using UV or visible light, which leads to problems related to light penetration into tissues.[15-17, 26] Light of wavelengths below 580 nm is usually unsuitable for biological applications since it is absorbed and scattered by tissues, resulting in a penetration depth less than 1 cm.[15-17, 26] This limits PDT application to tumors either on or just under the skin, or in the outer lining of internal organs/cavities.[29, 30] Additionally, given that this therapy only works at the irradiated zones, the method is inefficient against metastatic tumors, which are a frequent cause of death in oncologic patients.[15, 16]

To solve the limitation caused by the lack of penetration depth, several researchers proposed alternative strategies for PS activation, such as the use of near-infrared,[31, 32] two-photon,[33, 34] and X-ray irradiation for excitation.[35] Near-infrared light also presents light penetration limitations, low absorption efficiency, and may cause heat damage to tissues. On the other hand, X-ray excitation is unable to directly activate the PS and requires nanoparticles, which present low photo-conversion capacities, to convert high-energy radiation into photons of suitable energy.[29, 35]

Thus, developing a PS that can be activated intracellularly and selectively without the need for an external light source is a relevant and challenging topic worth studying that could increase the role of PDT in routine cancer therapy. Internal light sources have become attractive alternatives to external light sources

since they can solve the issue of light penetration in conventional PDT systems.[27, 29, 36] Recently, the exploit of chemiluminescence (CL) resonance energy transfer (CRET) and bioluminescence (BL) resonance energy transfer (BRET) has been studied to create self-illuminating PDT systems.[15, 37-42] In summary, the processes are based on a non-radiative energy transfer from donors (either CL or BL) to suitable acceptor molecules. These studies aim to use CL/BL donors to intracellularly activate the PS without the need for an external light source.[15, 37-42]

INTRODUCTION TO CHEMILUMINESCENT AND BIOLUMINESCENT SYSTEMS

CL is the conversion of thermal energy into excitation energy through a chemical reaction, usually in the presence of a catalyst and/or co-factor.[43-45] BL is a sub-type of CL in which the emission of light is catalysed by an enzyme in a biological system.[43, 46] This is a widespread phenomenon that can be observed in, among others, bacteria, dinoflagellates, worms, and insects. Several CL and BL systems have already been studied,[43, 45-49] enabling the development of new techniques that rely on a variety of intrinsic advantages: sensitivity, specificity, fast reaction rates, possibility of colour modulation, and high quantum yields. As such, BL and CL systems have gained several applications in the fields of sensing, pharmacy, biomedicine, and bioanalytics.[50-54] They are specially used for microbe detection, biosensing, bioimaging, determination of metabolites of interest, and as reporter gene systems.

Chemiluminescence

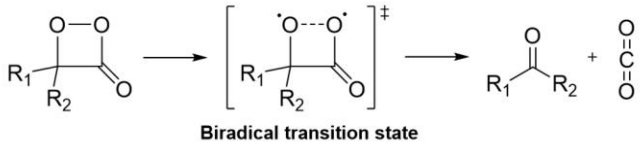
Nearly all CL reactions are characterized by the presence of a peroxide bond (O-O). This provides a way for a thermally activated ground-state reaction that results in excited-state products. [43, 46, 55] The peroxide-containing compounds can be divided into three main sub-categories, depicted in Figure 2: R_1, R_2, R_3, R_4-dioxetane-based compounds,[43, 46] R_1, R_2-dioxetanone-based compounds,[43, 46] and dioxetanedione.[56]

Figure 2. CL-capable cyclic intermediates containing peroxide bonds.

The ability to satisfy the energetic requirement is the most important factor when considering the suitability of a compound as a CL substrate – it determines whether the molecule will thermally decompose into an electronically excited-state product. Thermochemical calculations have shown that, for typical dioxetanes and dioxetanones, the heats of activation for their thermal decompositions vary between 70 and 90 kcal mol^{-1} and their activation energies range between 20 and 30 kcal mol^{-1}.[55] Therefore, the peroxide rings can provide enough energy for the chemical excitation of a fragment during thermal decomposition.

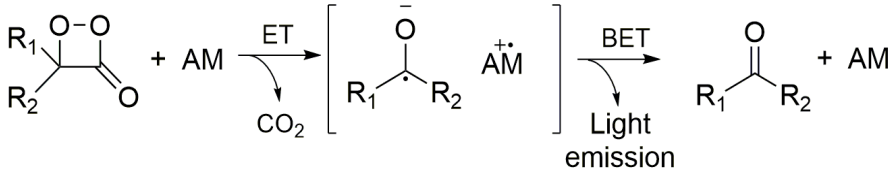
Two different reaction mechanisms have been proposed to explain the ground-state decomposition of peroxide-containing molecules: a stepwise biradical

mechanism and a mechanism that defends a partially/fully concerted decomposition in which the compounds are cleaved through a transition state.[57-61] The stepwise biradical mechanism (Scheme 1) is best supported by the literature than its counterpart. In this mechanism, the reaction starts with the homolytic cleavage of the peroxide bond, which results in a biradical transition state, and the C-C bond only breaks after this step. The participation of a biradical intermediate in the decomposition of simpler dioxetanones has been recently discussed. [61-63]



Scheme 1 - Stepwise biradical mechanism for the thermal decomposition of a CL-capable cyclic peroxide, here represented by dioxetanone.

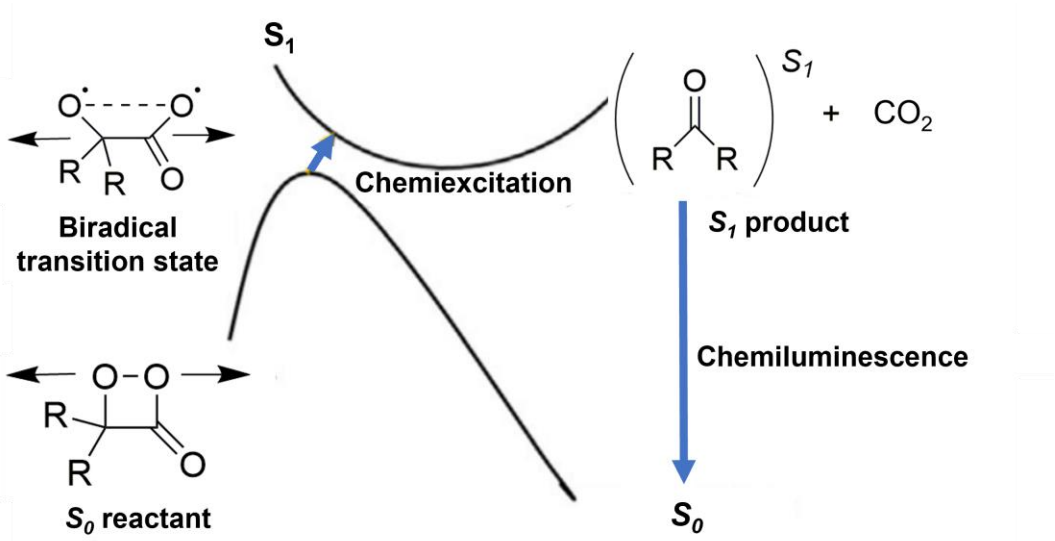
The effective generation of singlet excited states by these peroxides was originally explained by the chemically induced electron-exchange luminescence (CIEEL).[64, 65] In CIEEL, a radical ion pair is originated by an electron transfer (ET) from an oxidizable electron-rich moiety (an activator, AM), to the peroxide. The ion pair then undergoes back electron transfer (BET) from the carbonyl radical anion to the radical cation, generating singlet excited states through charge annihilation. Scheme 2 represents the intermolecular CIEEL for dioxetanones' CL. When the electron-rich moiety is part of the organic peroxide the mechanism can be termed intramolecular CIEEL, otherwise being termed intermolecular CIEEL.



Scheme 2. Representation of the intermolecular CIEEL for dioxetanones' CL.

Koo and Schuster's mechanistic theory started to be questioned when the CL of dimethyldioxetanone and diphenoyl peroxide, important examples of CIEEL, was re-examined.[66, 67] Studies revealed that these peroxides displayed low CL quantum yields, even though they had a supposedly efficient CIEEL decay, which disproved the basis of the theory. The presence of a catalytic activator (an oxidizable electron-rich moiety) only resulted in inefficient CL, unlike the CIEEL mechanism. Another study regarding polyacene endoperoxide and two different dioxetanones also supported these conclusions. [67]

Considering the failure of CIEEL, other mechanisms, in which neither the formation of a radical ion pair or a full electron transfer are involved, have been proposed to explain efficient CL reactions.[66, 68, 69] Instead of explaining the generation of S_1 states through full electron transfer, Charge Transfer-Initiated Luminescence (CTIL) mechanism is explained by gradual charge transfer and back charge transfer (BCT) between an ionized electron-rich moiety and the cyclic peroxide.[68-72] Nonetheless, even these revised forms of CIEEL are not yet fully in line with the available data, as theoretical results have suggested that charge-transfer (CT) processes should also be observed for CL systems with no electron-rich moieties.[58, 62] Additionally, the presence of electron-rich moieties is insufficient for the efficient formation of singlet excited states, both in intra- and intermolecular CL reactions.[55, 66, 73]



Scheme 3 - Schematic representation of the thermolysis mechanism for dioxetanone molecules and subsequent chemiexcitation.

CIEEL and CTIL were also not in conformity with the report by Pinto da Silva *et al*., in which the luminescent reaction of *Cypridina hilgendorfii* ("sea firefly") was studied.[74] The study of the key step of chemiexcitation allowed the team to obtain results indicating that neutral dioxetanone is responsible for chemiexcitation. Its thermolysis provides access to a long potential energy surface (PES) region of S_0-S_1 degeneracy (Scheme 3).[74] However, given that there is no clear correlation between ET/CT (between dioxetanone and the electron-rich moiety) and chemiexcitation, neither CIEEL nor CTIL can explain this imidazopyrazinone-based BL.[74] In the study, the researchers concluded that attractive electrostatic interactions between oxyluciferin moieties and CO_2 allow neutral dioxetanone to spend time in the PES region of degeneracy between singlet ground and excited states, thus explaining efficient chemiexcitation. On the other hand, anionic dioxetanone quickly detaches from CO_2 due to repulsive interactions, preventing access to the region of near-degeneracy.[74]

Following this, Magalhães *et al*. studied the chemiexcitation step responsible for the light-emission associated with Coelenterazine (Clz), a BL substrate that is part of a prototypical system for marine CL/BL.[75] The team found evidence that supports the identification of a neutral dioxetanone intermediate as responsible for efficient chemiexcitation. Again, the researchers proposed an explanation in which the dioxetanone spends time in a PES region of degeneracy between S_0-S_1, whereas for the anionic dioxetanone there is a quicker release of CO_2 due to the repulsion between Coelenteramide and CO_2 that prevents access to the region of degeneracy.[75] No evidence of a relationship between ET/CT and efficient chemiexcitation was found and, therefore, neither CIEEL nor CTIL could be used to explain the luminescence.[75]

Finally, a theoretical study focusing on dioxetanones supported the aforementioned reports and provided evidence suggesting that efficient chemiexcitation is the result of the reacting molecules reaching a PES region, since in inefficient chemiexcitation the molecules are unable to reach it.[76] The authors were able to hypothesize why this region only occurs in the thermolysis of certain dioxetanones: the main finding was that the access to the region of degeneracy between S_0-S_1 is a result of the increased interaction between moieties of the dioxetanones during the thermolysis (CO_2 and keto moieties), which appear to extend the biradical region.[76]. The increase attractive interaction seems to be caused mainly electrostatic interactions between the moieties of dioxetanone originated by charge separation . However, this electrostatic interaction does not appear to be so relevant for dioxetanes, and further study is required. Considering their results, the authors hypothesized that efficient chemiexcitation results from the interaction between the keto and CO_2 moieties (which control access to the PES region between S_0-S_1), instead of being just a result of ET/CT and charge annihilation, as defended by CIEEL and CTIL.[76] It is worth noting that, in energetic terms, the same factors leading to efficient singlet chemiexcitation can also lead to efficient triplet chemiexcitation, which is consistent with previous studies.[76]

In summary, despite decades of research, the mechanism responsible for the efficient chemiexcitation of cyclic peroxides in luminescent reactions is not fully understood. Long-standing theories, such as CIEEL and CTIL are not able to explain this phenomenon in all cases. More recent results indicate that efficient chemiexcitation is a result of reacting molecules having access to a region of PES where S_0 and S_1 are degenerate.[74-76] Access to this region of degeneracy seems to be the result of the increased interaction between the two moieties that compose the peroxide ring. Overall, these reports support the hypothesis that efficient chemiexcitation is not just a result of ET/CT and subsequent charge annihilation, but is instead based on the degree of interaction between the CO_2/keto and keto moieties (responsible for the access to the S_0-S_1 region of degeneracy).[74-76]

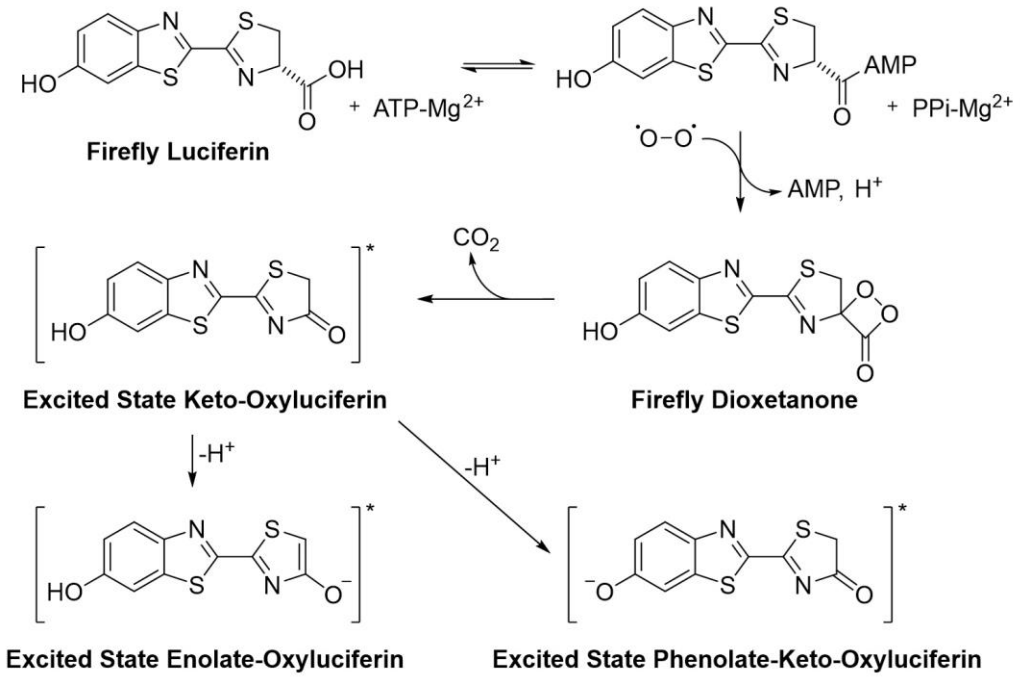
Bioluminescence

Light emission catalysed in a biological system by an enzyme is known as BL.[43, 46]

The study of several CL and BL systems, which ranged from simple dioxetanes/dioxetanones to complex BL substrates, allowed for the identification of three common key structural moieties: a peroxide bond, an electron-rich moiety, and an ionizable group.[46, 55]

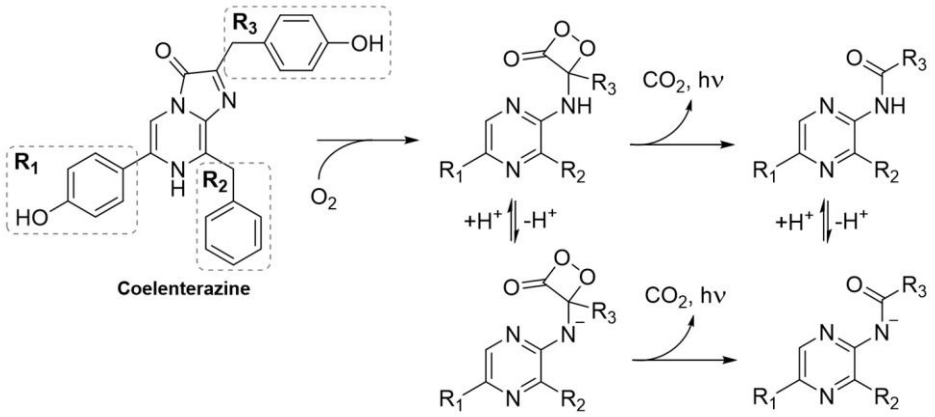
The first key structural moiety is the peroxide bond. When cleaved, it allows thermally activated ground-to-excited state chemiexcitation.[43, 46, 55] This type of feature is ubiquitous among BL systems, even though some differences can be found among them. In particular, the bacterial and dinoflagellate luciferins eject different fragments through a single bond-breaking step.[46] The second key structural moiety is an electron-rich group, responsible for tuning the activation energy of the peroxide ring decomposition (by charge/electron transfer).[43, 46, 55, 58, 66, 69] This can be found for nearly all BL cases, except for *latia* luciferin,[46] for which it is possible that the electron-rich moiety is provided by the enzyme, in the form of an aromatic amino acid. The electron-rich moiety can consist of different types of aromatic systems, as exemplified by firefly and coelenterazine dioxetanones.[68, 77, 78] It can be concluded that this electron-donating moiety is more relevant than the specific chemical composition. The third and last key structural moiety is an ionizable group, which is responsible for triggering the charge/electron transfer and sharply tune the activation energy for the thermal reaction.[58, 66, 68, 69, 77] These oxidizable groups are crucial for modulating the colour of the light emitted by the BL product.[79-82] More recently, the importance of these moieties has been debated: evidence suggests that some of them (ionizable groups) might not be required, whereas the role and function of others (electron-rich moieties) is not yet fully understood. [58, 83]

In the same way, luminol is the most studied CL substrate,[84-86] the firefly luciferin–luciferase reaction (Scheme 4) is, undoubtedly, the most studied and well-known BL system. Firefly luciferase (Fluc) catalyses a two-step reaction: it starts with an AMPylation reaction between D-luciferin and adenosine triphosphate (ATP) that leads to an adenylyl intermediate. In the second step, the adenylyl intermediate is oxidized by molecular oxygen, adenosine monophosphate is released, and firefly dioxetanone is formed.[43, 46, 87-89] Subsequently, the thermal decomposition of the peroxide results in the chemiexcitation of firefly oxyluciferin (the light emitter).[43, 68, 90] The Fluc/luciferin system is a pH-sensitive BL system. While the emitted light has a peak at 560 nm for basic pH, the maximum shifts to around 620 nm in acidic pH.[43, 46, 89] It is worth noting that the identity of the light emitter is still a matter of debate: while some researchers defend the participation of anionic keto species,[47, 68] others support the presence of the oxyluciferin enolate.[48, 91]



Scheme 4 - Reaction mechanism of firefly luciferin-luciferase BL.

Another example of BL is Clz, a luciferin that can be found among marine living organisms.[45] Clz, which has an imidazopyrazinone core, is essential to the BL of several marine species and is involved, as a substrate, in reactions catalysed by several luciferases, such as *Gaussia princeps* (Gluc) and *Renilla reniformis* (Rluc) luciferases.[92] Besides being a functional BL substrate, Clz can also be used for CL reactions involving molecular oxygen or ROS (such as superoxide anion),[92, 93] and is even routinely used as a probe for ROS.[94, 95] The reactions involving Clz (Scheme 5) start with an electron transfer from Clz to an oxidizing agent, leading to the formation of a radical intermediate.[92, 93] A fast radical coupling between the intermediate and a superoxide anion follows, forming the Clz dioxetanone. The thermal decomposition of this dioxetanone generates a singlet excited state of coelenteramide, with CO_2 loss.[92, 93] Coelenteramide, which is the light-emitting form, produces blue-green light with a spectral peak at 480 nm.[92, 96]



Scheme 5 - Reaction mechanism of Clz BL.

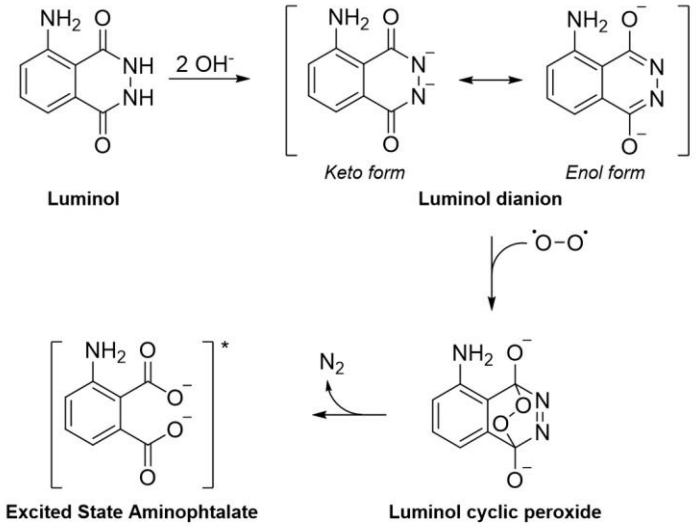
As in the case of firefly BL, there is still no full consensus between the true ionization state of coelenteramide undergoing BL emission.[81, 92] Coelenteramide is thought to occur in one of the chemical forms present in the equilibrium between the neutral, protonated, and deprotonated amino and hydroxyl groups, and the phenolate-amide tautomerism. However, two other forms can occur considering the nature of the emitter: the phenolate-NH_2^+ and the diphenolate. A study by Min *et al*. predicted the behaviour of the fluorescent and CL properties of coelenteramide in several conditions using time-dependent density functional theory.[97] The team determined the excited state equilibrium constants to estimate the relative stabilities of the different species of coelenteramide in the fluorescent and CL states and obtained the minimum-energy structures for each state for all possible conformations.[97] They concluded that CL states are “dark” states with low oscillator strengths that must evolve into bright fluorescent states and that the photoacidity of the phenol group in the molecules is considerably higher in the fluorescent state when compared to the CL state.[97] Finally, they concluded that the higher photoacidity permits the formation of phenolate coelenteramide in the fluorescent state instead of the CL state, enabling its role as the luminescent emitter.[97]

APPLICATIONS FOR CHEMILUMINESCENCE AND BIOLUMINESCENCE IN PDT

Designing PS that can be intracellularly activated, without the need for an external light source, is a challenging but rewarding research topic. Considering the limited depth penetration of external light sources, the range of applications for PDT could increase significantly when they are not dependent on said sources. CL and BL systems have been studied as intracellular excitation sources for PDT, with luminol (for CL-based systems), and firefly luciferase–luciferin and *Renilla* luciferase–Clz (for BL-based systems) being the best examples.

CL-based PDT systems

Light emission is the result of a large number of chemical reactions, and one of the most known and efficient examples is the oxidation of luminol (Scheme 6).[15, 84-86] Luminol is a well-studied system that has been thoroughly tested as a substitute for external laser activation in PDT.[15] Luminol undergoes oxidation in basic solution in the presence of hydrogen peroxide and catalysts (*e. g.* Fe^{2+}, Cu^{2+}, Co^{2+}, periodate ions, or hydrogen peroxidase). This CL reaction originates from the reaction of luminol with hydroxide anions, from which a dianion is formed.[15, 84-86] This dianionic intermediate reacts with oxygen to yield the corresponding cyclic peroxide. After releasing N_2, this peroxide evolves into the excited state of the aminophthalate ion, which emits blue CL with a maximum wavelength of 425 nm.[15, 84-86]



Scheme 6 - Reaction mechanism for the CL of luminol.

Laptev *et al.*, who first studied the luminol CL system, added hydrogen peroxide and ferrous sulphate to cultures of hybridoma cells to assess the efficiency of luminol in activating the PS hematoporphyrin (Hp).[15] They found that, when reacting, luminol could be turned into the excited state aminophthalate ion, which in turn could activate a conjugate involving Hp, resulting in nearly 100% of cytotoxicity, out of which around 15% was directly induced by luminol (indicating some biocompatibility issues).[15] Additionally, while the luminol system was able to lead to high cytotoxicity rates in the presence of the PS, the amount of Hp conjugate needed to attain LD_{MAX} with the CL-based system was 6.7 times higher than with an external source of radiation.[15] Thus, the system proposed by Laptev *et al.* required further optimization.

Yuan *et al.* also developed a new CL-based PDT[38] in which they used a luminol/hydrogen peroxide/horseradish peroxidase CL system to excite the PS oligo(*p*-phenylene vinylene) (OPV). Considering that luminol has a maximum at 425 nm and that OPV exhibits broad absorption ranging from 350 to 550 nm, the spectral overlap required for CRET is met.[98, 99] Additionally, CRET should be favoured by electrostatic interactions between the cationic OPV and the dianionic

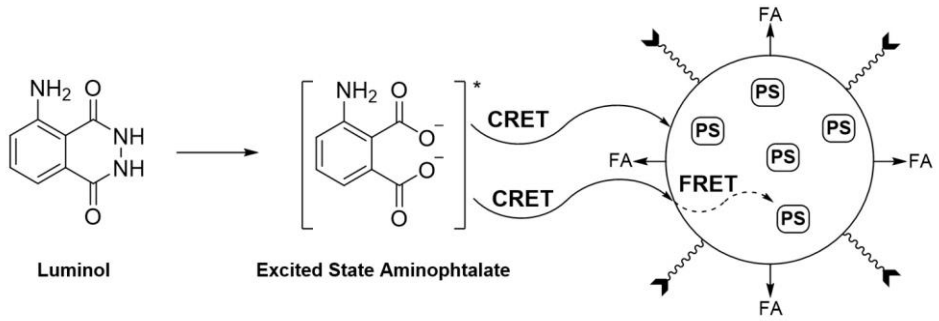
CL emitter.[38] To confirm that a CRET process takes place, they measured the luminescence of luminol in the presence of several concentrations of OPV.[38] The addition of OPV resulted in a large decrease of the luminescence intensity at 425 nm (corresponding to luminol) and, at the same time, an increase of the luminescence intensity at 550 nm (corresponding to OPV), which confirmed that CRET occurs in this system.[38]

The capacity of the luminol system to activate OPV was first evaluated *in vitro* in cervical cancer (HeLa) cells.[38] The use of the luminol CL system with increasing OPV concentrations resulted in a cellular viability lower than 10%. However, the luminol CL system was itself responsible for killing about 30% of the HeLa cells and was also cytotoxic to healthy human epithelial cells.[38] These results show that the luminol CL system has some biocompatibility limitations that might impede its clinical use, as observed in some studies.[15, 38] It is worth noting that these results, besides demonstrating that the CL system by itself is cytotoxic, also show that it has no specificity or selectivity for tumor cells.

The efficacy of this CL system was also evaluated by Yuan *et al.* as an *in vivo* excitation source in nude mice with HeLa cells-derived tumors.[38] They demonstrated that the system has, in fact, some potential in PDT, as around 30% of tumor inhibition was achieved with the CL-based PDT when compared to the CL system itself. Moreover, the addition of OPV alone did not cause any tumor inhibition. In this way, it was shown that CL-mediated PDT can result in *in vivo* tumor growth inhibition.[38] Remarkably, the CL-mediated PDT did not show any signs of side effects, as the mouse body weight growth was not inhibited in comparison with the control groups. The authors concluded that the luminol CL system had no apparent toxicity to normal healthy tissues, even though it presented a lack of specificity. However, tumor cells can be more susceptible to ROS-mediated damage than normal cells since they are characterized by their state of oxidative stress.[100] This being said, a lower amount of exogenous ROS is required to prompt cellular destruction in cancer cells. Considering the low cytotoxicity of the CL PDT system, this lack of toxicity might be the result of ROS generation in amounts that are insufficient to affect normal cells. Should the ROS output of the CL system to be increased, as would occur in a trial for clinical applications, toxicity for normal cells might also be observed. The use of a luminol signal enhancer impacted the anti-tumor activity of the CL-mediated PDT, resulting in a 55% tumor inhibition ratio, which pointed to signal enhancers as one way of increasing the effectiveness of CL PDT systems.[38]

More recently, the same research group proposed an electroluminescence-based PDT system,[101] and reported the preparation of a multifunctional nanoplatform for self-illuminating phototherapy.[102] The haemoglobin-nanoparticle platform was prepared coupling haemoglobin (Hb) to polymeric nanoparticles made of poly(styrene-co-maleic anhydride) and poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene]. The polymer-conjugated Hb can function simultaneously as the oxygen carrier, as the catalyst for the CL reaction of luminol with hydrogen peroxide, and as the PS for the generation of ROS.[102] Thus, the system does not require an external light source for the PS excitation and overcomes the problem of insufficient oxygen under hypoxia. Moreover, the system can be applied to control drug release and therefore be used simultaneously as PDT and chemotherapy.[102]

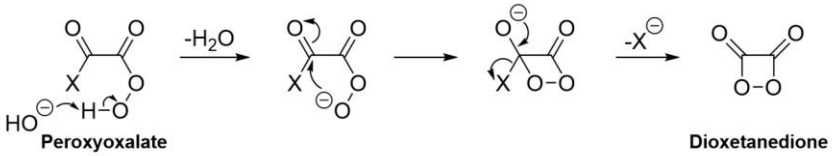
Another innovative report involving luminol was made by Zhang *et al*. The team constructed a “smart” nanoconjugate in which semiconducting polymer dots acted as a hydrophobic matrix to incorporate the PS *meta*-tetra(hydroxyphenyl)chlorin (*m*-THPC) (Scheme 7).[40] Amphiphilic Janus dendrimers were used as surface-functionalizing agents to conjugate horseradish peroxidase and aminated folic acid onto the surface of the polymer dots.[40] When adding luminol and hydrogen peroxide, the PS can be activated either by CRET from luminol CL to *m*-THPC or by CRET to the polymer dots, followed by fluorescence resonance energy transfer to the polymer dots. The photodynamic effect was evaluated by incubating polymer dot nanoconjugates with different types of cells (C6 glioma, MCF-7 breast cancer, and NIH3T3 fibroblast cells) *in vitro*, in the presence of CL substrates.[40] For a nanoconjugate concentration of 10 μg mL^{-1}, viabilities of 32%, 17%, and 72% were observed for the C6, MCF-7, and NIH3T3 cells, respectively. However, when the CL substrate is absent, a cell viability of over 95% is observed for all three cancer cell types.[40] These values showed that luminol can indeed activate the PS, inducing a photodynamic effect. Nevertheless, from a clinical use perspective, it could also be said that the cell viability was too low for healthy non-cancerous cells, while too high for tumor cells.[40] Moreover, given the overexpression of folate receptors by tumor cells, it is worth noting that the difference in cell viabilities between the cancerous receptors (C6 and MCF-7) and non-cancerous (NIH3T3) could be ascribed to the aminated folic acid present on the surface of the polymer dots.



Scheme 7 - Representation of the luminol CL-based system in conjugation with polymer dots. FA – folic acid.

Combining different forms of PSs with luminol CL resulted in a variety of *in situ* self-illuminating PDT systems including, among others, 5-aminolevulinic acid,[103] chlorin e6 conjugated with carbon dots possessing yellow emission,[104] or porphyrinic metal-organic frameworks (MOFs).[105] The covalent attachment of luminol to the PS is another strategy that enables the construction of a CL-PDT system through direct energy transfer from luminol to PS. Yesilgul *et al*. reported the development of the modular, unimolecular, erythrosine-luminol, which is capable of producing singlet oxygen in the presence of Cu^{2+} and hydrogen peroxide.[106] Xu *et al*. synthesized a chlorin e6-luminol-PEG conjugate (CLP), capable of self-assembling into core-shell nanoparticles, which can be exploited for imaging as well as specifically kill cancer cells.[107]

Another known CL reaction is that of peroxyoxalate, which rapidly transforms into the high-energy compound 1,2-dioxetanedione (Scheme 8).[108-110] The reaction ensues between active derivatives of oxalic acid and hydrogen peroxide. First, the nucleophilic attack by a peroxide result in the formation of the 1,2-dioxetanedione intermediate.[108-110] In certain conditions, while decomposing into two CO_2 molecules, this intermediate is capable of transferring energy to fluorescent energy acceptors, thus generating light. Philip and Maximuke were among the first to employ the CL reaction of peroxyoxalate as an internal light source, used it in conjugation with Photofrin II (a Haematoporphyrin derivative) to treat mammary adenocarcinomas in mice,[111] and observed some effectiveness in certain studied mice groups. They used exogenous hydrogen peroxide to trigger the CL reaction, however, large amounts of endogenous hydrogen peroxide are often found in malignant cells and inflamed tissues,[112] enabling CL even without the addition of exogenous compounds. These results suggested that a peroxyoxalate-based CL system could be a viable alternative to laser activation in PDT.[108-110]



Scheme 8 - Peroxyoxalate transformation into dioxetanedione. X = RO or Cl.

Romaniuk *et al*. studied polymeric oxalate as the substrate for CL reactions with tetramethylhaematoporphyrin (TMHP), acting both as PS and activator for the CL reaction.[113] Polymeric oxalate and TMHP were dispersed and stabilized with a surfactant. The dispersions could efficiently produce singlet oxygen and exert relevant cytotoxicity.[113] Mao *et al.* designed a nanoplatform for image-guided PDT by co-encapsulating bis [2,4,5-trichloro-6-(pentyloxycarbonyl)phenyl]oxalate (CPPO) with a specially designed PS (TBD) into pluronic F-127 and soybean oil.[114] The smartly designed TBD displayed bright NIR emission and efficient singlet oxygen generation. This system allows the surveillance of the tumor site via CL imaging and inhibits tumor growth through CL-based PDT singlet oxygen generation, thus accomplishing both tumor imaging and treatment.[114] The CL reaction of tumor hydrogen peroxide with CPPO produces singlet oxygen, which results in the photo-excitation of the PS TBD via CRET.[114] A system with a similar basis was developed by Yu *et al*., with the tumor hydrogen peroxide initiating the CL reaction of CPPO, which in turn activates the PS chlorin e6 via CRET.[115]

BL-based PDT systems

The first study aiming to assess the potential of BL for PDT was performed by Theodossiou *et al*. who, in 2003, used the Fluc-luciferin BL as an intracellular excitation source for Rose Bengal.[42] The PS is a water-soluble dye with a high singlet oxygen quantum yield (around 0.75).[42] The study was performed *in vitro*

using NIH3T3 murine fibroblasts as a model. The cells were transfected with a modified Fluc gene (Luc+, with cytosolic expression), and both luciferin and the PS were later added to the cell cultures. These results supported the use of firefly BL as an excitation source for PDT: the combination of the firefly BL system with the PS led to a 90% apoptosis rate, as opposed to the control groups, which exhibited a 100% survival rate.[42] Moreover, by using a singlet oxygen quencher, the authors concluded that the cytotoxic effect results mostly from the production of this particular ROS species.

One interesting aspect of the work by Theodossiou *et al*. is that it demonstrated that the addition of ATP to the cells is not required to induce the Fluc-mediated PDT, as the concentration of ATP in the fibroblasts is itself sufficient to trigger BL.[42] Additionally, it was shown that, upon uptake by the cells, both luciferin and Rose Bengal adopt a diffuse cytosolic distribution.[42] Knowing this is crucial, as BRET should only elicit sufficient PDT response if both the BL donor and the PS are co-localized within the cell. It is worth remembering that the Fluc enzyme was not added to the cell culture and instead, its gene was transferred into the cell, which means no information about the possible uptake of this enzyme by the model cells was obtained.[42]

This study was followed by the work of Schipper *et al*. who, in 2006, also tested the firefly BL system for application in PDT.[39] This team claimed that, despite the results of Theodissiou *et al.*,[42] there were still doubts regarding the photon output of the Fluc-transfected cells and questioned whether it was enough to induce a photodynamic effect when compared to doses typically associated with clinical PDT (around 50 mW cm^{-2} and above 1 J cm^{-2}).[39] The authors demonstrated that firefly BL is not able to generate enough *in vitro* photons to result in a cytotoxic effect from two different PS (Rose Bengal and hypericin).[39] The measured photon output only reached 2.3×10^{3} photons per second per cell, which is approximately 1.2×10^{-9} mW cm^{-2} orders of magnitude lower than the 50 mW cm^{-2} used in clinical trials.[39] Even assuming that maximal levels of photons were emitted during 24 h, the total amount of energy delivered would only be 1.03×10^{-7} mJ cm^{-2}, considerably lower than 1 J cm^{-2}, and higher than what is observed in clinical trials involving laser irradiation.

These results[39] contrast starkly with those presented by Theodossiou *et al.*,[42] and the differences between them have yet to be explained. Schipper *et al*.[39] suggested that the cell line used by Theodossiou and his team could have an unknown mutation, causing an increase in their sensitivity to PDT treatment. However, since no follow-up study was made regarding this, no data is available to support this suggestion. A simpler justification could come from the difference in luciferin concentration. While Theodossiou *et al*.[42] used concentrations of 500 µM, Schipper *et al*.[39] used a maximum luciferin concentration of 20 µM. Knowing that BL intensity is also dependent on luciferin concentration, could explain how Theodossiou *et al*. achieved higher light production than Schipper and his team.

In a recent work, Yang *et al*. reported a novel firefly BL-mediated PDT system based on a polymer nanoparticle made of biodegradable poly(lactic-co-glycolic acid).[116] The particle was loaded with the PS (Rose Bengal) and conjugated with Fluc. Spectroscopic characterization suggested that BRET effectively activated the PS to generate ROS, inducing oxidative stress.[116] *In vitro* photodynamic studies demonstrated that this BL-mediated PDT results in significant cytotoxicity towards

cancer cells.[116] The growth of subcutaneous tumors can be considerably inhibited while healthy, normal organs, such as the heart, liver, spleen, lung, and kidney, remain remarkably undamaged.[116] Another work on the use of firefly BL for PDT was performed by Yang *et al*., who conjugated carbon dots (with an excitation-independent emission) with protoporphyrin IX (PIX) to construct a PDT system.[117] In this case, carbon dots solved the limitations of PIX and served as an intermediate to bridge the excitation from the firefly BL to the PIX. This BL-mediated PDT resulted in the production of singlet oxygen in SMMC-7721 hepatocarcinoma cells through a fluorescence resonance energy transfer (FRET) process.[117]

Besides the firefly BL, the Rluc-Clz BL system (using the *Renilla reniformis* luciferase) has also been tested as an alternative intracellular excitation source. The first study on such a system was performed by Hsu *et al*. in 2013,[41] who conjugated the Rluc enzyme with carboxylate-containing quantum dots (QDs), which absorb the photons resulting from the BL reaction via BRET in the presence of Clz (**Erro! A origem da referência não foi encontrada.**).[41, 118] In this system, the QDs are responsible for PS activation by emission of light at 655 nm instead of the BL reaction.[41] The rationale behind this is that the emission of the QDs is easily tuneable when compared to that of the BL reaction, greatly increasing their versatility for PDT applications and allowing the system to be used with a larger number of PS.

The bioluminescent conjugate was used to intracellularly activate the PS *m*-THPC (already in clinical use) in mice transfected with human lung adenocarcinoma epithelial A549 cells.[41] The results were promising, as the mean relative volumes of PDT-treated tumors were 4.5 to 6 times lower (in comparison with the initial tumor volumes) than those from untreated animals on day 20. In the mice treated with the Rluc-QDs conjugate, tumor growth was significantly inhibited.[41] Additionally, the isolated tumor sizes of the group treated with PDT (Rluc/QDs/PS/Clz) were considerably lower than those of the PS/Clz (growth inhibition of 4.2%) and Rluc/Clz/QDs (growth inhibition of 23.3%).[41] Nevertheless, these results also showed that the Rluc-QDs conjugate has considerable cytotoxicity itself. This can be problematic, given that unwanted cytotoxicity to healthy cells can cause side effects, unacceptable in clinical applications.

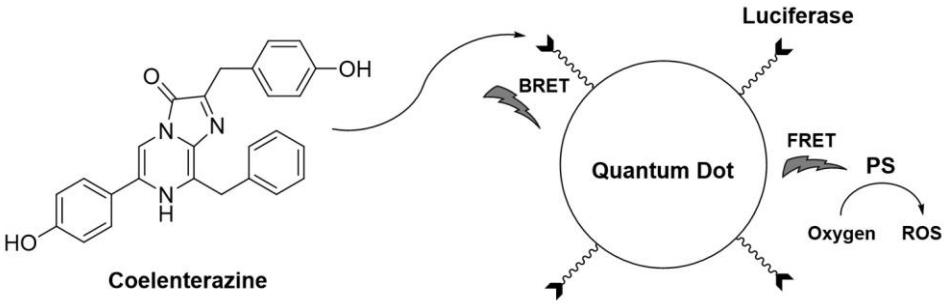


Figure 3. Schematic representation of the use of the Rluc-Clz BL system in conjugation with carboxylate-containing quantum dots to activate a PS.

The results of Hsu *et al*. also showed that tumors treated with BL-mediated PDT present considerably lower cell proliferation when compared to control groups (27.1±2.6% *versus* 87.4±2.2% / 92.2±2.6% /75.2±2%, respectively).[41] The Rluc-QDs conjugate also led to a decreased degree of vascularization of the tumors, causing their growth suppression.[41] This study also evaluated the effectiveness of BL-mediated PDT when compared to conventional irradiation by an external light source.[41] The team found that, for equal PS concentrations, the BL-mediated PDT reaction yielded an irradiation comparable to that resulting from externally applying 0.6-0.8 J cm^{-2}.[41] While 0.6-0.8 J cm^{-2} can be considered low, even more when compared to the light doses used in clinical PDT (higher than 1 J cm^{-2}), the efficiency of this BL-mediated PDT is enough to induce a photodynamic effect *in vivo*, as can be seen in the work of Hsu *et al*.[41] It is worth noting that the efficiency obtained by this complex, 0.6-0.8 J cm^{-2}, largely surpasses that obtained for firefly BL-mediated PDT (1.03 x 10^{-4} mJ cm^{-2}).[39, 41]

In summary, the study by Hsu *et al*. showcased the potential of the application of Rluc-QDs conjugates as an alternative and internal excitation source for PDT.[41] Despite that, the system could be further optimized, as the obtained photodynamic effect might not suffice for clinical application. The decreased efficiency of the system might originate from the fact that it is based on two energy transfer steps. First, we observe a BRET step, in which the energy is transferred from the BL reaction to the QDs. Subsequently, we observe a FRET process, in which the QDs transfer energy to the PS. A higher number of energy-transfer steps result in an increased number of points at which the overall efficiency of the therapy decreases.

Rluc-Clz BL-mediated PDT was also the focus of a more recent study by Kim and co-workers.[119] Their objective was to determine if BL, with its lower energy output, can actually be used in PDT as an alternative excitation source. They created self-illuminating Rluc-QDs, conjugated to intracellularly activate the PS (chlorin e6). The effects of the PDT on tumor growth in mice were studied *in vivo* using three different cancer cell lines: colorectal cancer cells (CT26), lung cancer cells (LLC), and melanoma cells (B16F10).[119] The authors calculated the efficiency of BRET (from the BL reaction to the QDs), which was between 60-65%. These values support the hypothesis that the low efficiency of the QDs-based PDT results from a loss of efficiency starting at the initial BRET step. In addition, the authors observed that the conjugates did not enter the cell, but accumulated at their external surface in high concentrations instead.[119] Interestingly, this study demonstrated that whereas chlorin e6 molecules (at a concentration of 100 μM) are activated 4 x 10^7 times per minute (by a flux from a 660 nm, 2.2 mW cm^{-2} laser), the Rluc-QDs complex increases activation to 3 x 10^8 times per minute.[119] These results support the conclusion that BRET energy (in the order of 100 mJ) could generate a stronger photochemical activation in the cellular membrane than energy from laser irradiation (of the same order, 100 mJ). Cell imaging and cytotoxicity tests demonstrated that this BL-mediated PDT system can lead to significant intracellular ROS, causing membrane damage and cellular death.[119]

Gluc (*Gaussia princeps* luciferase) was also used in a PDT system. Al-Ani *et al*. reported the preparation of a Gluc-LiDps (*Listeria innocua* DNA binding protein from starved cells) fusion protein, with chemical conjugation of Zn^{2+}-protoporphyrin IX (ZnPP) to lysine residues.[120] The Gluc-LiDps-ZnPP conjugate

could generate ROS via BRET between Gluc (470-490 nm) and ZnPP. An *in vitro* study demonstrated that the conjugate was efficiently taken up by tumor cells (SKBR3 and MDA-MB231 breast cancer cells).[120] In the presence of Clz, the conjugate inhibits the proliferation of SKBR3 cells due to high ROS levels, with the exposure to the conjugate resulting in significant suppression of the migration of the remaining cells,[120] which showcases the potential of the Gluc-LiDps-ZnPP conjugate as a nanoplatform for the development of an anticancer PDT.

ALTERNATIVES TO CONVENTIONAL CL/BL-MEDIATED PDT

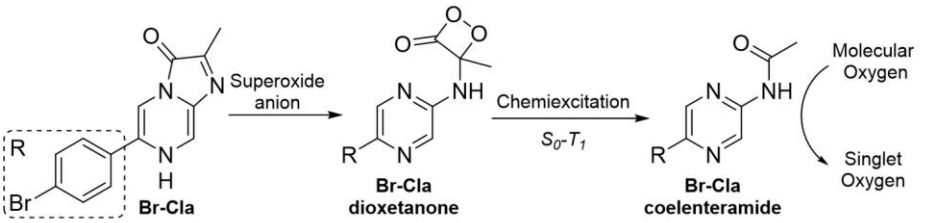
CL and BL have been extensively studied as alternative and intracellular light sources for PDT. However, these kinds of systems present limitations when considered for clinical applications.

The CL/BL substrates and the catalyst/co-factor must be present in the same microenvironment to generate the required light emission. However, it is not easy to ensure that all the components required for the therapy reach the same cellular space without reacting with one another during delivery. Moreover, given that the efficiency of energy transfer processes (such as CRET and BRET) is inversely proportional to the distance between the donor and the acceptor, the PS must be present in the same localization as the CL emitter to guarantee sufficient excitation. It is noteworthy that even if these conditions are met, the overall efficiency of the process still depends on several sequential steps: 1) CL or BL; 2) energy transfer from the CL/BL donor to the PS acceptor; 3) subsequent intersystem crossing of the PS; 4) ROS production.[38, 40, 41, 114] The existence of several steps will likely decrease the efficiency and reproducibility of PDT. Thus, before CL/BL-mediated PDT is available for clinical use, each individual step should be optimized to minimize the overall yield losses of the therapy.

In addition, one of the major advantages of PDT is its selectivity in terms of the targeted area, allowing the selective targeting of tumoral tissue.[16, 17] This comes from the fact that only the tissue that is irradiated by light is affected by the photodynamic effect. The substitution of laser light by CL/BL-mediated intracellular excitation as an activation source means this important advantage is lost, which can be observed in some of the studies mentioned earlier in this chapter (*e. g.* CL/BL systems lacking features that permitted tumor-selective activation).[38, 41] Considering that some of the components involved in CL/BL present significant cytotoxicity towards both tumoral and healthy cells (*e. g.* luminol), this is of particular concern.

Clz and its analogues have been studied as self-activating agents for PDT.[121-123] Recently, Clz-based alternatives overcoming the limitations imposed by conventional CL/BL-mediated PDT have been reported. One example of this is the work published by Pinto da Silva *et al*. in 2019.[121] The team developed a single-molecule Clz-based PS, which is capable of intracellular self-activation and is also tumor-selective since it is based on a CL reaction involving only a tumoral marker: superoxide anion (Scheme 9).[121] The PS is an analogue of Clz, known for its use as a superoxide anion probe.[94, 95] When Clz reacts with this radical in the absence of a catalyst/co-factor, it can be oxidized and lead to the formation of an unstable dioxetanone intermediate, which readily

decomposes into CO_2 and triplet excited state coelenteramide. Coelenteramide promptly transfers its energy to molecular oxygen, inducing the generation of singlet oxygen.[121] The direct chemiexcitation into triplet states is allowed due to the heavy atom effect, achieved by the addition of a halogen atom (bromine), which increases the rate of intersystem crossing.[121]



Scheme 9 - Schematic representation of the BL-based PDT using the Clz derivative, Br-Cla [6-(4-bromophenyl)-2-methylimidazo(1,2-*a*)pyrazin-3(7*H*)-one].

Since tumor cells are under oxidative stress, mostly caused by the overexpression of the superoxide anion, this ROS species can be considered as a cancer marker.[100] Considering that Clz leads to the formation of excited-state products when in the presence of this same cancer marker, it is possible to explore this to obtain a CL reaction that is only triggered by the presence of the superoxide anion, making the reaction intrinsically tumor-selective. Additionally, given the fact that the CL reaction can be used as a self-excitation mechanism, only the PS would need to be administered to the patients, eliminating the problem of delivering several reaction components to the same localization within the tumor.[121] Interestingly, the system developed by Pinto da Silva *et al*. is not about the production of ROS but rather about the exchange of superoxide anion into singlet oxygen: this provides another advantage, as superoxide is a major target of the cell antioxidant machinery while singlet oxygen is not. Thus, the resulting oxidative stress is able to bypass the defenses of the cell.[124]

The system proposed by Pinto da Silva *et al*. was based on the direct generation of triplet states via the Clz CL reaction,[121] which is known for the efficient production of singlet excited states.[45, 125] To promote the formation of triplet states, they increased the efficiency of intersystem crossing during the chemiexcitation step, which can be achieved by using heavy atoms.[126] The team prepared a Clz-based analogue in which the phenol group was substituted by a bromobenzene group (Br-Cla).[121] The *p*-cresol and benzyl moieties of Clz were substituted by a methyl group and a hydrogen atom, respectively, to simplify the structure.[121]

The *in silico* modelling of Br-Cla predicted that it should be present in a neutral form in acidic tumor microenvironments (pH 4.5-5 *versus* pH 7.4 for normal tissues).[121, 127, 128] Moreover, the theoretical calculations indicate that all the involved reactional steps should be exothermic, and so the S_0 CL reaction should be feasible. However, in normal tissue, when the Br-Cla core is ionized, the initial oxidation step is endothermic, meaning that the CL reaction is not favoured at a pH associated with healthy tissues.[121, 127, 128] Thus, the pH difference

between tumoral and healthy tissues is another factor that can provide selectivity for this system.[121]

Another advantage of Br-Cla is its conformity with Lipinski's rule of five,[129, 130] according to theoretical modelling. Lipinski observed that new drugs tend to have higher success rates in clinical trials when they have:[131] molecular masses lower than 500 Da; less than 5 hydrogen bond donors (sum of NH and OH groups) and 10 acceptors (sum of N and O atoms); and an octanol-water partition coefficient lower than 5 (lipophilicity).[121]

The ability of the superoxide anion to trigger the CL of Br-Cla reaction was tested measuring the CL output of the PS while adding different amounts of potassium superoxide (superoxide anion source). Light-emission was detected, which demonstrated the reactivity of Br-Cla towards superoxide.[121] Furthermore, to confirm the involvement of triplet states, the CL output was measured both under normal and inert atmosphere (in which O_2, a known triplet state quencher, was removed by bubbling the solution with N_2). The results showed a 26% higher CL output under inert atmosphere, indicating the involvement of triplet excited states.[121] The same test was conducted for normal Clz, however, N_2-bubbling did not have any effect on the CL output.[121] These results support the hypothesis that the addition of the heavy atom provided Br-Cla with triplet excited states, as opposed to commercial Clz. The production of singlet oxygen as a result of the CL reaction of Br-Cla was confirmed in fluorescent assays using 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) as a singlet oxygen sensor.[132] ABDA is a known singlet oxygen quencher and, given that Br-Cla induces its photobleaching, it allows the monitoring of singlet oxygen generation by fluorescent analysis.[132] CL reactions with increased amounts of Br-Cla significantly quenched ABDA's fluorescence, proving the Br-Cla-mediated generation of singlet oxygen.[121] Moreover, to confirm that these results were due to the CL reaction of Br-Cla and due to a non-specific interaction with the PS, ABDA's fluorescence with Br-Cla was measured both in the presence and absence of potassium superoxide. Br-Cla only induces quenching in ABDA's fluorescence when potassium superoxide is present, suggesting that the quenching is a direct result of Br-Cla's CL reaction.[121] The CL reaction of commercial Clz does not affect the fluorescence of ABDA, both in the presence or absence of potassium superoxide.[121] However, it should be noted that Clz itself induces some quenching of ABDA's fluorescence, which the authors attribute to non-specific interactions.[121]

To confirm the suitability of Br-Cla as a tumor-selective self-activating PS for PDT, the authors conducted *in vitro* cytotoxicity assays for both cancer (MCF-7 breast and PC-3 prostate cancer cells) and healthy (MCF-10A breast cells) cellular lines.[121] In aprotic solvents, Clz and its analogues can undergo CL triggered only by molecular oxygen,[75, 125, 133] whereas in aqueous solutions and biological media this only occurs in the presence of the superoxide anion,[92, 133] which is why, considering this radical is overexpressed tumor cells,[100] the reaction with superoxide anion should provide intrinsic tumor-selectivity to Br-Cla.

Br-Cla induced considerable cytotoxicity for MCF-7 and PC-3 tumor cell lines. Toxicity of more than 50% was observed with a Br-Cla concentration of 75 μM in PC-3 cells, whereas for MCF-7 toxicity was observed for concentrations as low as 0.1 μM.[121] Remarkably, Br-Cla showed better results than reference drugs such

as Tamoxifen (breast cancer)[134] and Metformin (prostate cancer),[135] which highlighted its potential as a PS for self-activating PS.[121] While cytotoxicity towards tumor cells is required, selectivity is also needed: when incubated with MCF-10A cells, Br-Cla showed no toxicity in concentrations between 0.1 and 100 μM, indicating a high selectivity for superoxide-overexpressing tumor cells.[121] However, *in vivo* studies are required given that overexpression of superoxide anion might also result from causes other than cancer, such as senescence and inflammation. Commercial Clz, did not induce any toxicity towards MCF-7 cells, and only induced slight cytotoxicity, at higher concentrations for PC-3 cells (but at levels largely below the 50% toxicity resulting from Br-Cla).[121]

In summary, the work by Pinto da Silva *et al*.[121] describes a single-molecule self-activating PS for PDT with tumor-selective anti-tumoral effects. The PS is directly chemiexcited into a triplet excited state via a CL reaction induced by the superoxide anion, a known cancer marker, culminating in the generation of singlet oxygen without the need for other excitation sources. Another factor, the fact that the process is favoured by the acidic pH of tumoral microenvironments, further improves the tumor-selectivity. Additionally, Br-Cla does not require additional catalysts/co-factors, facilitating its delivery process. The fact that it also showed cytotoxicity to several cancer cell lines while being safe towards normal cellular lines, turns Br-Cla into a promising anti-tumoral agent, even more effective than the reference drugs. This single-molecule PS provides a pathway to avoid the limitations associated with the need for an external light source (both in terms of tumor size and localization), providing an alternative PDT that is effective even in deep tissues or non-localized tumors.

Further building up on their work, Pinto da Silva *et al*. reported the syntheses of three new self-activating PSs that, like Br-Cla, worked through the generation of singlet oxygen via a CL reaction activated by the superoxide anion.[122] The performance of these new coelenterazine analogues as antitumor agents was also assessed *in vitro*: they performed better in co-treatment with reference drugs (Tamoxifen and Metformin) for breast and prostate cancers.[122]

The structures of these new single-molecule self-activating PSs (Clz-1, Clz-2, and Clz-3), also based on Clz, are depicted in Figure .[122] Similarly to Clz, the light-free activation of the new PSs is based on the production of triplet states via a CL reaction. Additionally, all three PSs were designed to incorporate bromine heavy atoms, in order to increase the efficiency of intersystem crossing (ISC) and generate more triplet states.[122]

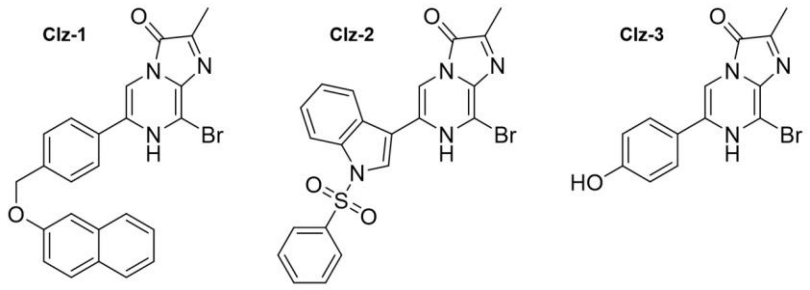


Figure 4. Clz derivatives Clz-1, Clz-2 and Clz-3.

The ability of the superoxide anion for triggering the reaction in the PSs was demonstrated when all the PSs emitted light in the presence of the cancer marker. Among these three PSs, Clz-3 had the best CL, and Clz-1 showed appreciable luminescence. In comparison, Clz-2 had an almost negligible CL.[122] It is worth noting that the intensity of the emission by the PSs was dependent on the amount of potassium superoxide used. Emission by Clz-2 increased with higher potassium superoxide concentrations, whereas emission of Clz-1 and Clz-3 decreased slightly, suggesting they might undergo excessive oxidation for superoxide anion concentrations above a certain threshold.[122] The production of singlet oxygen by the PSs was assessed through fluorescent assays with ABDA, revealing that all the PSs were able to quench the fluorescence of the singlet oxygen sensor. Clz-2 and Clz-3 showed moderate results, quenching around 20-25% of ABDA's fluorescence (for a PS concentration of 50 μM), while Clz-1 achieved quenching values considerably higher than 25%.[122]

The PSs' cytotoxicity towards breast and prostate cancer was evaluated *in vitro*, incubating them with MCF-7 and PC-3 cell lines, respectively.[122] Regarding breast cancer, the PSs require longer incubation times, as the best results were obtained with an incubation period of 72 hours. This agreed with the IC_{50} values obtained by the team (Table 1), which decreased considerably as the incubation progressed. For an incubation of 72 hours, the IC_{50} values of Clz-1 were similar to those presented by Tamoxifen, while those obtained for Clz-2 were up to three times lower than those of the reference drug.[122] Regarding prostate cancer, the PSs performed better than Metformin after 24 hours of incubation, suggesting that the new compounds were very active for a short time period for PC-3 cells.[122] The results showed that the new PSs were cytotoxic towards MCF-7 and PC-3 cancer cell lines, performing even better than the reference drug in some cases. The cytotoxicity towards MCF-10A (non-tumorigenic epithelial cell line) was assessed, with no cytotoxicity being observed, which supported the selective nature of the treatment.[122] Hence, the Clz-based derivatives possess potential to be safely used as PSs in PDT without the need for external light sources.

Table 1. IC_{50} values obtained for Clz-1, Clz-2, Clz-3, and the drugs Metformin and Tamoxifen in MCF-7 and PC-3 tumor cell lines (for treatments of 24 and 72 hours). IC_{50} values (μM) represent a half-maximal inhibitory concentration. * - Approximate estimate due to lack of data points.

	MCF-7 IC_{50}		**PC-3 IC_{50}**	
	24 h	**72 h**	**24 h**	**72 h**
Metformin	N.D.	N.D.	1.270 ± 0.261	0.813 ± 0.261
Tamoxifen	2.219 ± 0.194	11.07 ± 0.02	N.D.	N.D.
Clz-1	>100	12.18 ± 0.06	0.048 ± 0.426	12.11 ± 0.15
Clz-2	47.31*	3.00 ± 0.08	0.388 ± 0.459	1.647 ± 0.366
Clz-3	>100	49.59*	0.530	3.949

		± 0.525	± 0.362

Having observed the selective cytotoxic activity of the compounds towards breast and prostate cancer cell lines, the team continued studying the effects of co-treating these cell lines with the Clz-based PSs and the reference drugs (Tamoxifen and Metformin,).[122] Different concentrations of each PS, along with the reported IC_{50} dose for Tamoxifen (2.22 μM) and Metformin (1.27 μM), were used to co-treat the cells for 24 and 72 hours.

Co-treatment for the breast cancer cell line with an incubation period of 24 hours was the most efficient of all the studied combinations.[122] The most prominent effects were observed with higher concentrations of PSs (more than 10 μM). These results are quite promising, as the co-treatment with PS/drug was more effective than with the PSs themselves, or even than with the reference drug Tamoxifen.[122] Moreover, increasing the incubation time from 24 to 72 hours led to better results, which is in agreement with the IC_{50} for the individual compounds. After 72 hours of incubation, the cytotoxicity for all combinations was higher than that of the individual PSs and reference drugs, with a higher combination efficiency.[122]

Opposite to the co-treatment of breast cancer, co-treatment for prostate tumor cell lines was not very effective for an incubation period of 24 hours when compared to the PSs and Metformin alone.[122] At the very best, the co-treatment could be somewhat more efficient when compared to just Metformin. However, it is worth nothing that the PSs themselves already resulted in good individual responses. As for the incubation time, increasing it to 72 hours did enhance the co-treatment with Clz-3 and Metformin. When referring to higher concentrations of the PS, the combination yielded better results than the individual compounds.[122]

In summary, Pinto da Silva *et al*.,[122] synthesized three Clz-derivatives that presented selective cytotoxicity toward breast and prostate tumor cell lines. The potential of these PSs as co-treatments with the reference drugs was evaluated. For breast cancer, co-treatment yielded better results (for both 24- and 72-hours incubation periods) than the individual components. For prostate cancer (24-hour incubation period) co-treatment did not yield better results than PSs or Metformin alone. Only when the incubation time was increased did the combination of one of the Clz-based PSs with Metformin become better than the individual components.

As a final remark regarding the work, while the use of light-emitting reactions can be considered an alternative to external light sources and solves the issue of light's depth of penetration, the fact that several components have to be delivered to the tumor and co-localized complicates reaching the target. Also, it means that conventional PDT loses its main advantage, its selectivity towards tumor cells (as only irradiated areas undergo photodynamic effect). Last, considering that several energy transfer steps are involved in the process, CL/BL-based multi-component systems tend to present a somewhat low efficiency and reproducibility, which is not acceptable in PDT. The use of a single-molecule self-activating PSs, suggested by Pinto da Silva *et al*.,[121, 122] solves all the issues presented by normal CL/BL-mediated PDT. The use of a single-molecule means that there is only one component that needs to reach the target destination, facilitating treatment delivery. Even though the system does not require external light sources, it still retains its selectivity as the luminescent reaction of the PS is triggered by the

superoxide anion, a known cancer marker overexpressed in tumor cells. The cytotoxicity presented by the Clz-based systems was acceptable for its use in PDT and proved that, when activated, these PSs caused tumor cell death while not damaging normal cells. Furthermore, it was demonstrated that the PSs, which are effective by themselves, could be combined with reference drugs to potentiate the effect of the treatment, thus increasing the efficiency of the therapy.

CONCLUSION

Cancer is a global concern, and the cause of millions of deaths each year worldwide. Several types of therapeutics are employed to battle this condition, such as chemotherapy, radiotherapy, or immunotherapy, among others. One of such therapies is PDT, a modality known for its minimally invasive nature, selectivity towards tumoral cells, and limited side effects. However, the low depth of penetration provided by conventional light sources makes PDT effective only when considering more superficial tumors.

To solve this limitation, researchers have focused on creating strategies to improve the efficacy of PDT and allow it to be used in deeper tumors and even metastatic cancer. Chemiluminescence (CL)/bioluminescence (BL) reactions were exploited as alternative internal excitation sources for PDT. In these systems, the CL/BL reaction was used to excite the PS, eliminating the need for an external light source. However, the use of CL/BL reactions to excite a PS also has its limitations: these systems require different components to be present in the same cellular location; the efficiency of the energy transfer is limited given the participation of several components (which limits the efficacy of the photodynamic effect). The biggest advantage of PDT, its selectivity, is lost as most CL/BL reactions are not intrinsically tumor-selective. Even obtaining considerable cytotoxicity towards cancer cells, the residual toxicity towards normal cells (in some cases), the lack of selectivity and reproducibility, and the low efficiency in the energy transfer to the PS, ultimately mean that multi-component CL/BL-based PDT is not yet suitable for clinical application.

Because of the aforementioned limitations for CL/BL-based multi-component systems, new systems consisting of a tumor-selective and self-activating single-molecule PSs are being developed. These systems are based on the CL reaction of marine Coelenterazine (Clz). Without the need for a light source, the new PSs are chemiexcited directly into triplet states due to a CL reaction triggered solely by a cancer marker, which subsequently sensitizes the highly cytotoxic singlet oxygen. The use of a single-molecule system means that only one component needs to be delivered to the target area, facilitating treatment delivery. By being only activated by a cancer marker, these systems are intrinsically tumor-selective, meaning that even though the system is not activated by localized light irradiation it is still selective and thus cytotoxic only towards cancer cells. The cytotoxicity towards tumor cells is enough for the PS to be considered useful for clinical applications, while there is virtually no damage done to normal cells. The effectiveness of the system is comparable to that of reference drugs, and co-treatment with both potentiates the effects, leading to more effective treatment for the patient. Thus, these novel single-molecule PSs show great potential for

eliminating the light-related restrictions regarding tumor size and location that PDT currently presents, while maintaining its most relevant advantages (selectivity, minimally-invasive nature, and broad scope).

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