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REVIEWS

Latest trends on photodynamic disinfection of Gram-negative bacteria: photosensitizer's structure and delivery systems

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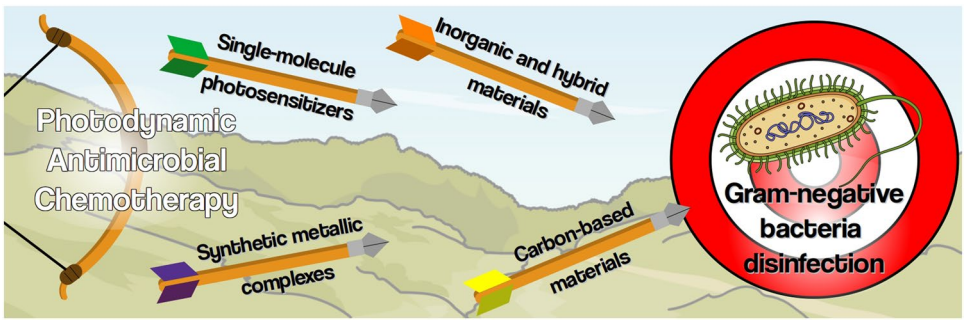
Abstract

Antimicrobial resistance is threatening to overshadow last century's medical advances. Etiological agents of previously eradicated infectious diseases are now resurgent as multidrug-resistant strains, especially for Gram-negative strains. Finding new therapeutic solutions is a real challenge for our society. In this framework, Photodynamic Antimicrobial ChemoTherapy relies on the generation of toxic reactive oxygen species in the presence of light, oxygen, and a photosensitizer molecule. The use of reactive oxygen species is common for disinfection processes, using chemical agents, such as chlorine and hydrogen peroxide, and as they do not have a specific molecular target, it decreases the potential of tolerance to the antimicrobial treatment. However, light-driven generated reactive species result in an interesting alternative, as reactive species generation can be easily tuned with light irradiation and several PSs are known for their low environmental impact. Over the past few years, this topic has been thoroughly studied, exploring strategies based on single-molecule PSs (tetrapyrrolic compounds, dipyrrinate derivatives, metal complexes, etc.) or on conjunction with delivery systems. The present work describes some of the most relevant advances of the last 6 years, focusing on photosensitizers design, formulation, and potentiation, aiming for the disinfection of Gram-negative bacteria.

Graphical abstract



Keywords Disinfection · Antibacterial alternatives · Photosensitizers · Delivery systems

1 Introduction

Antibiotics can be defined as small molecules with an antibacterial effect. Some of the first examples of antibiotic molecules were described at the beginning of the twentieth century, representing a cornerstone in the development of modern medicine. And although antibiotics may feel as a human achievement, antibiotics have been used by microorganisms as weapons in a chemical war fought since

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prehistoric times, aiming for the survival of the fittest and the control of nutrients [1]. As a counterpart to antibiotics, bacteria have developed resistance mechanisms that can be inherent to their physiology (i.e., thick cellular membranes and walls) or that are addressed for the resistance to a specific antibiotic (i.e., β-lactamic antibiotics degradation by β-lactamases enzymes). Although the transmission of genes related to antibiotic resistance is a possible natural phenomena, it is rare for it to happen in nature without selective pressure. However, the selective pressure in the environment, resulting from the widespread and thoughtless use of antibiotics in clinic and agriculture, has promoted the spread and propagation of bacteria resistant to antibiotics [2]. Furthermore, it has been found that bacteria are acquiring resistance to multiple antibiotics, especially on more challenging environments, such as hospitals and sewage water [3].

Although conventional antimicrobial drugs were described as "magic bullets" in the twenty first century, the increase of antimicrobial drug resistance requires a major and rapid intervention, to keep up with the medical advances developed so far [4]. Fighting antimicrobial resistance (AMR) has been recognized as a priority for public health, including food supply [5, 6]. The number of microorganisms exhibiting AMR, especially resistance to multiple antibiotics, has continued to increase in Europe. An estimation has predicted that deaths caused by antimicrobial resistance could rise from approximately 700,000 deaths a year to close to 10 million deaths per year by 2050, with a cumulative cost of 100 trillion US dollars [5, 7]. Alternative and more efficient antimicrobial strategies are urgently needed, especially against "ESKAPE" superbugs (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) [8]. In 2017, the World Health Organization (WHO) published a list of antibiotic-resistant priority pathogens (Table 1) [9], most of which are Gram-negative bacterial pathogens. Due to their thick impermeable outer membrane, Gram-negative bacteria are more resistant to the diffusion of small molecules than Gram-positive bacteria, resulting in an enhanced resistance to chemotoxic antibiotics. Furthermore, several Gram-negative bacteria have efflux pumps that prevent antibiotics to reach an effective concentration inside the cell [10].

Since resistance in bacteria is often mediated by plasmids carrying multiple resistance genes, treatment is frequently restricted to second-line and third-line antibiotics with high toxicity or poor efficacy, which causes patients harm and increases health care costs. Nowadays, the pipeline of chemotoxic antibiotics discovery and development is constrained to around 45 molecules which are under different phases of clinical trials, from which only one focuses in a new molecular target [1]. Then, although several molecules could reach clinical use, history so far has showed us that bacteria quickly adapt and overcome the new antibiotic agents, especially when the molecular target and the molecular mechanism are similar to already existing antibiotics. One of the best alternatives is the use of antibiotics cocktails, with two or more antibiotics with different molecular targets. One variation of this alternative would be the use of a single molecule with multiple molecular targets in bacteria [11]. As an example of an antibiotic alternative without specific molecular target, is the Photodynamic Antimicrobial ChemoTherapy (PACT) [12], which sometimes is referred as PhotoDynamic Inactivation (PDI) or as antimicrobial PhotoDynamic Therapy (aPDT).

Photodynamic therapy (PDT) requires the simultaneous use of a chromophore molecule, called photosensitizer (PS), oxygen, and light. Upon light irradiation, the ground

Table 1 List of antibiotic-resistant pathogens, against which the WHO recommends to focus the antibacterial research

Priority	Bacteria strain	Antibiotic resistance	Bacteria type
1. Critical	*A. baumannii*	Carbapenem-resistant	Gram-negative
	P. aeruginosa	Carbapenem-resistant	Gram-negative
	Enterobacteriaceae	Carbapenem-resistant, Extended spectrum β-lactamase (ESBL)-producing	Gram-negative
2. High	*E. faecium*	Vancomycin-resistant	Gram-positive
	S. aureus	Methicillin-resistant, vancomycin-intermediate and resistant	Gram-positive
	Helicobacter pylori	Clarithromycin-resistant	Gram-negative
	Campylobacter spp.	Fluoroquinolone-resistant	Gram-negative
	Salmonellae	Fluoroquinolone-resistant	Gram-negative
	Neisseria gonorrheae	Cephalosporin-resistant, fluoroquinolone-resistant	Gram-negative
3. Medium	*Streptococcus pneumoniae*	Penicillin-non-susceptible	Gram-positive
	Haemophilus influenzae	Ampicillin-resistant	Gram-negative
	Shigella spp.	Fluoroquinolone-resistant	Gram-negative

Gram-negative bacteria are underlined, showing their prominence among the list [9]

state PS (^{0}PS) gets into an excited singlet state (^{1}PS*). At this state, the ^{1}PS* can go back to the ground state either through short-lived fluorescence emission or through inter-system crossing, turning into an excited triplet state (^{3}PS*). The short-lived excited triplet state PS (^{3}PS*) can return to the ground state, either through phosphorescence emission (half-life up to 1 s) or through two different mechanisms. In the Type-I mechanism, ^{3}PS* transfers an electron to a reducible substrate (i.e., the lipidic components of cellular membranes), forming a short-lived radical PS (^{3}PS$^{\cdot-}$) that easily reacts with oxygen, leading to a cascade of reactive oxygen species (ROS), as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), or the hydroxyl radical ($OH^{\cdot}$). In contrast, in Type-II mechanism, the ^{3}PS* reacts directly with ground state molecular oxygen, with molecular oxygen being naturally in the triplet state ($^3O_2$). Both triplet species react and annihilate themselves, and through an energy transfer, the ground triplet state molecular oxygen forms a singlet oxygen reactive species ($^1O_2^*$). Then, although in both mechanisms oxygen is involved, Type-I depends on the intimate contact with the substrate, while Type-II depends on the availability of molecular oxygen into the media (Fig. 1). Both mechanisms produce ROS that induce the photo-oxidation of biomolecules, such as nucleic acids, lipids, and proteins, leading to eventual cell death [13].

The first report of a photodynamic effect was precisely an antimicrobial one, where Raab casually observed that acridine dyes and eosin affected the viability of *Paramecium caudatum*, a protist, when irradiated with light [14]. This serendipitous observation pushed the investigation of the usage of PDT as a treatment for cancer and tumors. However, the emergence of antibiotic molecules at the beginning of the XXth century halted the use of PACT as an antimicrobial alternative. One hundred years later, the emergence of MultiDrug-Resistant (MDR) bacteria has pushed the resurgence of PACT for the treatment of infections [15–18]. Because of the high reactivity of ROS, light-activated PS are able to neutralize bacteria and bacterial virulence factors, as demonstrated by Shrestha and collaborators, where chitosan nanoparticles loaded with Rose Bengal (RB) were able to inhibit the collagenase of *Clostridium difficile* biofilm [19], and the endodotoxins and lipopolysaccharides of *P. aeruginosa* [20], just to mention a well explored example of a photodynamic system. Then, PACT is not only able to decrease the survival of pathogenic bacteria but also able to decrease their pathogenicity and virulence. The speed of ROS production, and consequently, killing bacteria rate, together with the non-specific molecular target of ROS, is likely to decrease the development of resistance toward PACT [21, 22]. This is just one of the advantages of using PACT as an alternative for bacterial disinfection, as PACT is also able to cause localized impact on a site of infection thanks to a local irradiation. However, the need of light irradiation could be a double-edged sword, as some systemic infections (i.e., septicemia) are unlikely to be comfortably reached by light. Nevertheless, there are several potential environments where PACT could replace or complement conventional antimicrobial therapies (Fig. 2, [23]), not only limited to bacterial infections but also to fungal, viral,

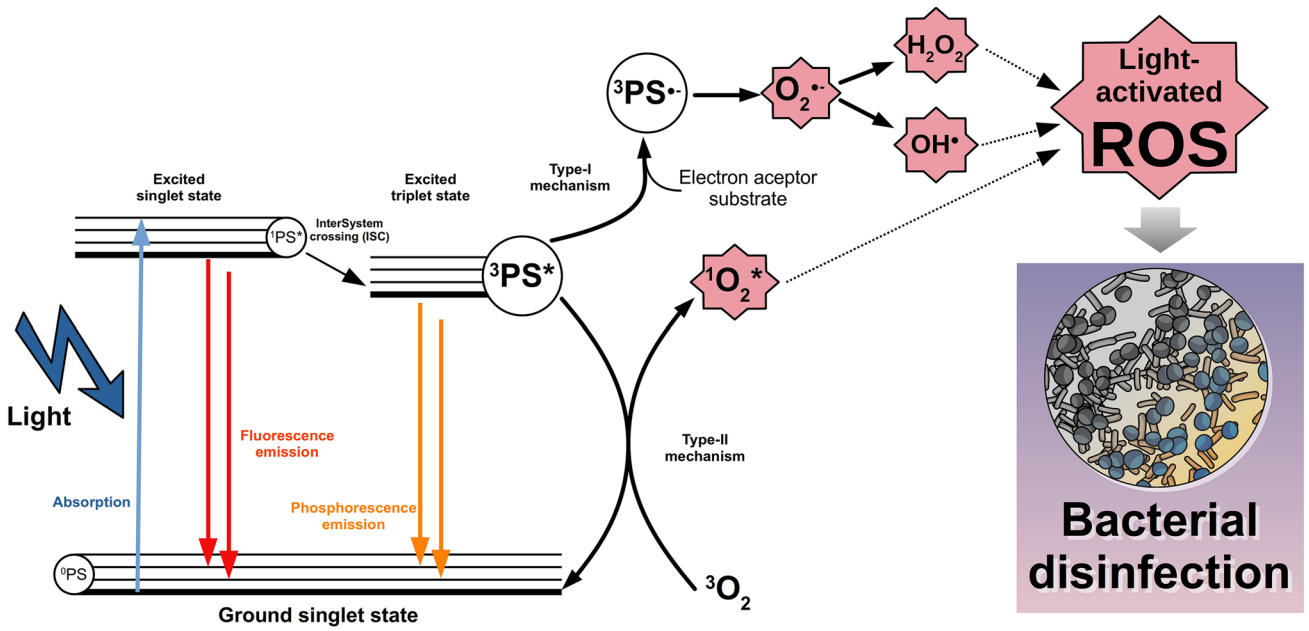

Fig. 1 Illustration of the photochemical mechanisms of different reactive oxygen species (ROS) produced during photodynamic action

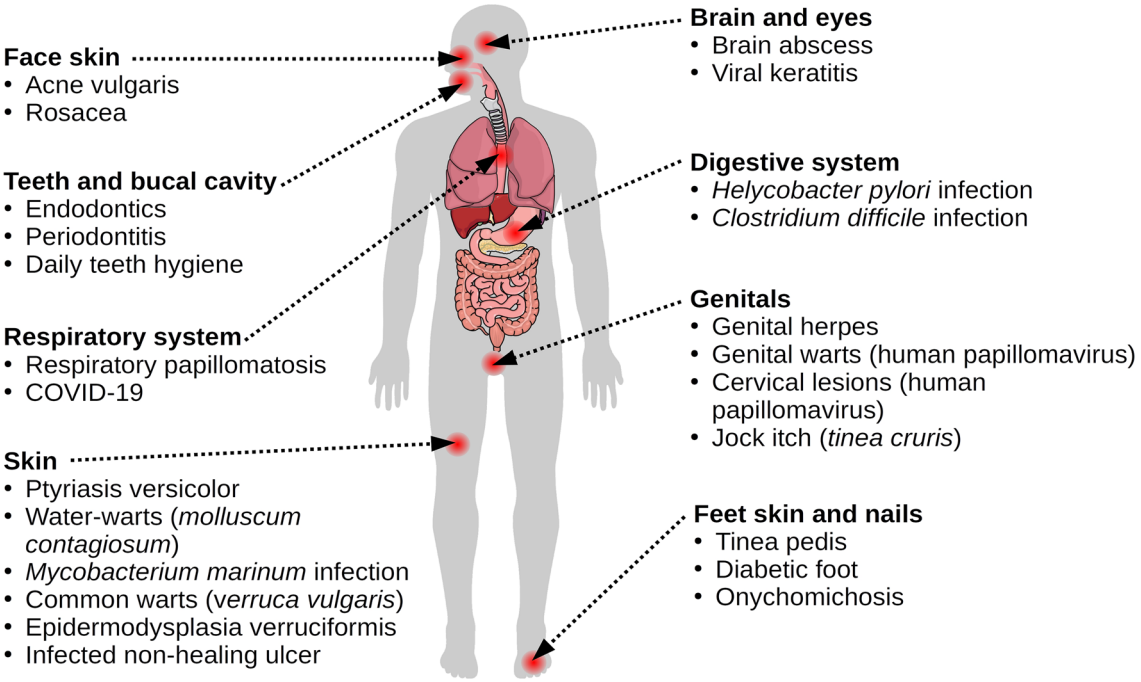

Fig. 2 Localized sites of infection, which could be potentially treated by PACT

and parasitic infections, which in advance are summarized in some late reviews [24–26].

Since the development of the initial antibacterial treatments, it was shown that bacteria had different susceptibilities toward chemotherapy, which relies on the physiology of bacteria. At the beginning of the "golden age" of antibiotics discovery, it was noted that penicillin was only efficient against Gram-positive bacteria, such as *Staphylococcus* and *Streptococcus* species, while remaining inefficient against Gram-negative bacteria, such as *Salmonella* and *Vibrio cholerae*, observation that turned the antibiotics discovery efforts toward more efficient molecules against Gram-negative bacteria [27]. Microbiologists usually divide bacteria based on the result of a dying process, namely Gram stain. In this dying process, bacteria are exposed to crystal violet, followed by a stringent washing process, and a second dying process with safranin. Gram-positive bacteria are able to retain crystal violet and present a deep purple color, while Gram-negative bacteria are unable to retain the crystal violet dye while retaining the safranin dye, appearing reddish under microscope. Crystal violet dyes the peptidoglycan cell wall, with the differential staining resulting from the thick peptidoglycan cell wall present in Gram-positive bacteria, while Gram-negative bacteria possess a thin peptidoglycan layer that easily washes away crystal violet. Furthermore, Gram-positive bacteria peptidoglycan is anchored to the cytoplasmic membrane by lipoteichoic acids, which confers negative charged moieties to the exterior of the bacterial envelope. Gram-negative bacteria's thin peptidoglycan wall is found between two cellular membranes, with the outer membrane being heavily decorated with negatively charged lipopolysaccharides (LPS). They prevent the passive diffusion of small molecules, relying on porin channels for the transport of molecules through the outer membrane (Fig. 3). Then, antibiotics require to be small, polar, and cationic compounds, which are able to interact with LPS to increase the chances to be transported through the outer membrane [28]. PSs used in PACT are not exempt to these solubility and charge challenges, and in the following sections we will explore some of the emerging alternatives toward Gram-negative disinfection, which, interestingly, not only relies on the presence of cationic charged molecules but also on delivery systems and the use of adjuvants to favor the permeation into the outer membrane of bacteria. It must be highlighted that most of the photosensitizers are tested both in the dark and under light irradiation, and researchers put special care to find molecules with a good photosafety window, where the effective concentration in the dark is higher than the effective concentration under light irradiation. Then, the toxicity of the photosensitizers and formulations hereby presented are not further discussed.

2 Photosensitizers structure

Most of the research efforts poured into PACT current pipeline still explores the effects of structural changes into the efficiency of the PSs. Although there is a wide variety of PS

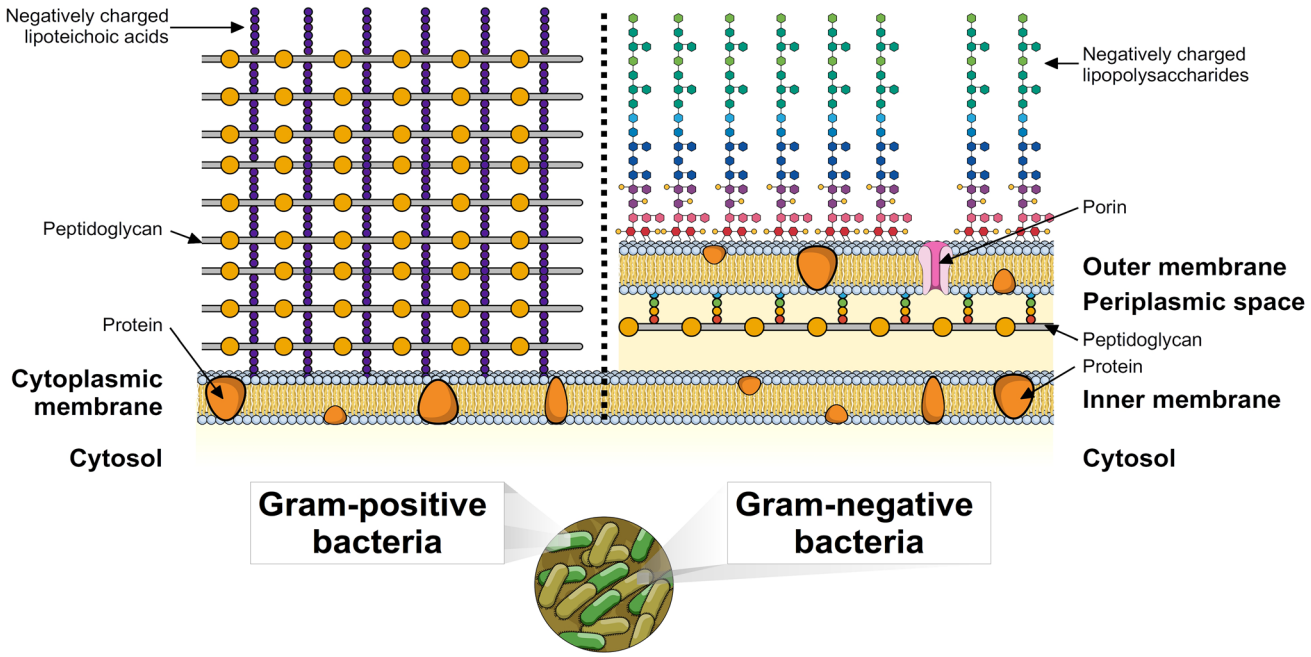

Fig. 3 Comparison of the cellular envelopes for Gram-positive and Gram-negative bacteria

structures, undoubtedly, there are some PS structures that are more popular than others. Some of them are the tetrapyrrolic derivatives, which are planar compounds, bearing 18 π electrons. Their core structure is susceptible to be modified, especially at their *meso* and *β* positions, which has allowed researchers to tune the chemical and photophysical properties of the desired compounds. The best known example of the tetrapyrrolic compounds is the porphyrin **1** (Fig. 4). Porphyrins, which name derives from the greek word *porphyra* (purple), are widely found in the nature, being key in life-sustaining processes, such as oxygen transport, photosynthesis, electron transfer, and iron scavenging. Although the aromatic core of the tetrapyrrolic compounds remains unchanged, in order to preserve the aromaticity and their photophysical properties, the reduction of one of the peripheral double bonds leads to the chlorin family **2**, while the reduction of both double bonds leads to the core structure of a bacteriochlorin **3**. The reduction of these bonds has consequences in their photophysical properties of the compounds, with chlorins and bacteriochlorins being more susceptible to absorb red light (600–800 nm) [29]. Besides these naturally occurring core structures, there are also some porphyrin-like synthetic compounds. One of the best known examples is the core structure of phthalocyanines **4**, which are intense blue-green compounds, with 18 π electrons and strong absorbance of red light (600–800 nm); however, phthalocyanines are prompter to aggregate than other tetrapyrrolic compounds [30, 31]. Lately, in the last few decades, a new core structure has attracted the attention of researchers, namely, the BODIPY (**5,** 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) derivatives, presenting advantageous characteristics, such as high visible light absorbance, resistance to photobleaching, and high light–dark toxicity ratios. However, they also present high fluorescence, and thus, low triplet state quantum yields, which researchers had solved through placing heavy atoms in the BODIPY core [32]. The differences in the structures of the PS are reflected in how they absorb light, as represented in Fig. 4. This entitles the use of specific wavelengths, while not preventing the use of white light, for the excitation of the PS, which has important repercussions in the experimental development of PACT.

As a side note, through the following sections, whenever the term "efficient inactivation" or similar is used, it refers to a decrease of at least 99.9% of bacterial survival or 3-log reduction. A technical resume of the described examples, along with relevant information (i.e., concentration of the PS, light source, light fleece, light dose, etc.), is summarized in Supplementary Material.

2.1 Cationic photosensitizers

Most of the antibacterial pipelines focuses on the development of novel cationic molecules [37], with this trend being valid for PACT photosensitizers as well. In this aspect, cationic PSs have been thoroughly explored as efficient PACT agents for the eradication of Gram-negative bacteria, while also remaining efficient against Gram-positive bacteria. As both types of bacteria have negatively charged moieties, the cationic charge promotes the binding to the bacterial envelope, which results in critical in the case of Gram-negative

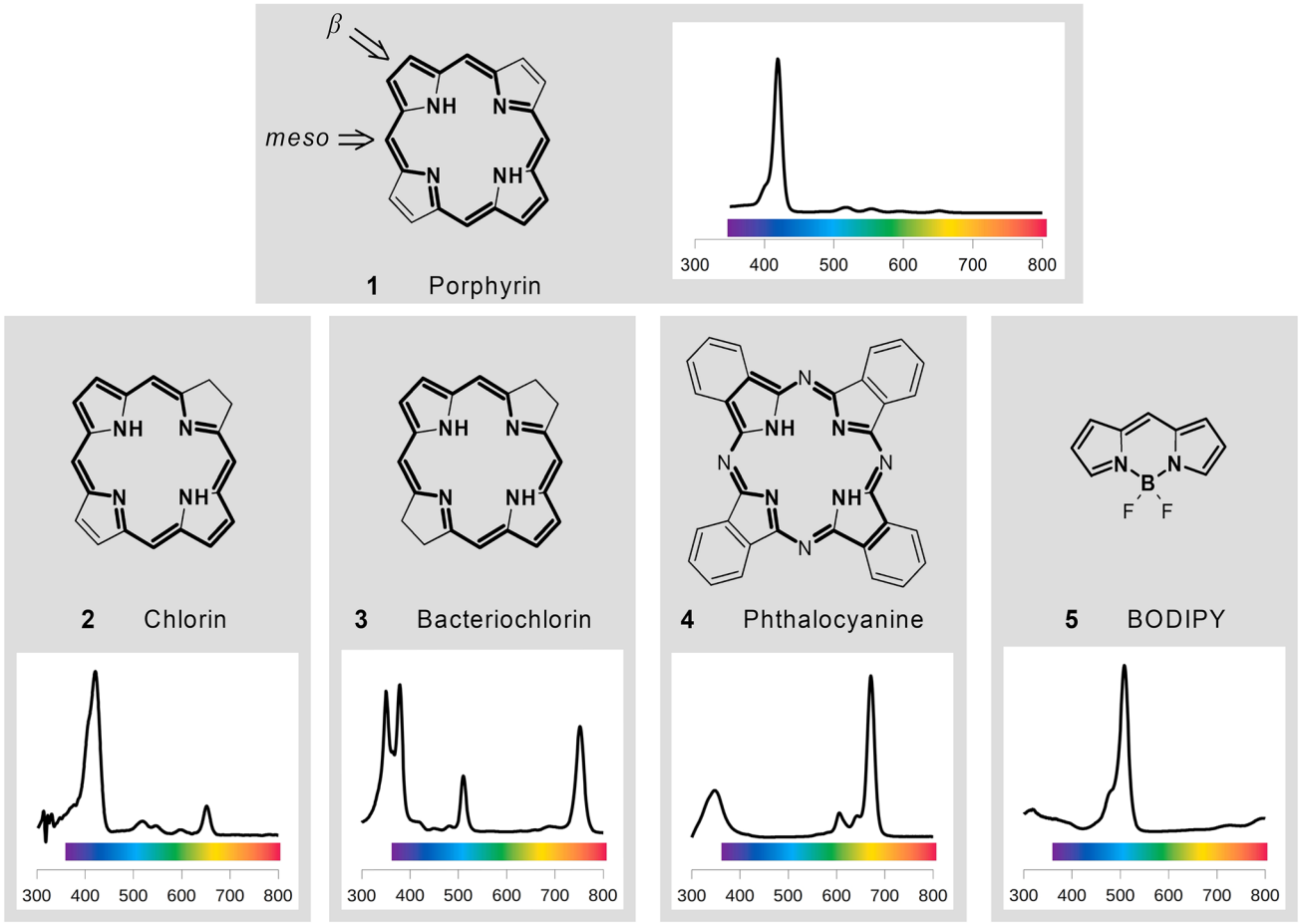

Fig. 4 Core structure of the most prominent PSs families, along with a representative UV–Vis absorbance spectrum [33–36]

bacteria, where small molecules cannot diffuse through the thick outer membrane. This has led to an extensive library of different PSs, with different core structures, as porphyrins, phthalocyanines, chlorins, bacteriochlorins, and others (Supplementary Material 1).

2.1.1 Cationic porphyrins

Porphyrin's relative easiness of synthesis and chemical tuning has provoked a huge number of relevant examples in the use of cationic porphyrins, understandably, relying in the presence of tertiary amines derivatives. One of the best studied examples of cationic porphyrins is **TMPyP** (**6**, Fig. 5), from which several applications are found in literature. In one of the most recent examples using **6**, and compared with its zinc(II) derivative **7**, Seeger et al. demonstrated efficient photo-inactivation against model *Proteus mirabilis* and *P. aeruginosa*, but also against their respective clinical isolates from canine otitis, as a result of light-driven production of singlet oxygen [38]. Although normally the non-metalated **TMPyP** or its zinc(II)-metalated derivative are analyzed, Skwor et al. have devoted to the analysis on the influence of the metalation (Cu^{2+}, Fe^{2+}, Pd^{2+}, and Zn^{2+}) in the antibacterial activity, finding that **8**, **TMPyP** derivative metalated with palladium(II), was the most efficient at eradicating *E. coli* [39], evidencing the upmost importance of the metal used, and consequences on the photo physics of the PS. Indeed, whereas copper and iron induce prompt quenching of the excited state, palladium(II) enhances spin–orbit coupling, and thus conversion to the triplet state and ultimately in fine singlet oxygen generation.

Some studies present evidence that the number of charges is not the only parameter that impacts the efficiency of a photosensitizing molecule. As an example, Calmeiro et al., synthesized a library of compounds that differ into the position of the cationic charge and their free-base state. Then, cationic porphyrins **9** and **10**, along with their zinc(II) derivatives **11** and **12** (Fig. 5), were successfully tested by Calmeiro et al. against *E. coli*, with its antimicrobial activity resulting enhanced by the presence of KI 100 mM (for further details on PACT potentiation effect due to inorganic salts, see Sect. 4.3, vide infra) [40]. Furthermore, a complete

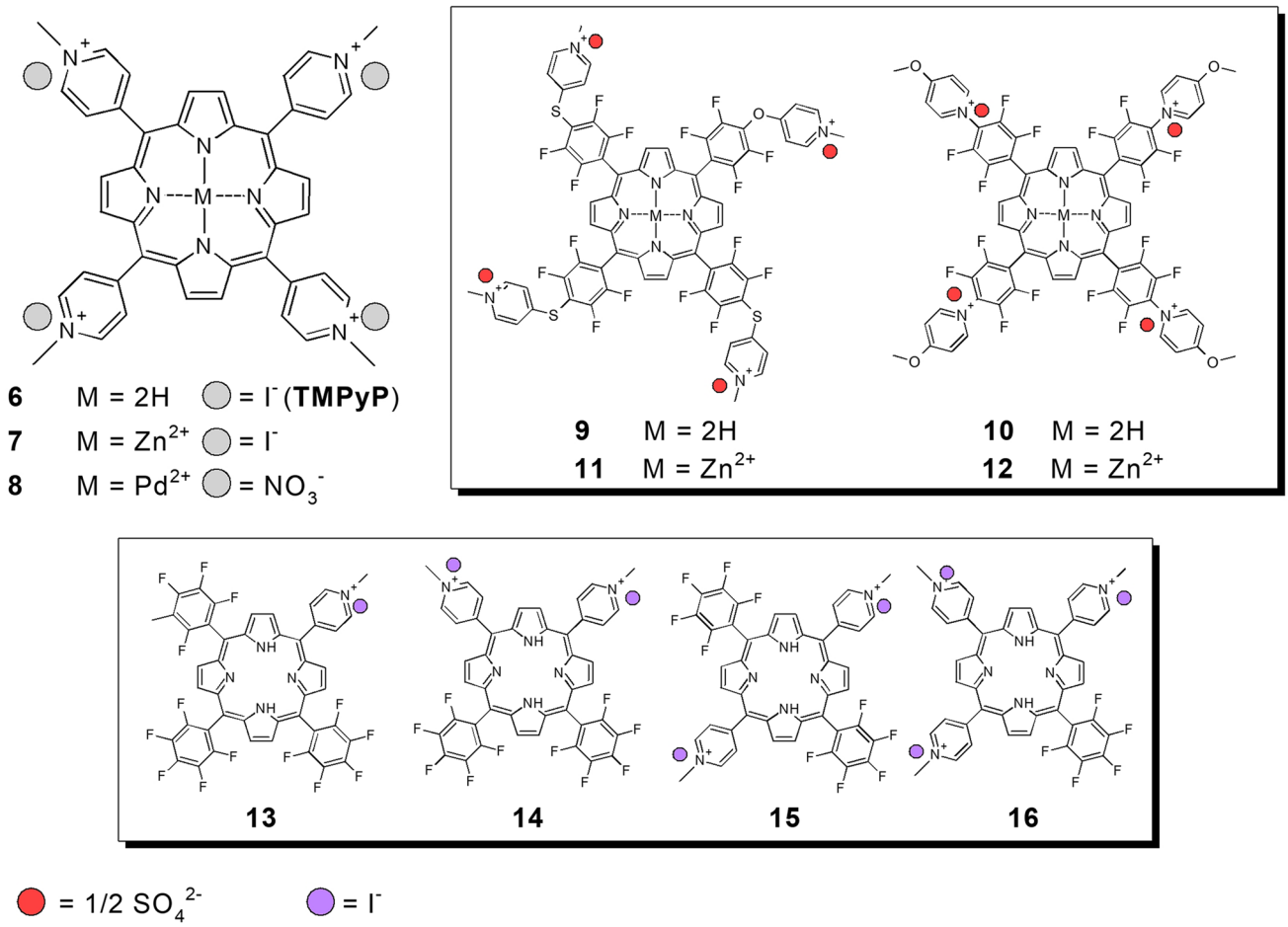

Fig. 5 Structure of some cationic porphyrins presented along this work

family of porphyrins bearing either non-cationic phenyl moieties or methylpyridinium-4-yl moieties (**13–16**, Fig. 5) was tested against *E. coli* cells. Interestingly, the compound **16**, with three cationic charges and a single pentafluorophenyl, was found to be the most efficient PS against *E. coli*, showing that other parameters, such as the charge number and distribution of the charges, must be taken into account for the design of novel PSs [41]. On regard of the efficient results obtained with **16**, it was further used by Marciel et al. for the efficient disinfection of *E. coli* in plasma and blood, with low evidence of hemolytic activity toward erythrocytes [42].

In another approach, several cationic *o*-pyridil porphyrins (**17–120**, Fig. 6) were compared, testing the influence of the alkyl's length chain on *E. coli* disinfection, where it was found that the compound **20,** bearing an alkyl chain with eight carbons, decreased the bacterial viability in around 97%, while compound **17** was only able to reduce bacterial viability by 20% [43, 44]. These results were compared with similar iron(III)-metalated porphyrins [45], obtaining similar results and bearing the same conclusions: for Gram-negative inactivation not only charges are needed but also lipophilicity is desirable. Then, we could hypothesize that the cationic charges ensure a tight interaction with the negatively charged LPS, while the lipophilicity ensures the penetration through the two cellular membranes; then, a highly cationized molecule could be bound tightly to the outside of the cell, preventing its internalization and preventing its photodynamic effect, as ROS are unable to diffuse through the bacterial envelope. Then, an ideal PS must have an equilibrium between its cationic charges and its hydrophobic nature.

As it is unlikely that current research has achieved the ultimate PS, several works aim to increase the structural diversity of the porphyrinic systems, aiming also for β-substituted porphyrins. Namely, Moura et al. presented four cationic porphyrins (**21–24**, Fig. 6), bearing imidazole units at a β-pyrrolic position, and analyzed their efficiency against bioluminescent *E. coli,* successfully reducing its bacterial density [46]. The results showed that only **27**, **23,** and **24** were able to decrease the bacterial survival of *E. coli,* although it was observed that **21** was a better $^1O_2$ producer than **24**. Further analysis leads to the conclusions that **21**'s

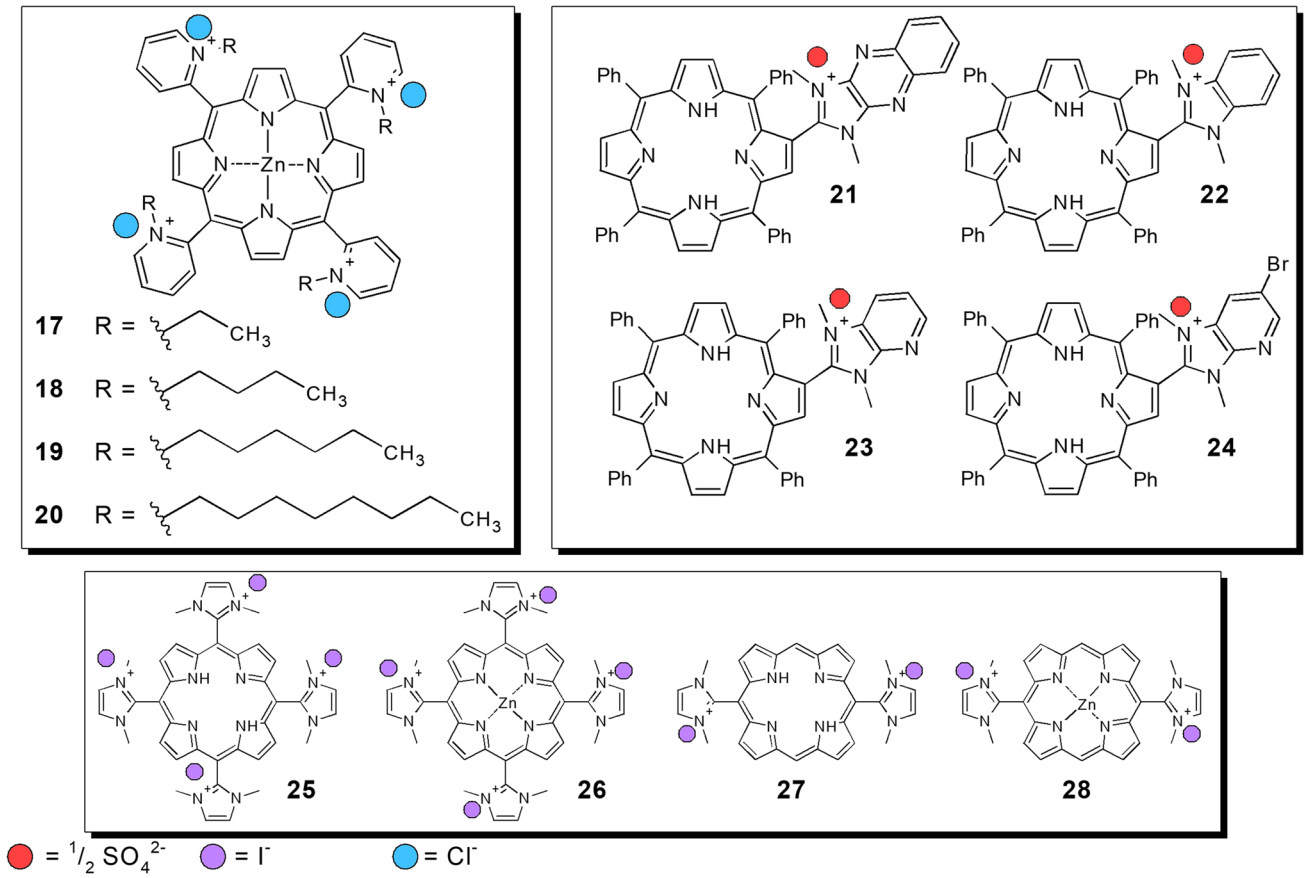

Fig. 6 Different structures of cationic porphyrins

lack of biological activity could be due to its higher hydrophobic nature, which could prevent its proper solubilization or even its interaction with the cellular membrane. In another example, Vinagreiro et al., focused on porphyrins *meta*-substituted with 1-methyl-imidazol-2-yl, synthesizing compounds **25** to **28** (Fig. 6). These compounds were successfully tested against model bacteria *E. coli* and *P. aeruginosa*, and given the efficient results obtained with compounds **26** and **28**, they were further successfully tested against multidrug-resistant *A. baumannii, K. pneumoniae,* and *E. coli* [47].

2.1.2 Cationic phthalocyanines and subphthalocyanines

Porphyrins are not the only PSs aimed for the inactivation of bacteria. Phthalocyanines are widely used against bacteria proliferation, with comprehensive reviews being recently published by Galstyan [48] and by Ribeiro [49]. In the present section we aim to present some of the most representative advances on the use of phthalocyanines against Gram-negative growth.

Returning to the previous investigation of Marciel et al. [42], the cationic phthalocyanine **29** (Fig. 7), bearing eight cationic charges, was used for blood disinfection. When researchers compared the obtained results of **16** (Fig. 5) and **29** (Fig. 7), although both PSs were able to efficiently decrease the bacterial concentration in PBS, when challenged in complex media (i.e., plasma or complete blood), the cationic porphyrin was more efficient than the cationic phthalocyanine, an effect attributed to the phthalocyanines tendency to aggregate in aqueous media, despite its eight cationic charges [42].

In another approach, Lourenço et al., developed four cationic phthalocyanines (**30–33**, Fig. 7), with four to sixteen cationic charges, which were tested against bioluminescent *E. coli* and *E. coli* biofilm, under white and red light. The best results were obtained under red light irradiation, for both planktonic and biofilm cells. Interestingly, the best result appears to be with the phthalocyanine bearing a single peripheral substitution and 8 cationic charges (**32**). This is interesting when compared with a bulkier phthalocyanine, bearing a double peripheral substitution and 8 cationic charges (**31**), which although having the best cellular uptake is unable to reach the efficiency of **32**. Furthermore, the methylated derivative **33**, bearing a double peripheral substitution and 16 cationic charges, although better than

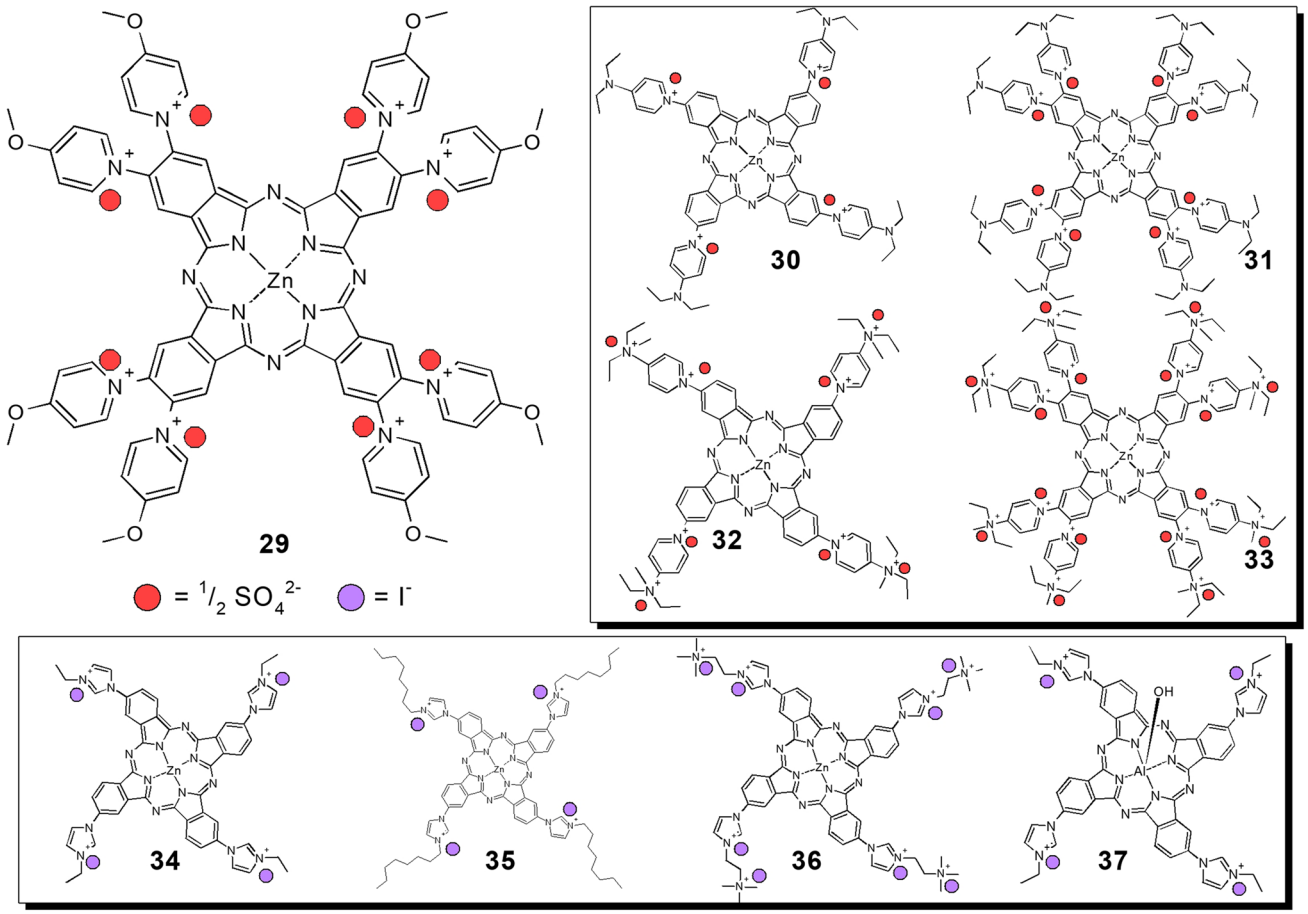

Fig. 7 Cationic phthalocyanines discussed along this work

31, is not better than **32**, which could be attributed to the size of the molecule, but also to a tight binding to the LPS, which could prevent its internalization into the cell [50]. In a similar effort, Aroso et al. prepared and analyzed the effect of the number of charges, the length of the alkyl chain, and the nature of the metal complexes (**34–37**, Fig. 7). The best antimicrobial effect against *E. coli* and *P. aeruginosa* was found with **34**, a zinc(II) phthalocyanine bearing four cationic charges and a two-carbon alkyl chain, in agreement with previous experiments where "less is more" [51].

In another example, Mantareva et al., prepared two lutetium(III) acetate cationic phthalocyanines and tested them against a strain of *P. aeruginosa*. The phthalocyanines differed in the position of the methylpyridinium-4-yl moieties, being it in the non-peripheral (**38**) or in the peripheral position (**39**, Fig. 8). This difference appears to be decisive, as the non-peripheral substituted phthalocyanine was an efficient photobactericide molecule, while the other only showed a modest reduction in bacterial survival [52]. In a related work, Dlugaszewska et al. prepared a magnesium(II) phthalocyanine (**40**, Fig. 8), bearing eight cationic moieties in the non-peripheral positions, which was able to disinfect planktonic bacteria of *E. coli*, *P. aeruginosa,* and *Serratia marcescens* under near-infrared light irradiation, but failed to destroy mature biofilm from the same bacteria [53].

Most of the differences found for several phthalocyanine compounds are due to the phthalocyanines tendency to aggregate in aqueous media. To overcome this, Ruiz-Gonzalez et al., prepared four cationic dendrimeric phthalocyanines, bearing either zinc(II) (**41** and **42**, Fig. 8) or ruthenium(III) (**43** and **44**, Fig. 8). Interestingly, it was found that the zinc-metalated phthalocyanines had a best performance against *E. coli,* under light irradiation at 635 nm [54]. The presence of hydroxyl moieties could prevent the aggregation of the phthalocyanines, as Meerovich et al. proposed with an octa-cholinyl substituted phthalocyanine (**45**, Fig. 8), which was able to successfully decrease the bacterial survival of *P. aeruginosa* planktonic cells, and biofilm structures, after red light irradiation [55]. Also, Lin et al. developed a monosubstituted phthalocyanine (**46**, Fig. 8), which was efficient at the inactivation of *E. coli*, needing only 8 µM of the compound to achieve a 5-log reduction [56].

Then, cationic phthalocyanines are a good alternative for PSs against Gram-negative bacteria, as demonstrated

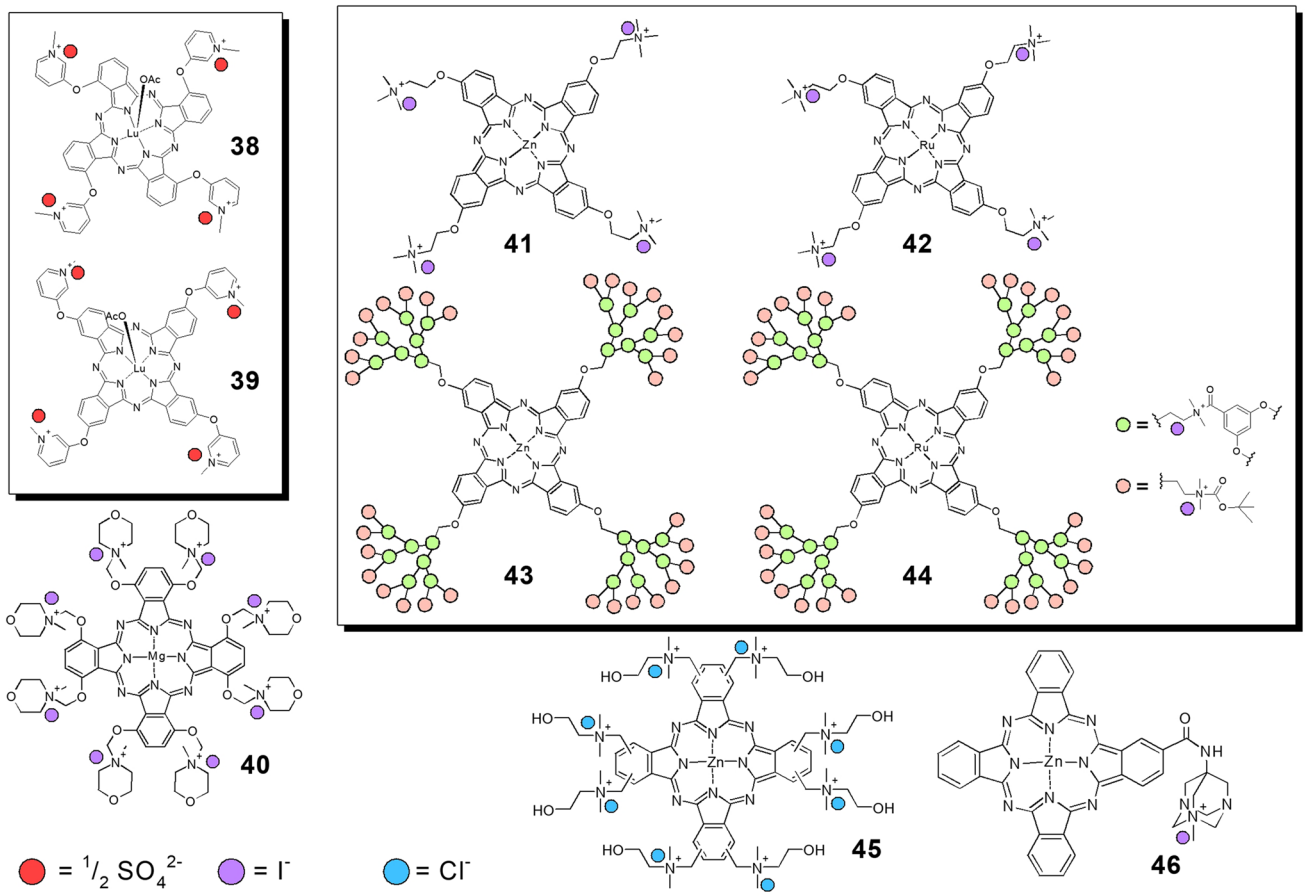

Fig. 8 Cationic phthalocyanines presented along this work

through several literature examples. New examples should consider not only the presence of cationic charges but also the presence of hydrophilic moieties to prevent aggregation in aqueous media, a factor that seems particularly detrimental for these compounds.

2.1.3 Cationic chlorins and bacteriochlorins

Although less common, there are some examples on the use of cationic chlorins and bacteriochlorins against Gram-negative growth. As an example, Meerovich et al., tested two different bacteriochlorins (**47** and **48**, Fig. 9) against *P. aeruginosa* planktonic cells and biofilm cultures. Both symmetric bacteriochlorins were able to efficiently reduce the bacterial survival of planktonic cells, while it required higher concentrations and higher light doses to eradicate biofilm cultures [55]. In the same work, Meerovich et al. compared the efficiency of these bacteriochlorins against the cationic phthalocyanine **45** (Fig. 8), evidencing that the bacteriochlorins were more efficient under similar conditions, possibly due to the difference of size, which permits smaller molecules to diffuse easily through Gram-negative membranes. In another example, Ziganshyna et al. tested a commercial bacteriochlorin tetrahydroporphyrin-tetratosylate **49** (Fig. 9), against several drug-resistant strains of Gram-negative bacteria, demonstrating a good inactivation, although high concentrations and high light doses (from a laser or a LED-source) were used [57].

2.1.4 Cationic BODIPY

BODIPY are pyrrolic derivatives which are easily synthesized and chemically tuned, which are under current intense research as efficient PSs for photodynamic therapy [58]. In a recent example, two cationic BODIPY (**50** and **51**, Fig. 9) were synthesized and tested against *E. coli* planktonic cells, achieving an efficient inactivation. Although structurally different, these two BODIPY's had similar effectivity, decreasing bacterial viability by ~ 2.5 logs. However, when 50 mM of KI was added to the mixture, the BODIPY with a choline moiety (**51**) was clearly the most efficient PS, decreasing bacterial viability by 7.5 logs, while **50** and 50 mM KI were only able to reduce it by 3.5 logs, when irradiated with white light [59]. After the successful results of **51**, it was analyzed

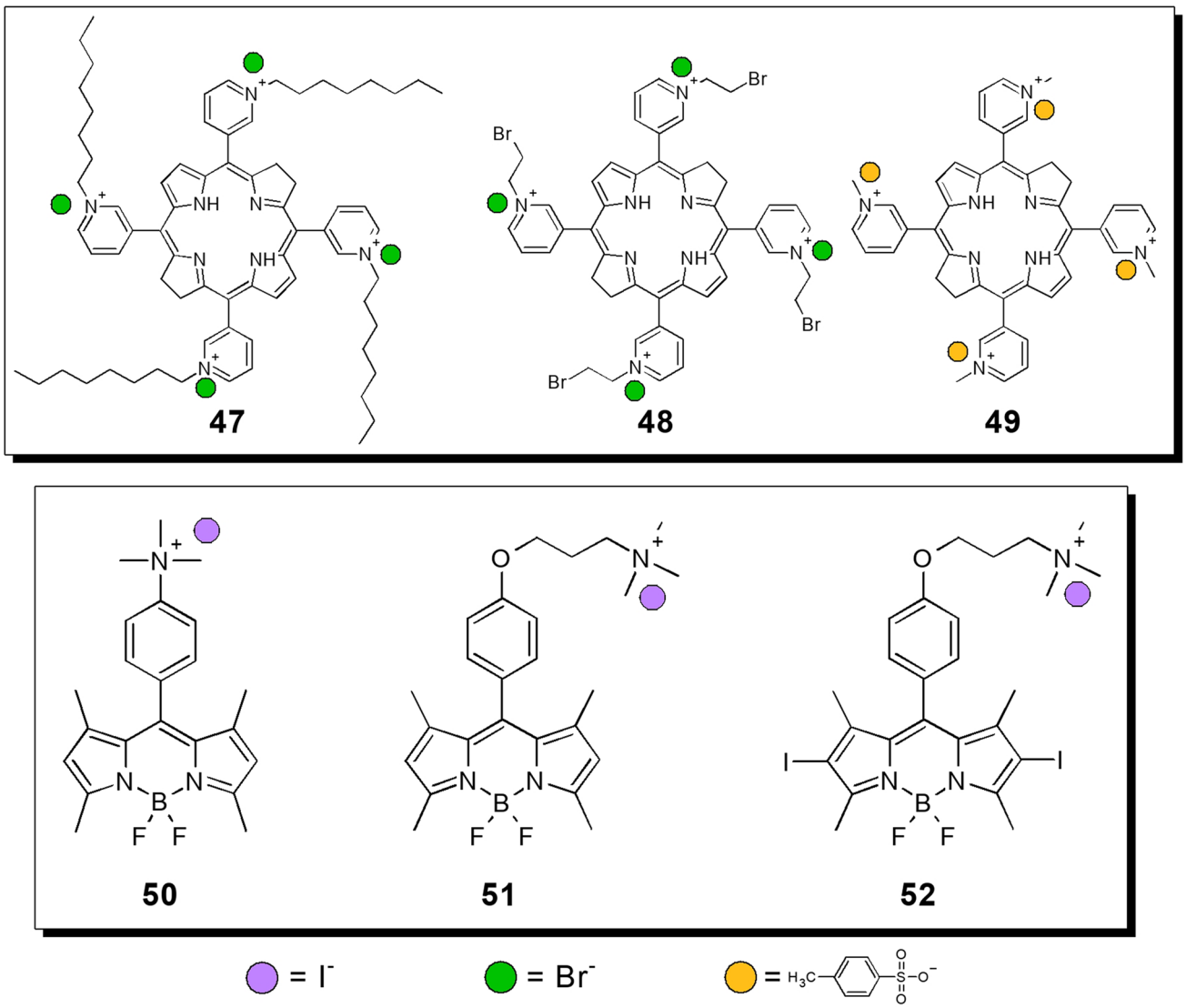

Fig. 9 Cationic bacteriochlorins and BODIPYs derivatives

in conjunction with its halogenated derivative **52** (Fig. 9), with both being efficient PSs against *E. coli*. Furthermore, the authors found that the presence of iodine in the BODIPY β positions increased its activity around 10 times [60].

2.1.5 Methylene blue and other phenothiazine dyes

Traditional phenothiazine dyes, such as Methylene Blue (**MB**, **53**, Fig. 10), were some of the first PSs. Up to this date **MB** is still under preclinical use, due to their well-known characteristics (i.e., absorbance at 550–700 nm, cheap and easy synthesis, low toxicity) and proven safety. Nevertheless, there are still novel applications and exciting results to be found, as it has been recently found that, besides its photobactericide activity, **MB** is able to overcome antibiotic resistance through the inactivation of some antibiotic's degradation enzymes. As an example, dos Anjos et al., used **MB** for the inactivation of three strains of *K. pneumoniae*, which were producers of β-lactamase, an enzyme that impedes the proper function of β-lactamic antibiotics. Furthermore, the use of **MB** in sublethal doses decreased the activity of β-lactamase, possibly reverting the sensitivity of the *K. pneumoniae* strains to β-lactamic antibiotics [61]. In a similar approach, different types of carbapenemases produced by *S. marcescens, K. pneumoniae,* and *Enterobacter aerogenes,* were photodynamically inactivated by **MB**, leading to a recovery of the sensitivity to carbapenem [62]. Additionally, there is evidence that **MB** can lead to a modest inactivation of several strains of *A. baumannii*, independently of their sensitivity toward carbapenem [63]. Then, **MB** appears as a suitable complement to traditional chemotherapy against drug-resistant bacteria. In another

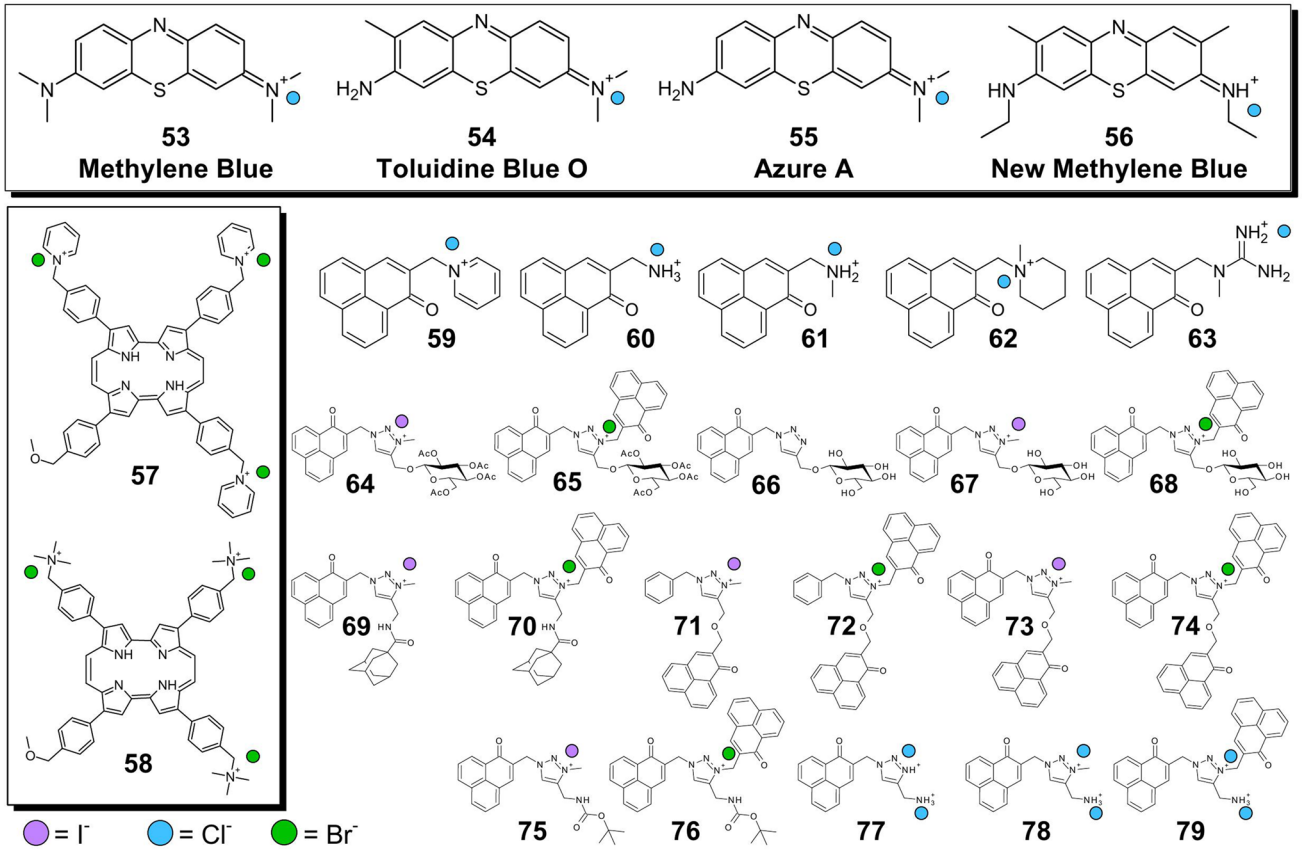

Fig. 10 Cationic bacteriochlorins and BODIPYs derivative

approach, different dyes, as **toluidine blue O (TBO) 54**, **azure A 55,** and **new methylene blue 56** (Fig. 10), had been tested against *K. pneumoniae*, resulting in efficient PSs against planktonic and mature biofilms [64].

2.1.6 Other cationic photosensitizers

Another kind of PS is porphycenes, which are isomers of porphyrins. Their unique aromatic structure provides them with different photophysical properties than those observed for the porphyrins, as a stronger absorption at 600–700 nm and a strong fluorescence [65]. Although the less, there are some examples of cationic porphycenes being used against bacteria, as Ruiz-González et al. research, whom synthesized two porphycenes (**57** and **58**, Fig. 10) and tested them against *P. aeruginosa* and *E. coli*, resulting in an efficient inactivation of bacteria [66].

Phenalenone derivatives have also acquired recent relevance as PSs. Phenalenone is an oxygenated polycyclic aromatic molecule, bearing a ketone moiety [67]. Previously described toxicity has started the development of phenalenone derivatives as photodynamic agents, but some recent studies are overtaking this challenge. As an example, Tabenski et al., compared **SAPYR 59**, the reference phenalenone molecule, against four cationic phenalenone derivatives (**60–63**, Fig. 10), bearing a quaternary amine, and testing their activity against *E. coli* after blue light irradiation,. The results obtained indicated an efficient bacterial inactivation, with the best result obtained with an imine-substituted derivative **63**, attributed to a foreseen ability to form hydrogen bonds with glutamate moieties on the bacterial membranes [68]. In a more recent example, Godard et al. prepared a complete library of phenalenone derivatives (**64–79**, Fig. 10), combining phenalenone's photosensitizing activity with the antimicrobial activity derived from triazolium salts. These compounds were tested against three Gram-negative strains, two strains of *E. coli* and one strain of *P. aeruginosa*. Some of the compounds were active against the three strains, and although the results are modest, the authors discuss about the increased effect on compounds with hydrophobic moieties, which are likely to increase the interaction with the lipidic membranes of Gram-negative organisms [69].

One can also cite fullerenes, spherical carbon-based molecules, that were just discovered in 1985. Thanks to their particular shape and structure, fullerenes absorb visible

light, have a high intersystem crossing yield, and are even able to produce light-driven ROS [70]. Due to their chemical characteristics, fullerenes are highly hydrophobic and tend to aggregate in aqueous media, and then cationic functionalization provides both with increased water availability and targets the membranes of Gram-negative bacteria [71]. In a recent example, three fullerenes (**80–82**, Fig. 11) with different degrees of cationization were synthesized. The fullerenes were fully characterized and tested against *E. coli*, under white and UV-A light. Interestingly, the most efficient fullerenes was **81**, possessing cationic and aliphatic chains. However, the aliphatic chains are susceptible of cationization, as it bears ten tertiary ammonium groups that may cationize in biological media. Besides the better biological development of **81**, the fullerene **82** was a better singlet oxygen producer, possibly due to their antenna-like moiety, and then resulting susceptible for tuning in order to achieve a better photodynamic effect [72].

2.2 Neutral and anionic photosensitizer

Although most of the current development of new PSs is focused on cationic molecules, there are still some examples where non-cationic molecules are used for efficient bacterial inactivation. In comparison with similar molecules, neutral and anionic PSs are less effective than their cationic analogs against Gram-negative bacteria, resulting in less results available in literature. As previously described, the efficiency of cationic compounds is related to their attachment to the negatively charged bacterial LPS, which promotes the uptake of the PS. It is noteworthy to mention that several of the molecules described along this section are parental molecules of the cationic porphyrins previously described, and several examples are partially described in Sect. 2.1. Furthermore, the use of materials and formulations (Sect. 3) or their potentiation with other strategies (Sect. 4) are discussed in the upcoming sections.

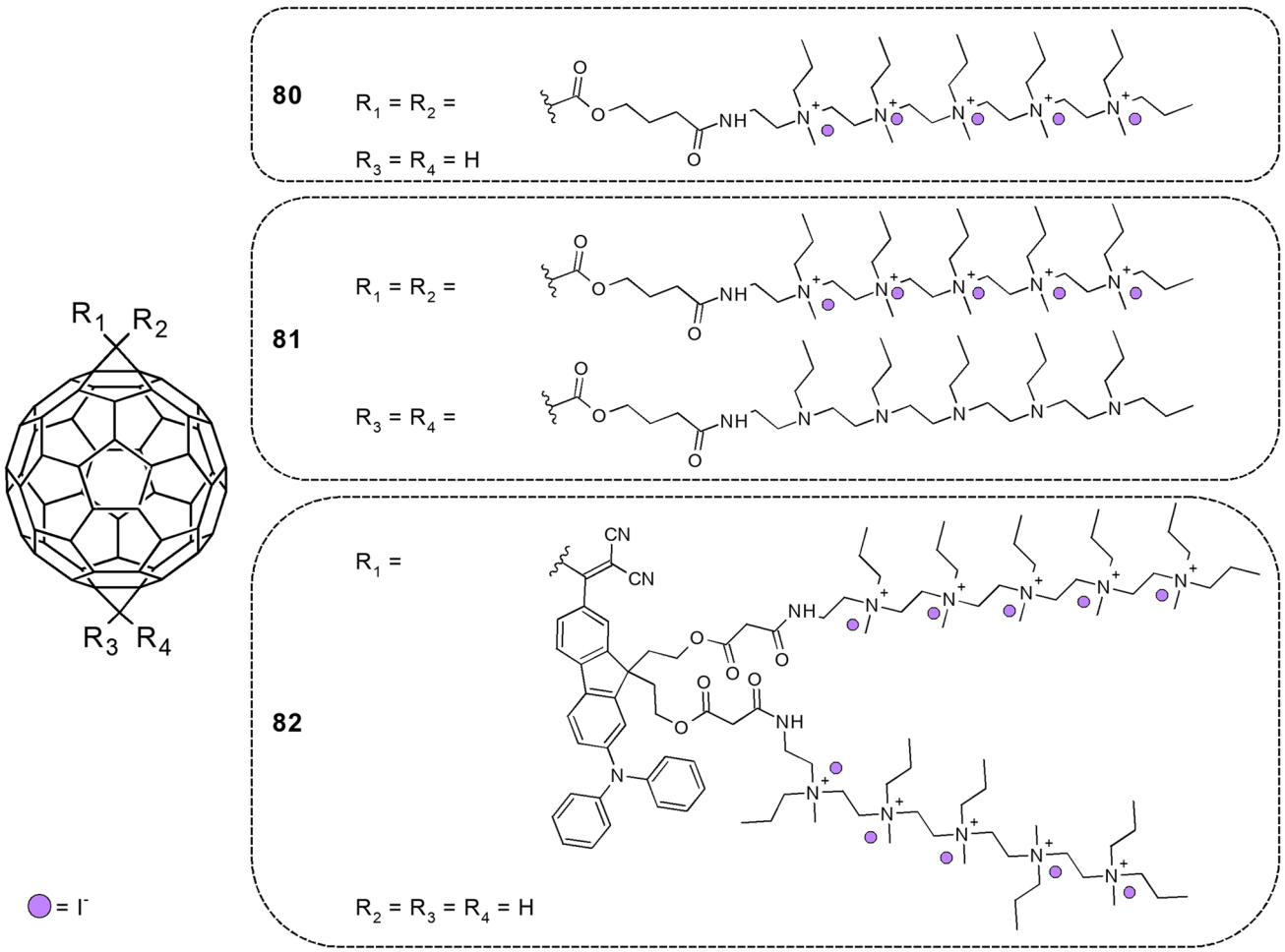

Fig. 11 Cationic fullerenes, as described in Ref. [72]

2.2.1 Porphyrins

As previously described, Calmeiro et al., prepared several porphyrins, including the non-N-methylated derivatives **83–86** (Fig. 12), which were tested them against *E. coli* in the presence and absence of KI [40]. Interestingly, while the neutral compounds failed to eradicate the bacteria, the addition of KI into the media increased the efficiency of photobactericidal activity up to 7-log reduction, through the permeabilization of the Gram-negative membranes. Significantly, in another example, Ferreyra et al., prepared a porphyrin (**87**, Fig. 12) and a chlorin (**88**, Fig. 12)**,** with the porphyrin being able to efficiently eradicate *E. coli*, which, despite its lack of cationic charges, was able to be uptaken by bacteria, reaching uptake levels similar as those found for cationic **TMPyP**. The authors proposed that this is due to the presence of their four basic amine groups on its periphery, permitting the acquisition of a cationic charge in biological media [73].

2.2.2 Phthalocyanines

Similarly, when neutral phthalocyanines are tested against Gram-negative bacteria, they failed to eradicate bacteria where its cationic counterparts (**46,** Fig. 8) succeeded [56]. Recently, Berezin et al., compared the efficiency of a neutral (**89**, Fig. 12) and an anionic phthalocyanine (**90**, Fig. 12) for *E. coli* inactivation, using Tween 80 1% as a membrane disruptor, but also as a de-aggregation agent for the phthalocyanines, which together with the use of EDTA 0.1% lead to excellent bacterial inactivation [74]. Similarly, Biyiklioglu et al., compared the efficiency of a silicon phthalocyanine (**91**, Fig. 12) and a boron subphthalocyanine (**92**, Fig. 12) against *E. coli*. Subphthalocyanines are homologs of phthalocyanines with only 14 π electrons in three aromatic macrocycles, fused around a central boron atom [75]. The researchers found that both were able to diminish the bacterial survival at the same concentration, with **92** requiring a lower light dose to achieve the desired biological effect. This correlates with subphthalocyanine's **92** superior singlet oxygen production [76].

2.2.3 Chlorins and bacteriochlorins

In a recent example, **chlorin e6 (93**, Fig. 12**)** was tested against biofilms made of two or three different bacterial species, namely, *Moraxella catarrhalis* (Gram negative)*, Haemophilus influenzae* (Gram negative), and *Streptococcus*

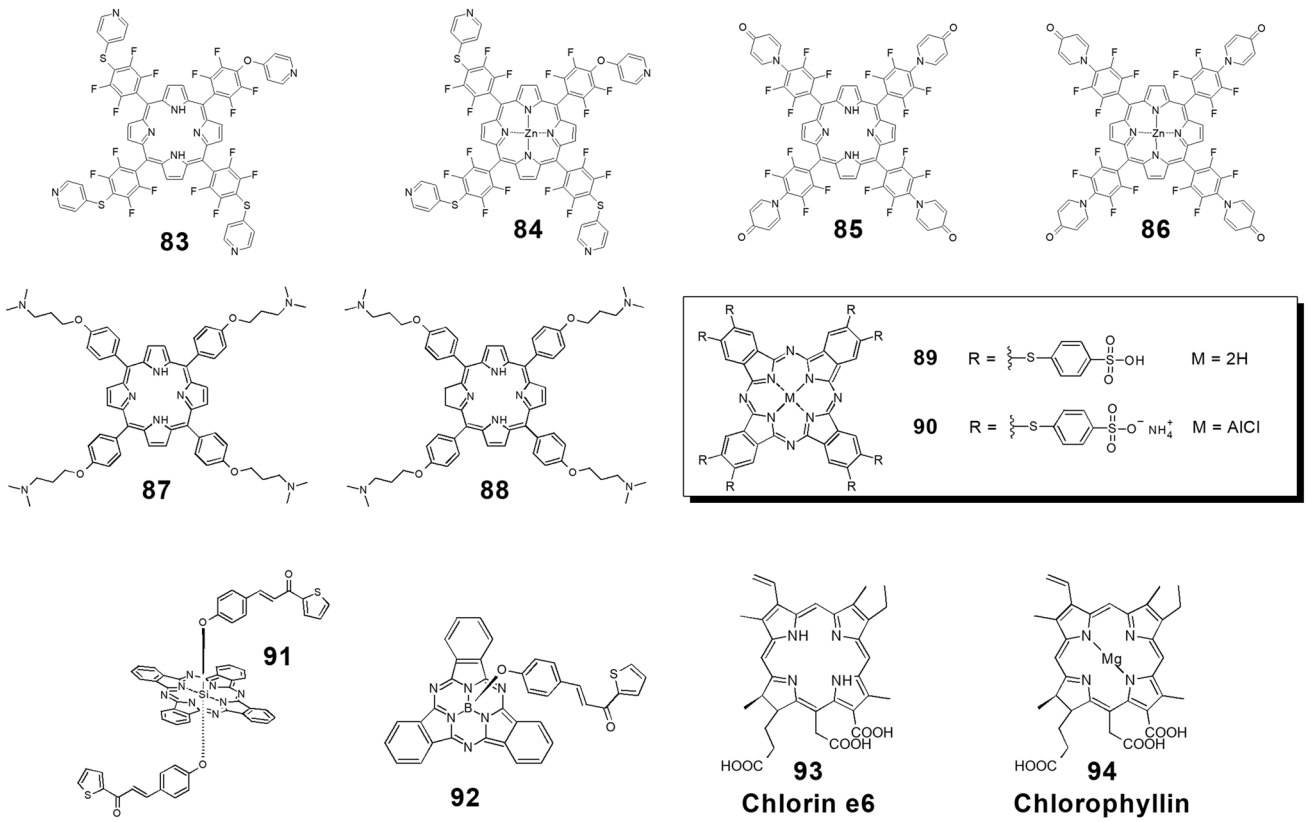

Fig. 12 Neutral and anionic porphyrins, chlorins, phthalocyanines, and subphthalocyanines

pneumoniae (Gram positive), mimicking the bacterial environment found in otitis media infections. The effectivity of **chlorin e6** against the biofilms depended on the bacterial strains, the **chlorin e6** concentration, the incubation time, and the light dose, with this work being a nice example of the application of PACT into more challenging and realistic conditions. Biofilms tend to be resilient communities that are impermeable to small molecules, providing resistance to antimicrobial therapies, and thus, the modest success of **chlorin e6** against bi and tri-species biofilm could be a fundamental stone for the use of photodynamic therapy, in combination with another antimicrobial, for the treatment of persistent otitis media [77]. Returning to the work of Ferreyra et al., they prepared a chlorin (**88**, Fig. 12), which was tested against *E. coli*, achieving efficient inactivation. When compared with the parental porphyrin (**87**, Fig. 12), both compounds had similar results against *E. coli* [73].

The lack of efficiency of neutral compounds against Gram-negative bacteria can be deduced as a consequence of the lack of interaction between the compounds and the cellular membrane, which eventually prevents the uptake into the cytoplasm. To deeply explore this, Krüger et al., approached this question using the neutral chlorin **chlorophyllin** (**94**, Fig. 12), against *Bacillus cereus*, *E. coli,* and a membrane-defective mutant of *E. coli*. Normally, **chlorophyllin** could only eradicate bacteria lacking a proper cellular membrane, with *B. cereus* being sensitive, while the wild-type *E. coli* resulted resistant to the photodynamic treatment. The authors demonstrated that an *E. coli* mutant, with a defective outer membrane, was susceptible toward the photodynamic effect of **chlorophyllin**, showing that neutral PSs are less effective toward Gram-negative bacteria due to their impermeable outer membrane [78]. Due to the complexity of these results, they could not be resumed into the Table of Supplementary Material.

2.2.4 BODIPY and dipyrrinato compounds

Like others example of cationic PS of whom neutral analogs have been also investigated, neutral BODIPYs have been also studied. In the previous example of Piskorz et al., the non-cationic derivatives of **95** and **96** (Fig. 13) were only able to reduce bacterial survival at high concentrations, as 50 and 500 µM [60].

In a recent example, Hohlfeld et al. prepared several neutral **BODIPY** compounds as an effort to understand the effect that chemical modifications had on their efficiency on bacterial disinfection. The tested compounds could be divided in two groups, bearing either a 2,3,5,6-tetrafluorophenyl (**97**–**101**, Fig. 13) or a 3-nitrophenyl (**102**–**106**, Fig. 13) moiety at the *meso*-position. Further functionalization allowed to assess the effect of different moieties (i.e., amine groups, alkyne groups, hydroxyl groups, etc.) in the inactivation of *P. aeruginosa*. The best results were obtained with the **BODIPY**s derivatives bearing a tetrafluorophenyl moiety and an unprotected thio-carbohydrate (glucosyl **99** or galactosyl **101**). Furthermore, the compounds were challenged in the presence of serum 10%, which significantly decreased the efficiency of the photodynamic treatments [79]. In a similar approach, the same researchers prepared several dipyrrinato-iridium complexes (**107**–**114**, Fig. 13), and tested them against *P. aeruginosa*, under similar experimental conditions. Several compounds were found to be effective against bacteria, and, similarly, their efficiency was decreased in the presence of serum 10% [80].

2.2.5 Other photosensitizers

Erythrosine (**115**, Fig. 14) is an anionic dye PS, whose efficiency against *A. baumannii* planktonic cells and biofilms has been investigated by Fekrirad and collaborators. Their research achieved an efficient inactivation of planktonic bacteria when incubated with 0.01% of acetic acid, and achieved biofilm inactivation when **erythrosine** was combined with 0.01% of acetic acid and 12.5 µM chitosan [81]. In a similar approach, Santos et al., increased the efficiency of **RB** (**116**, Fig. 14) and **eosin** (**117**, Fig. 14) against *Salmonella enterica* serovar Typhimurium, through the addition of KI prior irradiation with green light [82].

Although generally PSs are perceived as small molecules, some reports indicate that LOV (Light-Oxygen-Voltage receptor) proteins are susceptible to be used as PSs against bacteria [83]. In a recent report, three different proteins, SOPP3, SuperNova, and KillerOrange (Fig. 14), were found to be light-dependent antibiotics. These proteins are genetically modified derivatives of the protein anm2CP, a constitutively fluorescent protein found in jellyfish from the order Anthoathecata. These proteins were originally intended as in vivo reporters of oxygen-limited systems, and tend to be toxic toward *E. coli*, their heterologous host, when irradiated with light. Several experiments further confirm the toxicity of these proteins against *P. aeruginosa*, representing an interesting system that can easily be expressed together with targeting proteins, as lectin B (LecB), increasing their antibacterial efficiency [84].

2.3 Synthetic metallic complexes

As photodynamic applications against microorganisms gain popularity, researchers are likely to seek for alternatives away from already established research lines, diversifying the photodynamic systems used.PS We have previously addressed several metals, such as iron [45], iridium [60], palladium [39], ruthenium [54], and lutetium [52], are used to form complexes with PSs. However, the use of metals is not limited to the decoration of already established PSs, and

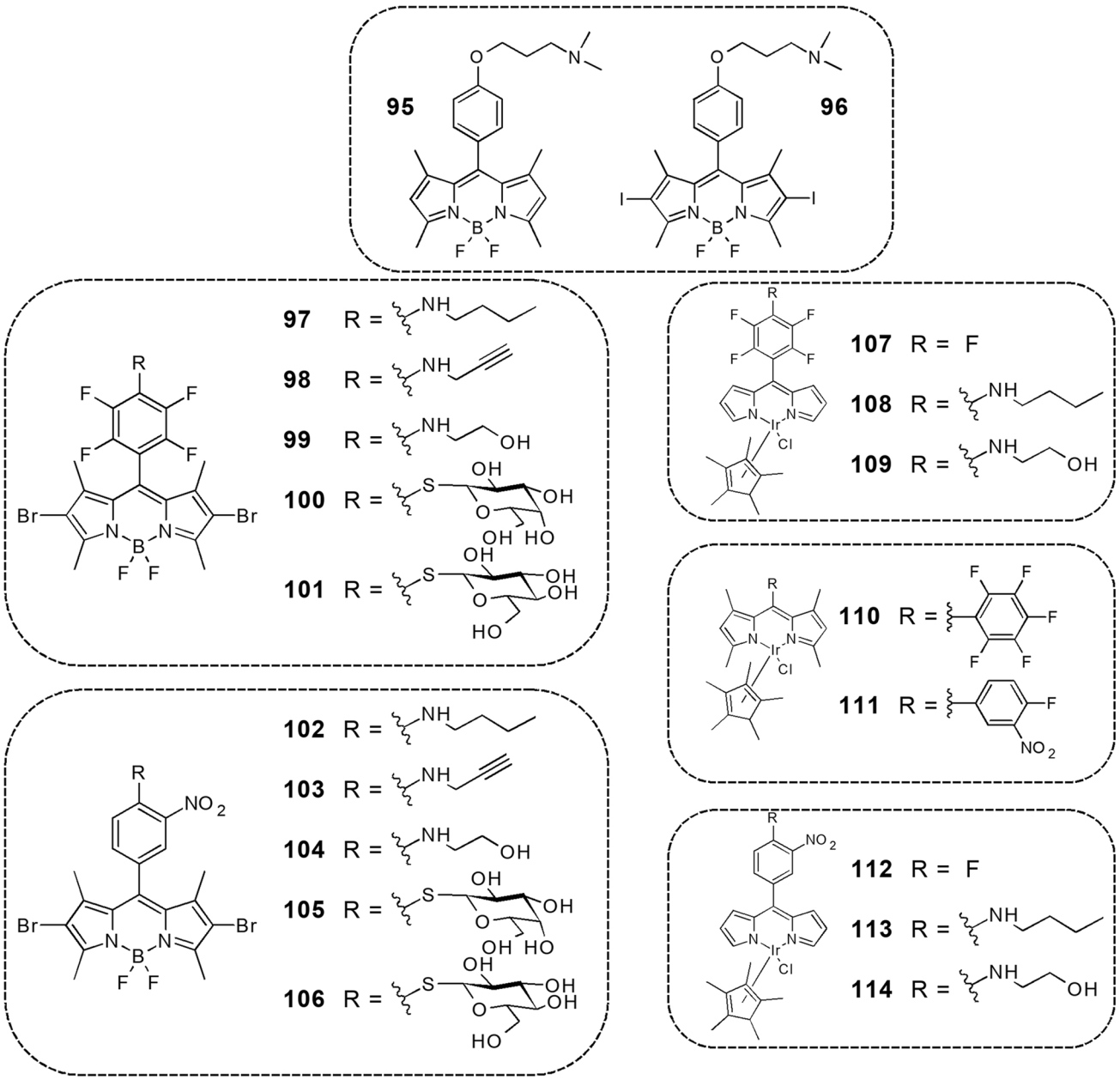

Fig. 13 Neutral BODIPY and iridium-dipyrrinate derivatives

in the following sections we will present several examples where metallic complexes permit the formation of efficient photosensitizing systems.

In a recent example, Hopkins and collaborators prepared a ruthenium(II) and platinum(II) complex (**121**, Fig. 15) with phenanthroline, which was then tested against *E. coli* under white light irradiation. The results obtained indicated that the complex acts as a PS, resulting more efficient than cisplatin, a model platinum complex [85]. Intending to provide an increased bioavailability of the ruthenium complexes, Soliman and collaborators synthesized a ruthenium-polylactic acid complex (**122**, Fig. 15), which was used to form polylactic acid nanoparticles. The nanoparticles were fully characterized, but when tested against bacteria, they were incapable to eradicate Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, which was explained through the low internalization of the nanoparticles [86]. Similarly, Le Gall et al. prepared several ruthenium polypyridyl complexes (**123**–**139**, Fig. 15). Then, although their binding to *E. coli* and *P. aeruginosa* was demonstrated through luminescence, the compounds were unable to decrease bacterial survival of Gram-negative bacteria. The data about these compounds

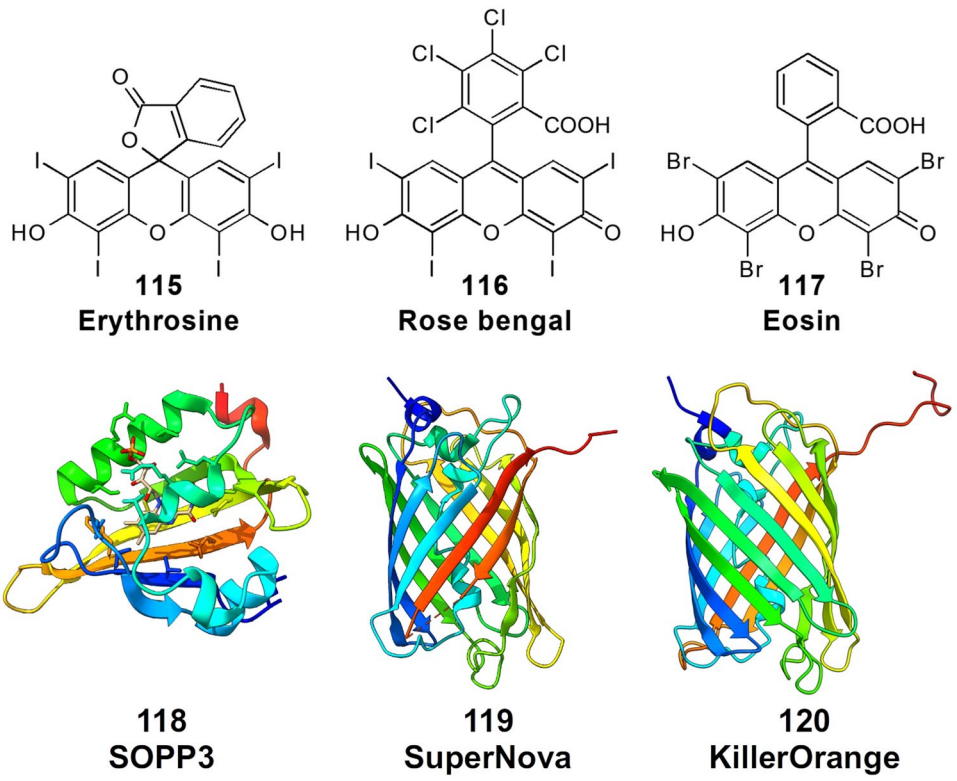

Fig. 14 Neutral dyes and proteins with photosensitizing properties. The crystal structures were obtained from the Protein Data Bank (PDB), entries 6GPU (SOPP3), 3WCK (SuperNova), and 4ZFS (KillerOrange)

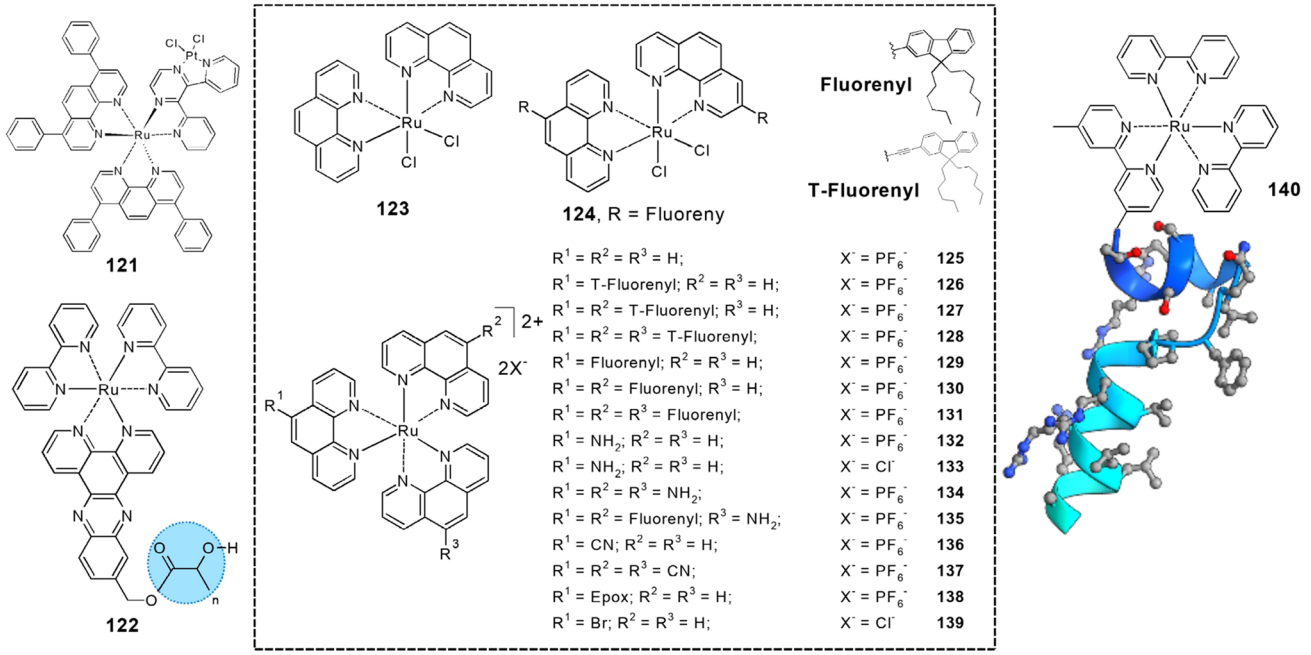

Fig. 15 Some metallic complexes described in this work. The buforin II structure was extracted from the PDB entry 4KHA

are not in the Table, as the methodology is not presented in the publication [87]. In order to increase the binding between the ruthenium complexes and bacteria, another approach has been taken by Pierce et al. This group of researchers attached buforin II, an antimicrobial peptide, to a ruthenium complex (**140**, Fig. 15), and then tested it against several model strains, successfully photo-inactivating the model *E. coli*, but also against clinical isolates of drug-resistant *P. aeruginosa, E. coli, A. baumannii,* and *K. pneumoniae* [88]. Then, metallic complexes are an interesting option for the

disinfection of Gram-negative bacteria, but they also rely in an intimate contact with bacteria, which could enhance their internalization into the cell.

3 Materials with photosensitizing properties and delivery systems

As previously stated, AMR is a huge health concern for governments and scientists, with an imperative need for stronger policies, encouraging the development of novel technologies to fight microbial presence in sensitive environments, such as hospitals and food processing facilities. One ever-growing topic is the development of self-disinfecting surfaces, such as microbial contamination in surfaces, at hospital and food processing environments, which can provide a platform for the development of pernicious bacterial biofilms. The development of materials with self-disinfecting properties can be achieved with photosensitizing molecules, additionally addressing the low aqueous solubility of several PSs. Several examples of photosensitizing materials use either carbon based or inorganic scaffolds, with all the systems having assets and drawbacks; an efficient antimicrobial material should indeed exhibit a good equilibrium between stability, preserved photophysical properties of PS, bactericidal efficiency, and safety toward the host.

3.1 Materials based on biopolymers and carbon-based molecules

During the past decades, in line with the emergence of green chemistry, and due to their low cost and renewability, bio-sourced polymers have appeared as an interesting alternative for drug formulations and scaffolds. As for PACT applications, an interesting review about the use of bio sourced polymers has recently been written [89], but the ever-growing interest of bio-sourced polymers makes a difficult task to pace up with the literature. Thus, in the following sections we present some of the most recent advances of materials based on biopolymers.

3.1.1 Cellulose materials

Among all the biopolymers, cellulose, a major structural compound in plants, is particularly used. Indeed, this natural polysaccharide is constituted of a linear chain of several hundred to many thousands of β(1 → 4) linked D-glucose units, with a large amount of hydroxyl groups available, which can undergo a large range of chemical modifications that give rise to different materials. These properties permit cellulose to be addressed as an alternative raw material for different PS formulations.

For example, some studies reported the development of antibacterial cellulosic fabrics. The study published last year by Fayyaz et al. evaluated the photodynamic activity of three different tetracationic porphyrins, and their zinc derivatives (**6**, **7**, Fig. 5, 141–144, Fig. 16) impregnated on cellulosic fabrics, and tested against *E. coli* and *P. aeruginosa* [90]. Not only all the modified fabrics displayed a photo-antibacterial activity against all strains investigated, but also, accordingly to the washing and thermal stability of the materials, these fabrics could be efficiently used for biomedical textile applications. In another study, Nzambe Ta Keki et al. investigated a neutral metalated porphyrin (**145**, Fig. 16) covalently attached onto Kraft pulp fibers [91]. The authors clearly demonstrated here that their system was efficient toward Gram-negative bacteria, observing a decrease of more than 4 logs in bacterial survival, as a result of light irradiation.

More recently, a new strategy to obtain cellulose-fibers-based materials has been developed, such as electrospun cellulose microfibers developed by Wang et al. [92]. Indeed, electrospinning is a simple and inexpensive polymer formulation method, which can be applied to both natural and artificial polymers, accommodates a wide variety of PSs, and allows to generate materials with favorable features, such as high surface area-to-mass ratio, or a wide range of morphologies and high porosity. Thus, the authors investigated porous cellulose diacetate electrospun microfibers loaded with protoporphyrin-IX (PPIX, **146**, Fig. 16) PS against *E. coli*. Protoporphyrin is a natural PS, being a biosynthetic intermediate of the heme group, essential for biological processes as the transport of oxygen. The different characterizations of the obtained material showed that PPIX was uniformly distributed on the microfibers, and that due to the pores size and shape of the material, *E. coli* was less prone to adhere. However, the modest antimicrobial photodynamic efficiency against *E. coli* could be improved, once again, through the addition of 100 mM KI, enabling up to 6 logs of bacterial reduction.

Through these examples, the authors have also emphasized the importance of the morphology of the material as well as its capacity to adhere to bacteria, while preserving the PSs capacity to produce ROSPS. Those parameters are even more important for materials without cationic charges, which are recognized as important factor for the interaction with Gram-negative bacterial outer membrane. Similar kind of observations has been done for chitosan-based photo-antibacterial materials.

3.1.2 Chitosan-based materials

Chitosan is a polymer derivative from chitin, a polymer that composes the shells of shrimps and insects. This polymer is composed of β-(1 → 4) glucosamine and N-acetyl

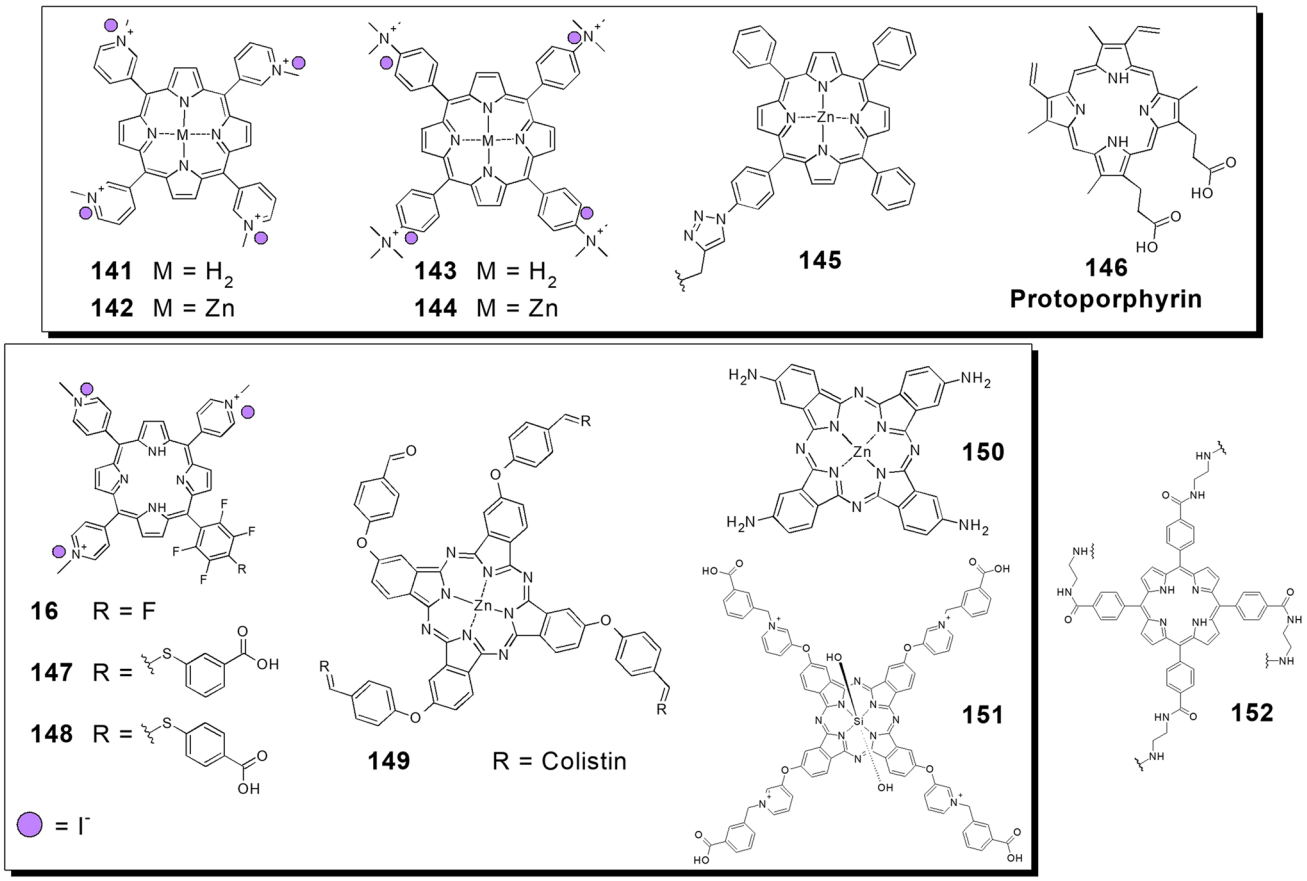

Fig. 16 PSs conjugated to some biopolymers, described along this work

glucosamine monomers. Indeed, this polysaccharide has evidenced its suitability for PACT application, due to its low toxicity, biodegradability, bacterial association, film-forming ability, and intrinsic antimicrobial activity, due to its numerous pendant amino groups. Castro et al. have, for example, investigated the antibacterial efficacy of **16** derivatives (**147** and **148**, Fig. 16), conjugated to chitosan (films) or TiO_2 (powder), against *E. coli* [93]. The PS immobilization on chitosan was less efficient than on TiO_2, and the interaction of PS with chitosan was evidenced by UV–visible absorption spectra shift. Moreover, **16** showed the same $^1O_2$ production in solution and immobilized on chitosan, probably due to aggregation-driven attenuation, or due to increased photostablility of the immobilized porphyrins. The antibacterial efficiency of the systems was found to be linked to their singlet oxygen production, which relies on both PS structure and PS-material interaction, being decreased for both chitosan and TiO_2 materials.

Chitosan, in its polycationic form, can be formulated as a hydrogel. Hydrogels are cross-linked polymer chains, swollen with water, which can be loaded with small molecules, either through entrapment or grafting through its amino groups. Bayat and Karimi have used these properties to prepare different chitosan hydrogels. In a first example, they associated a zinc phthalocyanine/colistin conjugate **149** (Fig. 16) and chitosan, increasing the bioavailability of the phthalocyanine, while also providing a targeting moiety [94]. Additionally, they observed enhanced PACT efficiency against *P. aeruginosa*, resulting from the synergy of the photodynamic effect and the antibacterial agent targeting, which has been observed previously by Le Guern et al. [95]. The second hydrogel contained a zinc phthalocyanine **150** (Fig. 16)**,** difloxacin, and chitosan, producing $^1O_2$ with good efficiency, under visible light irradiation, resulting in a good candidate for PACT applications [96]. More recently, another study analyzed a hydrogel bearing a silicon phthalocyanine **151** (Fig. 16), published by Strokov and Galstyan. When analyzed, the hydrogel kept the phthalocyanine's photophysical features, namely, absorption and emission wavelength, fluorescence quantum yield, while showing an increased $^1O_2$ production. This led to an efficient *E. coli* inactivation, probably due to the stronger interaction with bacteria cells and the enhanced singlet oxygen production of the material [97]. In another example, Yin et al., demonstrated that inactivation of drug-resistant bacteria *E. coli* can be achieved through incorporation of upconverting

nanoparticles (UCNPs) doped with **MB** in chitosan hydrogel [98]. Their most efficient system (UCNPs@MB:QCS 2:100) against *E. coli* achieved up to 95% of bacteria killing efficiency, after 20 min of 980 nm irradiation at 1 W/cm^2.

3.1.3 Other biopolymers materials

A recent study published by Kumari et al., deals with the self-assembly of a DNA-Porphyrin (**152**, Fig. 16) hybrid nanonetwork [99]. In this work, the DNA-porphyrin hybrid nanonetwork was less efficient than the free porphyrins to inactivate *E. coli* strains, probably due to the negatively charged DNA backbone, which would prevent the uptake of the scaffold. However, the systems also showed reduced toxicity toward mammalians cells, that is a great asset for future applications.

Another very interesting natural biopolymer is protein keratin. Wool keratin has demonstrated to have great potential in regenerative medicine, but bacteria colonization and biofilm formation should be prevented. Ferroni et al. associated wool keratin 3D scaffolds with encapsulated Azure A (Fig. 10), in order to form photo-activable porous sponges [100]. The obtained materials exhibited good efficiency against *P. aeruginosa* under light irradiation, and no toxicity toward mammalian skin cells, while possessing a suitable 3D structure, pore size, and degradation rate, resulting in a suitable candidate for tissue engineering.

3.1.4 Micelles, liposomes, vesicles, and microemulsions

Lipidic materials are able to spontaneously form self-assembled micelles and liposomes, which have been widely exploited in therapeutic applications [101]. For example, Rout et al. investigated the use of eucalyptus oil microemulsion as a carrier for delivering TBO (**54,** Fig. 10) [102]. They evidenced that, in addition to a synergic antibacterial effect against *P. aeruginosa*, this formulation increased the bioavailability of TBO, as the micelles increased the penetration into the viable layers of the skin, when compared with the free TBO; additionally, TBO's stability was enhanced.

In another study, Sharma et al. described cationic vesicles encapsulating MB against *E. coli* [103]. These vesicles are made with hexadecyl pyridinium cuprate as cationic component, and sodium oleate as the anionic one, with different ratios. The ratio 70:30 in the cationic and anionic components demonstrated a complete inactivation of bacteria within 5 min of irradiation, with the enhanced vesicles attachment being the key factor for the improved efficiency. Moreover, the role of the metal was also evidenced, demonstrating enhanced $^1O_2$ generation, as compared against the MB without a formulation. MB has also been encapsulated in cationic liposomal formulations containing dimethyldioctadecylammonium chloride, dipalmitoylphosphatidylcholine, and cholesterol, by Boccalini et al. [104]. MB-loaded liposomes showed a higher efficiency, in bacterial toxicity against *E. coli* and penetration into the bacterial biofilm, compared to the free MB. Moreover, an enhanced inactivation of LPS, a major pro-inflammatory endotoxin of Gram-negative bacteria, was also observed.

More recently, Pourhajibagher et al. encapsulated curcumin (**153**, Fig. 17) in silver sulfadiazine nanoliposomes, developing a photo-activable antimicrobial system, efficient against *A. baumannii* [105]. Curcumin is a natural PS, extracted from the root of the turmeric plant, and with a strong background in traditional Asian medicine, supported with increasing evidence of a wide range of therapeutic effects [106]. In this case, the curcumin-loaded nanoparticles showed negligible toxicity against eukaryotic cells, while efficiently photoeradicating *A. baumannii*. In another example, Sobotta and collaborators, encapsulated two chlorins (**154** and **155**, Fig. 17) inside lipidic vesicles and tested them against *E. coli*. However, at the highest concentrations tested, the treatments were unable to decrease the bacterial survival of *E. coli* further than 2.23 log, while demonstrating efficiency inactivation of Gram-positive bacteria [107].

In addition to liposomes, vesicles, and microemulsions, PS can also be encapsulated in micelles, as evidenced by Wang et al. in 2021, who prepared chlorin-e6 polyethylenimine-based micelles, with enhanced photodynamic inactivation of *E. coli* [108]. The $^1O_2$ generation production of chlorin-e6 was preserved inside the micelle, while the

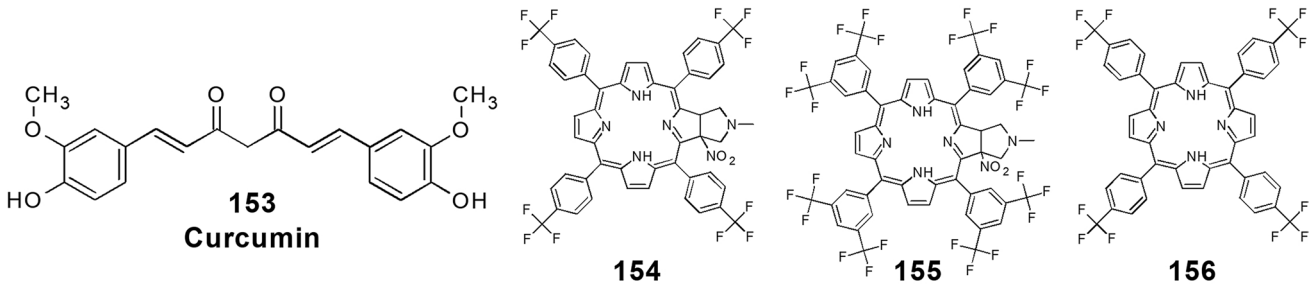

Fig. 17 PSs associated to some lipidic systems, described along this work

water solubility of the PS was improved without the need of any organic solvents. The authors also evidenced that cellular uptake was enhanced when chlorin-e6 was transported through micelles, with both enhanced uptake and singlet oxygen production, leading to the high antibacterial efficiency observed.

3.1.5 Other systems

More recently, Contreras et al. have also investigated Photodynamically Active Fibers (PAFs) based on poly(L-lactide) co-(glycolide) and poly(ε-caprolactone) loaded with MB [109]. Moreover, The MB-encapsulated electrospun fibers showed a good structure stability, as well as a controlled released of MB over 3 weeks. In addition, up to 2.5 logs viability reduction was observed for *E. coli* under light irradiation. Moreover, dark toxicity was drastically reduced compared to free MB. Electrospun fibers containing poly(ε-caprolactone) were used to develop innovative membranes, that were associated to a metal–organic framework (MOF), loaded with RB [110]. The authors combined all the assets of the different components for their molecular system: poly(ε-caprolactone) allowed the membrane formation, while MOF, with their intrinsic porous structure, provided high chemical and thermal stability. After visible light irradiation, these hybrid nanofibrous membranes exhibited excellent ROS generation and antimicrobial activity against *E. coli*.

Another interesting approach is the one developed by Castro et al. They developed two systems based on 2-hydroxyethyl methacrylate, and **156** (Fig. 17), either as a co-polymer or as a porphyrin-loaded polymer [111]. The obtained co-polymer was more efficient in terms of $^1O_2$ generation, than the **6**-loaded polymer, probably due to the dispersion of the porphyrin on the polymer net, avoiding PS aggregation. Moreover, a reduction of approximately 99.9% of *E. coli* was observed in the case of the co-polymer, which could be reused at least three times, without losing its efficiency. The porphyrin-loaded polymer was more efficient than the free PS against *E. coli,* while requiring higher concentrations than for the co-polymer treatment (co-polymer > loaded polymer > free porphyrin). This example perfectly illustrates the primordial importance of PS formulation.

3.2 Inorganic and hybrid materials

As previously described, in addition to formulation to reach the highest photo-activable efficiency of PS, scientists consider other factors when designing photo-activable materials. As an example, they aim for the development of environment-friendly systems, which would be economic, reusable, recyclable and cost–benefit permissive. This can be obtained through the use of inorganic solid matrices as scaffolds for PS molecules.

3.2.1 Magnetic nanoparticles

This could be addressed, for example, by immobilization of the PS on magnetic iron oxide nanoparticles, which can be visualized and guided in water and organic solvents, by means of an external magnetic field. Besides, magnetic nanoparticles have been widely used for the transport and delivery of drugs, demonstrating their biocompatibility, safety, stability, while also representing, and ecological alternative [112, 113]. As a sidenote, for the nanoparticles suspension studies, ζ-potential is the surface charge of the nanoparticle, which determines the behavior of the suspension. Thus, it is usually desirable to obtain values above ± 20 mV, as the repulsion forces prevent the quick sedimentation of the suspension [114]. As an example, Scanone et al. have developed silica-coated Fe_3O_4 nanoparticles grafted with either a **156** (Fig. 17) [112] or BODIPY derivatives **157** and **158** (Fig. 18) [115]. Here authors particularly investigated the effect of charge in the whole system and nicely evidenced that the most efficient against *E. coli* was the one that exhibited the most positive ζ-potential and the highest $^1O_2$ quantum yield. Indeed, the authors observed a 3-log reduction of *E. coli* after 30 min of irradiation (visible light; 90 mW/cm^2). Moreover, the system could be reused at least three times without losing its efficiency [112]. This example illustrates the importance of an equilibrium between stability, charge, and ROS generation, for systems used in PACT applications. In the latter study, with BODIPY derivatives, the authors investigated the impact of heavy atom on ROS production and the overall efficiency of the photo-activable antimicrobial material. The bromine substituted system **158** reached a reduction of 5 logs in *E. coli* survival after 15 min of irradiation, versus 1.7 logs in the same conditions, for the non-brominated system **157** [115].

Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) had been also used for antimicrobial materials. Indeed, they are well-known as effective PhotoThermal Therapy (PTT) agents that could be associated with PS in order to reach combined PTT–PDT systems [116]. In this line, Bilici et al. investigated the bactericidal effect of indocyanine green (**ICG**, **159**, Fig. 18) grafted on 3-amino-propyltrimethosilane-coated SPIONs against *E. coli, K. pneumoniae,* and *P. aeruginosa*, particularly focusing in the influence of the charge on cellular uptake. Interestingly, the system without ICG exhibited a positive ζ-potential, whereas, when loaded with ICG, ζ-potential was negative; nevertheless, the cell internalization studies for both systems did not show significant difference. Efficiency against bacteria was dependent of the strains, with *K. pneumoniae* being the most sensitive strain to the treatment. In another approach, Zang et al., developed Fe_3O nanoparticles for PTT-PDT, coating them with chitosan and decorating them with an anionic porphyrin, TCPP **160** (Fig. 18). The authors showed that the

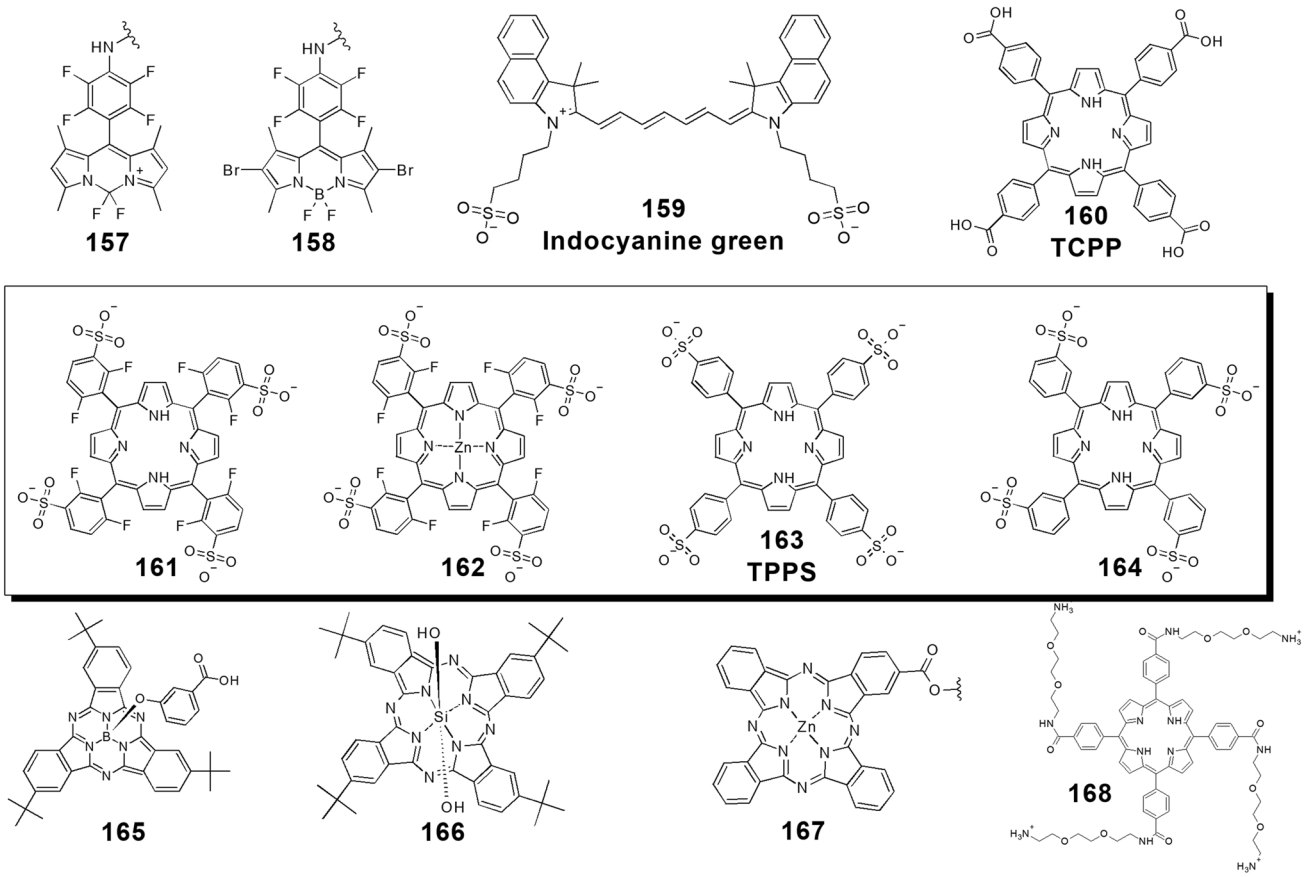

Fig. 18 PSs associated to inorganic materials, described along this work

introduction of metal nodes prevented the self-aggregation of the porphyrin and improved $^1O_2$ generation [117]. By using both PTT and PDT, the authors reached a 98% of *E. coli* reduction of bacterial survival, while PTT or PDT only reached 45% and 42%, respectively.

3.2.2 Titanium oxide nanoparticles

Titanium oxide (TiO_2) nanoparticles are nanomaterials with interesting APDT applications, thanks to their low toxicity, high stability, and excellent biocompatibility, as already underlined in the work of Castro et al. [93]. One can also cite the work of Sułek et al., who investigated visible-light-activated TiO_2-based materials, modified by tetra sulfonated porphyrin derivatives (**161**–**164**, Fig. 18), and formulated as transparent colloidal solutions [118, 119]. However, although only 1–2-log decrease in survival of *E. coli* was monitored after 20 J/cm^2 of blue or green light (420 nm or 530 nm), the authors improved the efficiency of their systems with KI potentiation, leading to a reduction of up to five logs under visible light irradiation.

In another example, Ozturk et al., developed TiO_2-based nanomaterials loaded with a subphthalocyanine **165** (Fig. 18), which showed promising results against *E. coli* [75]. They evidenced that their system had inhibitory and bactericidal effects on *E. coli* at 20 and 30 J/cm^2 light doses, respectively. The systems combined the activities of both TiO_2 (intrinsic) and subphthalocyanines (light activated), while preventing PS aggregation. Unfortunately, no photophysical data of the PS were measured, preventing a comparison with Sułek's system in order to propose a structure–activity relationship.

As a sidenote, the toxicity of TiO_2 nanoparticles is currently in debate, with increasing evidence that TiO_2 nanoparticles accumulate into the tissues, with prospective negative effects into the health. However, the main concern involves the use of TiO_2 as a supplement in foodstuff, in order to preserve their organoleptic properties (i.e., color, odor), and their interaction with the digestive system, while there is still little evidence of their accumulation when administered into the skin, not permitting us to reach a conclusion on its safety for PACT applications [120].

3.2.3 Mesoporous silica nanoparticles or supports

Another material currently used in biomedical application is silica nanoparticles. In this framework, while aiming to improve antimicrobial systems and bacteria cell wall interaction, as well as developing a theranostic materials, Grüner et al. have fully described UCNP, coated with a mesoporous silica shell, loaded with a silicon phthalocyanine (**166**, Fig. 18). Moreover, they further functionalized their nanoparticles with (3-aminopropyl)triethoxysilane and CH_3I, HOOC–TEG–COOH, or HOOC–TEG–NH_2 and CH_3I. These complex systems were analyzed for their efficiencies at photo-inactivating *E. coli* [121]. The intrinsic toxicity of UCNP was enhanced by the mesoporous shell of the particles, which improved the binding of the particles to the bacterial membrane. A synergic effect was observed on *E. coli* inactivation, with it being dependent on the number of cationic charges incorporated into the systems, permitting the binding to the bacterial membrane, and promoting its disorganization. Tang et al. described as well a very smart system that combined silicon nanoparticles, loaded with chlorin e6 as PS, and glucose polymer, to promote their transport through the ATP-binding cassette transporter [122]. Their systems showed an antibacterial efficiency of up to 96% against *P. aeruginosa* under visible light irradiation.

In another example, Sun et al. developed a complex platform for bactericide applications associating a polymeric matrix made up with poly(ε-caprolactone) and the vegetal-protein zein, coating MB-loaded mesoporous silica nanoparticles [123]. Moreover, their surface was further modified with trichloro-(1H,1H,2H,2H-heptadecafluorodecyl) silane, and this nanocomposite exhibited enhanced surface hydrophobicity and bacterial repellency. Indeed, thanks to the synergistic effects of PACT and antibacterial adhesion, the hybrid nanomaterials showed a better disinfection rate against *E. coli* (up to 97%) than the PS alone. Another nanohybrid material, combining the effect of different materials has recently been described by Kuthati et al. [124]. In this study, the authors associated curcumin as PS, with silver nanoparticles, immobilized on copper-impregnated mesoporous silica nanoparticles. Their whole system exhibited a positive ζ-potential, up to + 35 mV, which allowed good binding to *E. coli*, while showing an efficient membrane disorganization, resulting in an efficient photo-active disinfectant material against *E. coli*.

3.2.4 Silver NP

Currently, PSs have been combined with silver nanoparticles (AgNPs), as the silver bactericidal effect is widely known. In this field, AgNPs exhibit a pronounced antimicrobial effect, especially against Gram-negative bacteria, being a potential complement to PACT. The mechanism of AgNPs bactericidal effect relies in the destruction of bacterial membranes, thanks to the release of Ag^+ ions. Moreover, the conjunction of PS and AgNPs has resulted in a synergistic effect of the bactericide effect of the Ag^+ and ROS. Several examples can be found in the literature, using TMPyP [125] or MB [126] as PS. Similar observations were made with a phthalocyanine **167,** which was immobilized to a cellulose fabric and the decorated with AgNPs [127]. In this study, Chen et al. hypothesized that the strong electric field around the silver nanoparticles can promote the optical absorption of **167** and singlet oxygen generation, as already observed by other groups. Recently, Macia et al. have proposed an improvement of this interaction between AgNPs and PS, comparing the effect of the shape, in RB-decorated silica-coated silver nanocubes (Ag@SiO_2-RB Ncs) or silver nanospheres (Ag@SiO_2-RB Nss) [128]. They evidenced that the intrinsic electromagnetic hotspots produced by the lightning-rod effect in anisotropic metal nanoparticles permitted a better bactericidal efficiency. A viability decrease of *E. coli* of 6 logs was observed in the case of Ag@SiO_2-RB NCs, compared to 4 logs in the case of RB-Ag@SiO_2 NSs, whereas their hydrodynamic diameters and ζ-potential were within the same range.

3.2.5 Graphene quantum dots

In another example, AgNPs were combined with Graphene Quantum Dots (GQD) [129]. GQDs are zero-dimension carbon nanomaterials that exhibit excellent photoluminescence, water dispersibility, biocompatibility, and low cytotoxicity properties, as well as the capacity to photogenerate ROS. In this study, the authors aimed to combine both photodynamic and photothermal effects, finding an increased ROS generation by the GQD in the conjugate, which could be attributed to GQDs stabilization onto AgNPs surface. Furthermore, the antibacterial effect against *E. coli* was enhanced, when compared to the free GQDs, resulting from the synergistic effect of enhanced PDT, efficient PTT, and the unique properties of AgNPs.

GQDs can also be used as a two-photon PS. Normally, PSs get excited through one-photon absorption, requiring the absorbance of a single high-energy photon; in two-photon absorption, PSs get excited through the absorption of two low-energy photons, which together replace the energy of a single high-energy photon. This is advantageous, as it permits the efficient excitation of PSs using wavelengths where the skin is transparent (600–800 nm), increasing its biological value [130]. As an example, the use of GQDs by Kuo et al., leads to an efficient elimination of *E. coli,* after ultra-low-energy (800 nm) irradiation from a femtosecond laser during only 15 s [131]. In order to further improve the specificity and the antibacterial efficiency of their GQD, the authors coated them with LPS, a major component of the

outer membrane of *E. coli*. More recently, Huang et al. also modified GQD surfaces in order to enhance their efficiency in PACT. Indeed, they developed spermidine co-doped polymeric GQDs, that could be photo-activated by very short LED irradiation (1 min.), in order to accelerate wound healing and thus reduce the risk of recurrent infections [132]. They evidenced that their positively charged materials could directly interact with the cell membrane of bacteria, thereby disrupting the membrane's integrity, but have not observed a significant photothermal effect on the inactivation of bacteria, as the temperature during the irradiation, just increased from 25 to 30 °C.

GQDs have also been combined with organic PS, such as MB [133] or as TCPP-loaded zirconium-based metal–organic framework [134]. In the first example, the sulfur-doped GQDs improved the singlet oxygen generation of MB, by increasing both the lifetime of the triplet state of methylene blue and the efficiency of internal system crossing from singlet-MB to triplet-MB, increasing the bactericidal efficiency against *E. coli* [133]. In the second example, the authors developed a novel textile material, with GQDs being grafted onto the cotton fiber surface, via amide bond after chemical modification, and a MOF being synthesized in situ. They achieved an enhanced $^1O_2$ generation, thanks to Förster resonance energy transfer (FRET), from GQDs to MOF, and due to the singlet oxygen diffusion through the MOF's porous structure. Furthermore, their material was able to achieve 6-log reduction of inactivation of *E. coli* and *P. aeruginosa* [134].

3.2.6 Carbon quantum dots

As GQD, Carbon quantum Dots (CQDs) represent a promising alternative for photodynamic applications. CQDs are mostly prepared by bottom-up synthetic strategies and have spherical shape of up to 10 nm, whereas GQDs are typically derived from graphene/graphite, or other graphitic 3D material, by top-down synthetic approaches [135]. Knoblauch et al., has recently studied brominated CQDs and evidenced that they were able to generate ROS, via both Type-I and Type-II mechanisms, which led to growth inhibition of *E. coli* [136]. Unfortunately, the authors also observed dark toxicity, due to the pH-triggered release of reactive nitrogen species, both under dark and UV exposed conditions. In addition, it is noteworthy to mention that, once again, this material exhibits negative ζ-potential, while still being efficient against bacteria. Nie et al. also recently published two studies focused on CQDs synthesized from citric acid and 1,5-diaminonaphthalene in ethanol, using a one-pot solvothermal method, either used as such [137] or trapped in polyacrylonitrile electrospun nanofibers [138]. In both cases, materials were more efficient against Gram-negative *E. coli* than against the Gram-positive *S. aureus*. The authors hypothesized this can be due to the physical interaction between the CQDs and the bacteria, which was favored by the rod-shaped *E. coli* whereas *S. aureus* tends to form grape-like clusters, preventing ROS diffusion toward bacteria residing within the interior of these clusters. These systems exhibited positive ζ-potential and were able to produce only singlet oxygen as ROS.

3.2.7 Other carbon-based materials

Single-wall carbon nanotubes (SWCNT) and Multiple-Wall Carbon Nanotubes (MWCNT) have also been successfully used as antimicrobial materials against Gram-negative strains, having attracted attention as drug delivery systems, especially as vectors in PACT or PDT. The potential broad-spectrum antimicrobial action of carbon nanotubes also enhances their efficacy of antibacterial therapy [139]. In recent studies, RB conjugated to MWCNT [140] or MB conjugated to SWCNT [139], evidenced an enhanced photodynamic efficiency against *E. coli*, disregard the charge of the PS, and due to a greater interaction with bacteria. In the same line, Yu et al. reported in 2019 a supramolecular self-assembly of a polycationic porphyrin (**168**, Fig. 18) and graphene nanoribbons, grafted with poly(ethylene oxide) chains to afford excellent dispersibility in aqueous solution [141]. Their new nanocomposite combined both PDT and PTT, being photo-activable at 660 nm and 808 nm, respectively, for the treatment of bacterial infection. It was demonstrated to be efficient against a wide range of bacterial strains, including *E. coli* and *P. aeruginosa,* while being photostable and with minimal toxicity against human cells.

It is noteworthy to underline that MB has been loaded in non-natural hydrogels, with good results toward bacteria, as the polyacrylamide hydrogel grafted with cationic phenothiazinium derivatives, developed by Spagnul et al. [142].

4 Strategies for PACT potentiation

Along this work we have described several improvements in the development of photosensitizing molecules, systems and scaffolds. Noteworthy, several of these strategies failed by themselves, and researchers craftily overcame these difficulties through the use of potentiation strategies and targeting methods. These methods are further discussed in the following sections, shedding light on these strategies.

4.1 Potentiation with antibiotics

In previous sections, we have discussed the concomitant use of PSs with some well-known antimicrobial molecules, such as colistin [94] and difloxacin [96]. This conjugation can increase the target selectivity and, eventually, the efficacy

of disinfection, by disturbing the membrane integrity of microorganisms.

Another strategy is the conjugation of PS with well-known antibiotics. As an example, Nieves and co-workers demonstrated the use of gentamicin as a targeting unit in a covalent conjugation strategy [143]. Their conjugate (**169**, Fig. 18) showed a significant inactivation of *E. coli* strains at sub-micromolar concentrations.

Recently, the use of verapamil, a small molecule acting as a multidrug resistance modulator, demonstrated to have an incidence on PS uptake. Verapamil inhibits the activity of the Multidrug and Toxic compound efflux (MATE) pumps, by binding to their active site. Sułek et al., have demonstrated that the photodynamic activity of TMPyP (**6**, Fig. 5), TPPS (**163**, Fig. 17) and Cl_2TPPS (**170**, Fig. 19) is increased when verapamil is added into the media. The authors showed that verapamil leads to a small increase in the cellular uptake of the porphyrins; however, this is reflected in 1–2-log additional reduction of *E. coli* viability. Then, they attributed this effect to verapamil preventing the efflux of the porphyrins out of the cell during the photodynamic inactivation, which implies that porphyrinic compounds are effluxed out of the cell by MATE pumps [144]. Furthermore, De Aguiar Coletti et al. have tested the synergistic effect of the combination verapamil/MB on biofilms of *S. aureus* and *E. coli* [145]. For *E. coli* biofilm, the combination of 215 μg/mL of verapamil and 200 μg/mL of MB, and a light dose of 44 J/cm^2 enhanced the biofilm reduction by 3.4 log.

4.2 Potentiation with oligopeptides and amino acids

Other authors have used antimicrobial peptides (AMPs) for targeting bacterial cell membranes by electrostatic interactions, resulting in the disruption of membrane integrity. As an example, polymyxins are non-ribosomal lipopeptides, used for the treatment of infections caused by Gram-negative bacteria, with colistin being one member of this family [146]. Colistin conjugation to PS has demonstrated an increase in the PS uptake by Gram-negative bacteria [147]. In recent years, Le Guern et al. have developed several conjugates with polymyxin B (**171**–**173**, Fig. 20), obtaining enhanced antimicrobial activity against Gram-negative bacteria [95, 148, 149]. Indeed, a cationic porphyrin was attached to a polymyxin B derivative, using thiol-maleimide click chemistry, and the obtained conjugate presented an enhanced PACT efficacy against *P. aeruginosa* and *E. coli*. Similarly, the previously described **149** (Fig. 17) bears three colistin molecules via imine formation [94], being embedded into chitosan hydrogels, and resulting in efficient inactivation of *P. aeruginosa*. Additionally, other authors have developed a probe associating MB and polymyxin B, as a theranostic agent for bacterial infections [150, 151]. However, we should pinpoint that researchers need to be careful when selecting an antibiotic for PS conjugation, as although colistin and polymyxin are efficient antibiotics, they are considered as "last resource" molecules, due to their inherent toxicity and proneness to generate AMR.

Previously, we have described the use of AMPs as targeting moieties against bacteria, taking advantage of their own antimicrobial activity. However, these are not the only examples using amino acids and oligopeptides as targeting molecules. As an example, PPIX was bound to the $(KLAKLAK)_2$ peptide (**174**, Fig. 20) [152], presenting an excellent activity against both *S. aureus* and *E. coli*. In another example, the AMP Aurein 1.2 was concomitantly used with MB, chlorin-e6 and curcumin, and tested as a disinfection treatment against several bacterial strains, showing modest

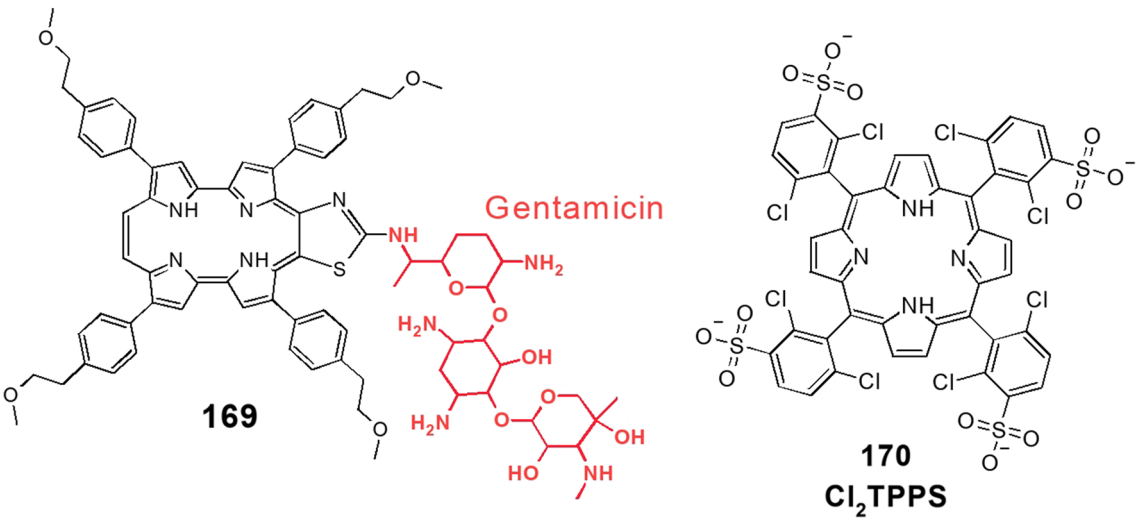

Fig. 19 Some of the PSs potentiated with antibiotics, described along this work

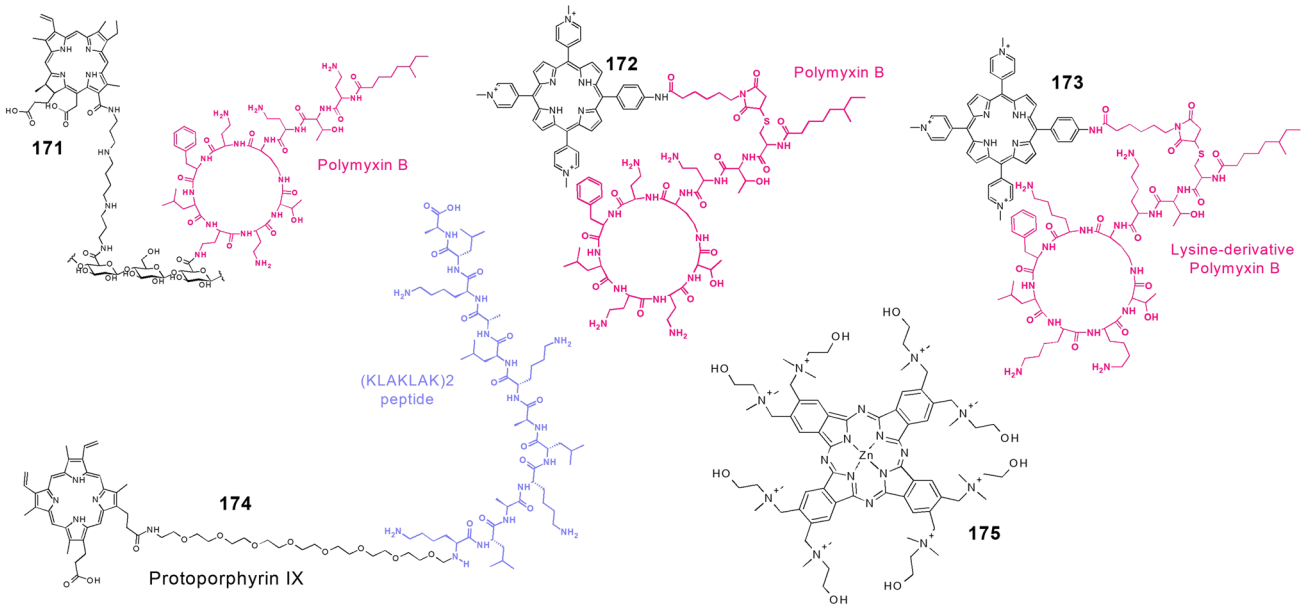

Fig. 20 PSs conjugated with peptides or AMPs. Finally, the structure of a cationic phthalocyanine used to predict the internalization of photosensitizers in Gram-negative bacteria

results against *E. coli* bacteria, contrasting with the excellent results obtained against Gram-positive strains [153].

4.3 Potentiation by potassium iodide

The use of inorganic salts as potentiation agents in PDT and PACT has been previously addressed but not fully described [154]. Some of our previous examples have shown that some of the neutral and anionic PSs are only efficient against Gram-negative bacteria when KI is present into the media [40, 59, 82]. The use of KI started as a serendipitous observation, but now it is widely been explored as a potentiation of PACT alternative.

Trying to shed some light into this issue, Wen et al., demonstrated that RB, a photosensitizer mostly inactive against Gram-negative bacteria, efficiently eradicates *E. coli* and *P. aeruginosa* when complemented with KI. Furthermore, they provided insight into the mechanistic of this potentiation, being found that anionic iodine (I^-) is released as a function of irradiation, when RB is present. Then, the authors hypothesized that I^- reacts with singlet oxygen, forming the radical iodide and the superoxide anion, forming a cascade of reactive oxygen species that lead to bacterial death [155]. This agrees with observations from other researchers, as Santos et al., [82], who hypothesized that the remarkable results obtained against *Salmonella* are due to the stabilization of the singlet oxygen with the ion I^-, leading to the formation of peroxyiodide and other radical species. Then, PACT potentiation with KI is cheap, non-toxic, and uses a widely available inorganic salt, and the good results obtained so far foreseen a deeper exploration in the years to come.

5 Conclusions

In the present work, we had explored some of the most recent publications on the photodynamic applications for Gram-negative eradication. It is widely known that cationic photosensitizers are more efficient against Gram-negative bacteria, as they are more likely to bind to the negatively charged lipopolysaccharides that conform their outer membrane. So a general assumption would be that the greater the number of charges, the best the photobactericide effect. But most of the examples described here have demonstrated that this is not necessarily true, sometimes finding that "less is more." Although there is barely any literature on this regard, some studies indicate that the binding of the cationic photosensitizer **175** (Fig. 20) to the outer membrane is an instantaneous process [156], which could indicate an electrostatic interaction. Through computational analysis, the authors have predicted that the photosensitizer binding to the anionic moieties is just the first step before a self-promoted uptake. Although this computational prediction was done with a very simple photosensitizer, this can provide an insight into the way other types of photosensitizers are internalized by Gram-negative bacteria. But this may be limited by the size of the photosensitizer, which sometimes is not taken in account by researchers, who, trying to add the most cationic moieties, end up with bulkier photosensitizers.

In this aspect, small core molecules, such as BODIPY, phenalenones, and subphthalocyanines, are strong candidates of a new generation of photosensitizers, permitting to design cationic non-bulky molecules. It seems relevant that some very efficient molecules possess both cationic and lipophilic moieties, thus suggesting that asymmetric photosensitizers are strong candidates for Gram-negative disinfection.

Something that in most of the cases is underrepresented is the biological effect of other photophysical mechanisms, such as Type-I and Type-III. Most of the researchers look forward for highly efficient singlet oxygen systems, and while it is true that singlet oxygen is an efficient cytotoxic molecule, we should not underestimate the potential of other light-driven ROS. So along with new molecules, it is likely to see in the future systems with more diverse photodynamic mechanisms.

Although it seems that the quest for the "perfect photosensitizer" still has a long way to go, several of the results shown here demonstrate that even photosensitizers that are not impressively efficient can be potentiated through the use of formulation systems or through other enhancing systems, such as the use of inorganic salts, their synergy with antibiotic molecules, and even their conjugation with antimicrobial peptides.

Infectious diseases caused by Gram-negative bacteria are still a concern challenge in daily life, but PACT has demonstrated to be an effective bactericide alternative. Challenges presented, such as low internalization and binding toward Gram-negative bacteria, are currently being addressed not only through the development of new photosensitizer structures but also through the development of delivery and potentiation systems. Currently, there are not active clinical trials of photosensitizers aiming for the disinfection of Gram-negative bacteria, but this panorama is likely to change in the next few years, given the increasing rate of publication of articles related to photodynamic disinfection.

We conclude that further efforts need to be poured into this topic, as the results are already promising and fruitful. Then, not only scientists but also politicians should focus on the devotion of more resources and politics, inverting the current trend and leading to an ever-growing pipeline of antibacterial alternatives.

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Author contributions SL-L contributed to conceptualization, funding acquisition, project administration, and supervision; NM-C was involved in software; NM-C; T-SO, and SL-L were involved in investigation, visualization, writing—original draft, and writing—review and editing.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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