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Antimicrobial Photodynamic Therapy to Kill Gram-negative Bacteria

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Abstract

Antimicrobial photodynamic therapy (PDT) or photodynamic inactivation (PDI) is a new promising strategy to eradicate pathogenic microorganisms such as Gram-positive and Gram-negative bacteria, yeasts and fungi. The search for new approaches that can kill bacteria but do not induce the appearance of undesired drug-resistant strains suggests that PDT may have advantages over traditional antibiotic therapy. PDT is a non-thermal photochemical reaction that involves the simultaneous presence of visible light, oxygen and a dye or photosensitizer (PS). Several PS have been studied for their ability to bind to bacteria and efficiently generate reactive oxygen species (ROS) upon photostimulation. ROS are formed through type I or II mechanisms and may inactivate several classes of microbial cells including Gram-negative bacteria such as *Pseudomonas aeruginosa*, which are typically characterized by an impermeable outer cell membrane that contains endotoxins and blocks antibiotics, dyes, and detergents, protecting the sensitive inner membrane and cell wall. This review covers significant peer-reviewed articles together with US and World patents that were filed within the past few years and that relate to the eradication of Gram-negative bacteria via PDI or PDT. It is organized mainly according to the nature of the PS involved and includes natural or synthetic food dyes; cationic dyes such as methylene blue and toluidine blue; tetrapyrrole derivatives such as phthalocyanines, chlorins, porphyrins, chlorophyll and bacteriochlorophyll derivatives; functionalized fullerenes; nanoparticles combined with different PS; other formulations designed to target PS to bacteria; photoactive materials and surfaces; conjugates between PS and polycationic polymers or antibodies; and permeabilizing agents such as EDTA, PMNP and $CaCl_2$. The present review also covers the different laboratory animal models normally used to treat Gram-negative bacterial infections with antimicrobial PDT.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

Keywords

Antimicrobial photodynamic therapy; gram-negative bacteria

ANTIMICROBIAL PHOTODYNAMIC THERAPY – BRIEF INTRODUCTION

The spread of multi-resistant bacterial strains is one of the most worrying threats to public health in recent years and has arisen due to the excessive use of antibiotics [1]. In view of the prediction of the “end of the antibiotic era” [2], antimicrobial photodynamic therapy (antimicrobial PDT) is starting to be considered as a promising alternative approach to resistant infections and has the further advantage of not leading to the selection of resistant strains [3–5].

Antimicrobial PDT is particularly good for dental [6–11] and dermatological [5, 12, 13] applications, involving the light irradiation of a tissue containing microorganisms that were previously exposed to a photosensitizing dye (PS). This PS should be able to generate reactive oxygen species (ROS) in the presence of light and oxygen [3]. In addition, the PS is often derived from the tetrapyrrole aromatic nucleus found in many naturally occurring pigments such as heme, chlorophyll and bacteriochlorophyll [14].

In order to be suitable for antimicrobial PDT, the ideal PS should possess low levels of dark toxicity and the presence of absorption bands in the so-called optical window (600–900 nm) for sufficient tissue penetration of light [14]. Moreover, the PS should have relatively high absorption bands (>20,000–200,000 $M^{-1}cm^{-1}$), that in turn will diminish the dose needed to deliver the desired effect. The PS should also have a high yield of excited electronic triplet state and of singlet oxygen [14].

The generation of ROS as a consequence of PDT follows two main pathways characterized by different photochemical mechanisms called “type I” and “type II”. The PS should be excited by visible light of the correct wavelength (wavelength absorbed by the PS) to enter a long-lived triplet state. This particular state of the PS can then interact with molecular oxygen by energy transfer (Type II) or by electron transfer (Type I) cprocesses. Type II mechanism will lead to singlet oxygen production while type I will form superoxide anion that can go on to form more reactive ROS such as hydroxyl radicals [15] (Fig. 1).

After being excited to the short-lived singlet state the PS can lose energy by fluorescence, heat conversion or can undergo intersystem crossing to the long-lived triplet state. In case the PS is a fullerene, energy loss by fluorescence is negligible, and in the absence of oxygen fullerene triplet states lose energy by phosphorescence [14].

Antimicrobial PS should be able to kill multiple classes of microbial cells at relatively low concentrations and low fluences of light. The PS should also be reasonably nontoxic in the dark and should show selectivity for microbial cells over host mammalian cells. In fact, the microbial uptake process of PS with cationic substituents such as quaternary ammonium groups is rapid when compared to the uptake of these PS by host mammalian cells, which slowly occurs over time [16]. Therefore, if light is delivered soon after applying the PS to the infected area, microbial cells can be killed without causing harm to the host tissue [6, 17].

PDT APPLICATIONS FOR GRAM-NEGATIVE BACTERIA

Gram-negative bacteria are responsible for many life-threatening infections in humans, especially in elderly people, and they are often innately resistant (especially *P. aeruginosa*)

to the most commonly used antibiotics, making the search for new antibacterial drugs and alternative therapies, such as PDT, very important [18].

While Gram-positive bacteria present a thick and porous cell wall of inter-connected peptidoglycan layers that surround a cytoplasmic membrane [19, 20], Gram-negative bacteria have an outer membrane, a thinner peptidoglycan layer and a cytoplasmic membrane [19]. In other words, Gram-positive bacteria possess a porous layer of peptidoglycan and a single lipid bilayer, while Gram-negative bacteria have a double lipid bilayer sandwiching the peptidoglycan layer plus an outer layer of lipopolysaccharide, which results in a low degree of permeability for lipophilic small molecules [14]. The cell wall of fungal yeasts such as Candida shows an intermediate permeability between Gram-positive and Gram-negative bacteria (Fig. 2).

The outer membrane of Gram-negative bacteria plays an important role that is related to resistance to many antibiotics that are highly effective against Gram-positive bacteria, e.g. macrolides, novobiocin, rifamycin, lincomycin, clindamycin and fusidic acid. This explains the higher prevalence of Gram-negative infections in the modern hospital environment [21]. Accordingly, to perform antimicrobial PDT, the PS employed needs to penetrate the cell walls of the bacteria and end up in the plasma membrane or in the cytoplasm; however, the membrane barriers of the bacterial cell limit the simple diffusion of PS into the bacterial cytosol [22]. Therefore, PDT-killing of Gram-positive bacteria is definitely much easier to accomplish than that of Gram-negative bacteria.

Thus, it is more difficult to obtain highly potent PS to mediate PDT of Gram-negative bacteria since their membrane barrier prevents the uptake of anionic and neutral PS [18, 23, 24]. Nevertheless, different approaches have been documented and these aim to efficiently kill Gram-negative bacteria via PDT. Several of these approaches involve the optimization of the chemical structure of the PS and are listed further in this review.

OUTER MEMBRANE-DISORGANIZING AGENTS

As stated above, the primary difficulty of killing Gram-negative bacteria using PDT is to achieve a good penetration of the PS inside the bacterial cell wall. However, different approaches aim to eliminate this problem by, for example, creating positively charged (cationic) PS or by coupling or combining the PS with positively charged entities such as poly-L-lysine [25], polyethylenimine [26] and polymyxin B nonapeptide (PMBN) [27].

The PS molecule, for instance, has to bind to the bacterial cell [28], most often to the cell plasma membrane [29] so the PDT killing effect can take place [27]. Gram-positive bacteria and yeasts are affected by neutral or anionic metal-free porphyrins [30], while Gram-negative bacteria are not. This resistance to photosensitization by Gram-negative bacteria with anionic porphyrins was widely reported in the literature of the 1980's [28, 31–33]. PDT of both Gram-negative *Escherichia coli and P. aeruginosa* with high concentrations of hematoporphyrin derivative (HPD) or deuteroporphyrin (DP) combined with high intensities of illumination did not result in any bacterial inactivation [28, 31, 33, 34].

In addition, *E. coli* was only sensitive to porphyrin and light after suffering a pretreatment with toluene, which then induced susceptibility of this Gram-negative bacteria to PDT with hematoporphyrin derivative [35]. It is only when the inner membrane of *E. coli* is exposed that porphyrin can bind to this membrane [29, 35]. Knowing this and the fact that the polycationic agent polymyxin nonapeptide can disturb and disorganize the outer-membrane structure of Gram-negative bacteria [36, 37], Nitzan *et al.* (1992) were able to successfully kill *E. coli* and *P. aeruginosa* with PDT mediated by deuteroporphyrin (DP) [27], what represented a true advance in photodynamic inactivation of Gram-negative bacteria.

Nevertheless, neither of these results [27, 38] resolved the problem of Gram-negative bacterial resistance [18].

One simple approach to turn Gram-negative susceptible to PDT is to pre-treat them with ethylene diamine tetraacetic acid (EDTA). It is known that Gram-negative wild-type cells treated briefly with EDTA lose up to 50% of their lipopolysaccharide into the medium and become very sensitive to hydrophobic agents [18]. Bertoloni *et al*. [34] pre-treated Gram-negative *E. coli* and *Klebsiella pneumoniae* with EDTA and performed PDT with hematoporphyrin, stating that cells retained their resistance to hematoporphyrin and light exactly as before exposure to EDTA. However, Bertoloni *et al*. [24] showed that with non-porphyrin sensitizers (phthalocyanines) and a pre-treatment with tris-EDTA that altered the outer membrane of the bacteria, a successful response could be obtained for *E. coli.* The response was better with Zinc phthalocyanine than it was with zinc monosulfonated and disulfonated phthalocyanines.

In fact, cationic molecules can more easily bind to the cell wall of Gram-negative bacteria, which is negatively charged due to teichoic acid residues, for example [19]. The negatively charged LPS molecules also have a strong affinity for cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), the binding of which is required for the thermodynamic stability of the outer membrane [23].

Again, considering the physical arrangement of the lipopolysaccharide layer of the Gram-negative bacteria outer membrane, treatment with low concentrations of polycations that tend to bind tightly to the highly negatively charged surface and to displace divalent cations can be effective [18]. As previously stated, the combined exposure to PMBN, DP and light inhibited *E. coli* and *P. aeruginosa* cell growth [27]. In addition, it was stated the disappearance of plasmid super-coiled fraction of *E. coli* when post-treated by PMBN and DP [39].

Besides PMBN [40] and EDTA [41], polycationic agents such as protamine and a lysine polymer with 20 residues (Lys20) can disorganize the outer membrane, thus rendering various Gram-negative bacteria extremely sensitive to hydrophobic antibiotics, dyes and detergents [36, 37]. Both PMBN and Lys20 sensitize the bacteria by a mechanism similar to that of EDTA-treated cells; however, Lys20 releases about 30% of lipopolysaccharide into the medium, while PMBN does not cause any release of lipopolysaccharide [18].

Finally, a disturbance in the outer membrane of Gram-negative bacteria must occur so porphyrins and phthalocyanines can act in their inner membrane. In that way, the permeabilizing agent PMNP can disrupt the outer membrane and allow the penetration of porphyrin, consequently enabling the photosensitization of Gram-negative bacteria [18]. In addition, through the same mechanism, EDTA treatment combined with phthalocyanines inactivate those bacteria and consist in a promising photodynamic therapy [18].

A patent discloses the method of combining photosensitizing dyes “such as methylene blue, methylene green or toluidine blue, combined with pyrrolnitrin and optionally combined with a surfactant material, such as SDS, polymixin B, cetrimide or benzalkonium chloride along with different wavelengths and light doses to constitute a photodynamic therapy that advantageously acts as a broad spectrum antimicrobial treatment” [42]. According to the patent, this kind of PDT may be utilized before surgical operations and, in fact, this photosensitizer/pyrrolnitrin/surfactant combination comprises an invention useful to destroy gram-positive and gram-negative bacteria, as well as fungi, viruses and spores, being even useful when acting upon biofilms [42].

PHOTOSENSITIZERS WITH CATIONIC CHARGES

It is well known that Gram-positive bacteria can be inactivated with PDT [22, 34, 43]; however, as discussed above, Gram-negative bacteria are far more resistant to this therapy [18]. To overcome this limitation, besides permeabilizing the outer membrane with PMBN [27] or Tris/EDTA [24] to allow non-cationic PS to be used, some cationic PS may also be employed.

A pronounced polycationic charge that is present in many naturally occurring antibacterial peptides [44] allow them to bind to negatively charged bacteria and subsequently disturb the outer-membrane permeability barrier [45]. Consequently, an efficient photodynamic inactivation can be achieved with the Gram-negative bacteria *E. coli* and *P. aeruginosa* when incubated for 30 minutes with the cationic water-soluble zinc pyridinium phthalocyanine (PPC), but not if incubated with a neutral tetra-diethanolamine phthalocyanine or a negatively charged tetra-sulphonated phthalocyanine [46].

Other reports show that the incubation of *E. coli* with a cationic phthalocyanine in the dark caused alterations in the outer membrane permeability and increased the uptake of hydrophobic compounds. Interestingly, adding Mg^{2+} to the cells previous to incubation with this PS inhibited these alterations in the membrane and also avoided the PDI of the bacteria [47]. Among antibacterial polycations we may cite polymixins [48], protamine [49], insect cecropins [50], reptilian magainins [51], various cationic leucocyte peptides (defensins [52], bactenecins [53], bactericidal/permeability increasing protein [54]), polymers of basic amino acids [55] and polyethyleneimine [56].

A polycationic covalent conjugate between the PS chlorin$_{e6}$ (c_{e6}) and poly-L- lysine (pL) average molecular weight 2 kDa, 20 lysine residues) were able to efficiently promote photo-inactivation of both Gram-negative and Gram-positive bacteria after short periods of incubation and illumination with red light [57]. To be clinically effective photodynamic inactivation (PDI) needs to be performed with a PS that can kill both classes of bacteria, so it can be employed prior to the identification of the infectious agent [25]. In that way, the cationic pL-c_{e6} was much more effective than the neutral acetylated pL-c_{e6}-ac and the anionic succinylated pL-c_{e6}-succ conjugates against Gram-negative and Gram-positive bacteria [57]. Moreover, c_{e6} attached to a pL chain of 20 amino acids giving a conjugate with an approximate 1:1 substitution ratio was effective to kill both Gram-positive *Actinomyces viscosus* and Gram-negative *Porphyromonas gingivalis* through PDI [57]. Consequently, this efficacy was lost if the conjugate was rendered neutral by polyacetylation, or anionic by polysuccinylation.

As previously said, polycationic dyes need to gain access through the outer membrane to more sensitive parts of the cell [25]; however, the efficacy of this process depends on the size of the polycationic chain [25]. Conjugates with eight, thirty-seven lysines and free c_{e6} can efficiently inactivate *Staphylococcus aureus;* but only the conjugate with thirty-seven lysines could kill *E. coli.* It is plausible that 37-lysine can interact with the outer membrane of *E. coli*, perhaps causing the loss of some LPS and rendering the remaining LPS more permeable, allowing the conjugate to penetrate. On the other hand, the 8-lysine conjugate did not provoke the same effect, which was probably due to its insufficient polycationic character [25].

NOVEL PS FOR ANTIMICROBIAL PDT OF GRAM-NEGATIVE BACTERIA

One of the most studied groups of PS consists of porphyrin derivatives, which are described in inventions and may act as photodynamic agents, since these derivatives generate reactive oxygen species such as singlet oxygen or oxygen free radicals when irradiated with

appropriate wavelengths and in the presence of oxygen. Consequently, these compositions are suitable for curative or prophylactic treatment of several medical conditions including infections with Gram-negative cocci (e.g *Neisseria* sp.) and Gram-negative bacilli (e.g *E. coli*) [58–60].

The halogenated xanthenes additionally constitute a family of potent photosensitizers, since they also become photoactivated upon shining visible light on the treatment site that was previously exposed to these compounds [61]. These medicaments are in turn suitable for intracorporeal administration and thus were employed to achieve photodynamic therapy in human or animal tissues. In three distinct inventions, the primary component of given medicaments is a halogenated xanthene or a halogenated xanthene derivative. Furthermore, this xanthene molecule is more preferably Rose Bengal or a functional derivative of Rose Bengal [61–63].

”These medicaments are broadly suitable for improve PDT of various conditions related to microbial, viral, fungal or parasitic infections of human and animals. Such therapy is claimed to be useful for bacterial and antibiotic resistant bacterial infections, including those caused by Gram-positives and Gram-negatives, *Streptomycetes* sp., *Actinomycetes* sp., *Staphylococci* sp., *Streptococci* sp., *Pseudomonas* sp., *E. coli, Mycobacteria* sp. and others. In addition, the medicaments can be applied through conventional intracorporeal administration modes and even to tissues exposed during surgery, such as endoscopic surgery or other endoscopic procedures.” [61–63].

Biologically active phenoselenazinium compounds can be used directly or in combination with a polymer to promote *in vivo* PDT for infections like gum abscesses, gum disease, gingivitis and plaque biofilms. In fact, the compounds presented in this invention retain advantages to antimicrobial treatments, once they are able to inactivate antibiotic resistant bacteria such as MRSA. The compounds are also highly effective against Gram-positive and Gram-negative bacteria, besides fungal infections and quiescent/stationary bacteria [64].

As expected, the susceptibility of bacteria to phenothiazinium mediated PDT depends on whether the bacteria are Gram-positive or Gram-negative. New methylene blue and di methyl methylene blue, for example, were proven to be efficient at inactivating MRSA [65]. Biologically active methylene blue derivatives are also effective in deactivating a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, MRSA and fungal infections [66–68].

In addition, these phenothiazinium dyes are active against quiescent/stationary bacteria, have high selectivity with minimum damage to the host tissue, besides presenting low levels of photobleaching. Following illumination the tetra-n-pentyl-3,7-diaminophenothiazin-5-ium compound led to >3 log reduction in CPU/mL for both log phase, Gram-negative (*E.coli, P.aeruginosa*) and Gram-positive bacteria (*S. aureus*, MRSA) [66–68].

On the other hand, naturally occurring and synthetically available siderophore structures can be conjugated chemically with photoactive compounds such as chlorin e_6 to improve their penetration into bacterial cells, via microbial proteins that recognize and transport iron-loaded siderophores. In that way, PS that otherwise could not cross the cell wall and membranes can then be transported inside the bacteria [69] allowing Gram-negative bacteria to be susceptible to this particular approach.

Actually, the siderophore-transporting systems of microbes are specific to individual classes of bacteria and fungi. Therefore, siderophore-conjugates with PS are not taken up by mammalian cells, what makes them a good alternative for antimicrobial PDT, since they are not harmful to the host and are truly specific for pathogenic microbes. Thus, the application

of this method presents a highly efficient treatment of pathogenic Gram-positive and Gram-negative bacteria such as *P. aeruginosa, E. coli, Streptococcus pyogenes, S. aureus*, as well as for other antibiotic resistant microbial infections including infections that occur in chronic wounds [69].

Another patent describes a method and composition that utilizes Safranin O in conjunction with light irradiation, preferably around 530 nm in order to destroy microbes, especially bacteria. The Safranin O containing compound must be introduced to the treatment area and then, after a sufficient period of time, the light must be delivered to this area. This is an effective PDI approach for Gram-positive and Gram-negative bacteria, particularly good for areas surrounded by complex media such as blood serum, blood or saliva [70].

As mentioned above *E. coli* was only sensitive to porphyrin and light after suffering a pretreatment with toluene, which then induced susceptibility of this Gram-negative bacteria to PDT with hematoporphyrin derivative [35]. In addition, positively charged (cationic) PS, including porphyrins and pthalocyanines, promote efficient inactivation of Gram-negative bacteria without the need of modifying the natural structure of the cellular envelope [46, 71].

Nevertheless, the utility of known porphyrin-based antimicrobial agents is limited since these compounds are generally unable to differentiate target microbial cells from non-target host cells [72]. Finally, not all microbial infections are suitable for PDT, because some infection sites may not be accessible to light [72].

Considering the limitations of porphyrin-based PS as well as the difficulty of light penetration into the tissues, new methods for killing or attenuating the growth of microbial agents are needed. That is why a patent was issued in 2007 and describes the use of a compound which formula is based on porphyrin dyes but does not include exposing the compound to a photodynamic therapy light source or sonodynamic therapy ultrasound source [72]. In other words, the medicaments described in this method have an intrinsic antimicrobial activity, being toxic to bacteria, mycoplasmas, yeasts, fungi and viruses and non-toxic to host mammalian cells [72].

Another recent patent describes a method that involves a PS which has a composition with at least one photoactive ingredient in a chemically reduced state [73]. This PS is applied to a tissue or substrate in a way that its photoactive ingredient is chemically altered to a photoactive state. This oxidation process makes the compound able to inactivate the corresponding pathogen when stimulated with light of the correct wavelength [73].

By being in a transportable state (lipophilic or reduced state), these compounds mentioned above can more easily pass through certain barriers, which allow them to have increased anti-microbial activity against Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *Serratia marcescens*, black pigmented anaerobes such as *P. gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Aggregetobacter actinomycetemcomitans,* etc. [73].

De novo synthesis of bacteriochlorins is also described in a patent. The bacteriochlorins are useful for several purposes, being active agents in PDT [74]. In fact, the progressive $2e^-/2H^+$ reduction of the porphyrinic macrocycle effectively changes the HOMO/LUMO gap of the electron in the molecular orbital between porphyrins, chlorins and bacteriochlorins. After reduction, absorption in the red or near infrared region is largely increased (Fig. 3) [74]. At these wavelengths the light penetration into tissue is maximal and, accordingly, PDT mediated with the compounds described in this patent may be useful for treating opportunistic infections, infected burns, periodontal diseases, etc. [74].

In fact, chlorins differ from porphyrins by having one pyrrole ring reduced at the β-positions. Porphyrinic compounds can be effective PS for performing PDT in opportunistic infections, and particularly in soft tissues [75]. The infecting agents of such infections include *S. aureus, E. coli* and *P. aeruginosa*, and in the most preferable application the total light energy used for irradiation is between about 500 Joules and about 10,000 Joules [75].

A new invention describes a way to enhance the antimicrobial photodynamic activity of a PS when it is used in combination with an effective amount of chitosan [76]. Chitosan has been used and/or suggested for a wide variety of purposes, including flocculation of bacteria, yeasts and microfungi and is known as an antimicrobial material. In addition, PS incorporated to a chitosan membrane can provide a significant photokilling of *E. coli* [77]. Thus, combining chitosan to photosensitizers such as Rose Bengal, methylene blue and chlorin e_6 leads to the efficient inactivation of Gram-positive and Gram-negative bacteria and fungi. *S. aureus*, methicillin-resistant *S. aureus*, *Streptococcus epidermidis, S. pyogenes* and *P. aeruginosa* can be killed with the help of this particular invention [76].

Several improvements are continuously made in PDT. A relatively new patent describes an improved treatment for infections related to orthopedic conditions and surgery [78]. Actually, the method described in this invention comprises a PS selected from toluidine blue O, methylene blue, dimethylene blue or azure blue chloride that can be employed to both hard and soft tissues. Even military medical procedures are mentioned in the text, illustrating a particular utility for this therapy [78].

For this particular invention toluidine blue O is more preferably used in the form of "tolonium chloride" [78]. The given light delivery system plus the PS presented are proven to be effective against "Gram-positive and Gram-negative bacteria, including strains of *Peptostreptococcus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Actinomyces* sp., *Bifidbacterium* sp., *Coorynebacterium* sp., *Eubacterium* sp., *Lactobacillus* sp., *Propioibacterium* sp., *Pseudoramibacter* sp., *Nieserria* sp., *Veillonella* sp., *Actinobacilus* sp., *Campylobacter* sp., *Cantonella* sp., *Centipeda* sp., *Desulphovibrio* sp., *Enterococcus* sp., *Escherichia* sp., *Fusobacterium* sp., *Haemophilus* sp., *Porphyromonas* sp., *Prevotella* sp., *Selenomonas* sp., and *Treponema* sp." [78].

Hydrophilic cationic and anionic photosensitizers have been found to inactivate pathogenic bacteria. In a recent invention photosensitizers are formulated in calcium phosphate nanoparticles formulations for antibacterial PDT. The calcium phosphate nanoparticles including stabilizers cium phosphate nanoparticles including stabilizers "provide excellent storage stability and therapeutically effective amounts of photosensitizer for intravenous or topical administration" [79]. These formulations were tested against *S. aureus* and Gram-negative *P. aeruginosa* demonstrating a very high percentage of killing [79].

It has been said that certain edible or ingestible food dyes are equal to or even superior to synthetic chemical photodynamic agents. They are of non-toxic nature, which definitely configures an advantage. In addition, they have the ability to be safely consumed and their breakdown is always to safe and environmental friendly products [80]. By that means, an invention teaches how to treat an infected animal or decontaminate a surface, for example, by using a safe natural or synthetic food coloring agent that has photodynamic properties [80].

This same patent presents one or more food dyes or colors as photodynamic agents. They include natural or synthetic compounds, which may be defined as: if "natural", something that exists in or is formed by nature, and if "synthetic", something artificial or formed through a chemical process by human agency [80]. The PS may be selected from the group of "chlorophylls, carotenoids, flavenoids, quinonoids, coumarins, indigoids, curcuminoids,

befalins, acthocyanins, cyanines, indocyanines, phthalocyanines, rhodamines, phenoxazines, phenothiazines, phenoselenazines, fluoresceins, porphyrins, benzoporphyrins, squaraines, corrins, croconiums, azo compounds, methine dyes, and indolenium" [80].

Still according to this patent [80], "natural food dyes include but are not limited to annatto extract, anthocyanins, B-carotene, beta APO 8 Carotenal, black currant, burnt sugar, Canthaxanthin, caramel, carbo medicinalis, carmine, carmine blue, carminic acid, carrot, chlorophyll, chlorophyllin, cochineal extract, copper chlorophyll, copper chlorophyllin, curcumin, curcumin/CU-chloro, grape, hibiscus, lutein, mixed carotenoids, paprika, riboflavin, spinach, stinging nettle, titanium dioxide, turmeric, aronia/red fruit, beet juice, paprika extract, and paprika oleoresin. Synthetic food colors include but are not limited to allura red, amaranth, black PN, carmoisine, fast red E, erythrosine, green S, patent blue V, ponceau 4R, quinoline yellow, Red 2G, sunset yellow, and tartrazine. Examples of Lake food colors include but are not limited to Lake allura red, Lake amaranth, Lake brillian blue FCF, Lake carmosine, Lake erythrosine, Lake indigo carmine, Lake ponceau 4R, Lake quinoline yellow, Lake sunset yellow, and Lake tartrazine" [80].

These natural or synthetic dyes may be used to perform treatment or elimination of planktonic and biofilm microorganisms, including *Pseudomonas* sp., *E. coli, Campylobacter* sp., *Salmonella* sp., *Listeria* sp., *Staphylococcus* sp., *Aspergillus* sp. and *Fusarium* sp. [80]. Knowing that focal infections are characterized by high intensities of different bacteria and, consequently, high concentrations of their extracellular signal molecules sensing the cell density, another interesting approach is to target PS to the extracellular signal molecules secreted by the bacteria instead of on the bacteria themselves [81].

One of the characteristic signal molecules synthesized by Gram-negative bacteria is acyl-homoserine lactone (acyl-HSL, member of the autoinducer family AI-1) [81]. HSLs are common for all Gram-negative bacteria, whereas the cell density signal molecules (members of the autoinducer family AI-2) communicate between Gram-positive and Gram-negative bacteria. An efficient binding to the HSL-moiety of the acyl-HSL guarantees a broad spectrum of Gram-negative bacteria to be knocked out by light-stimulated PS that are attached to the acyl-HSL. According to the invention, the PS can also be packed into special liposomes with lipid chelators or multiple coupled to dendrimers in order to be especially suitable antimicrobial photodynamic therapy [81].

Moreover, a patent has stated that phthalocyanines substituted in specific positions of only one ring of the macrocycle with cationic groups or with protonatable groups are effective in inducing *in vitro* photoinactivation of viruses, fungi and bacteria. As disclosed by the invention, these metal-phthalocyanines can inactivate the Gram-negative bacteria *E. coli* [82].

In addition, novel tetra-substituted phthalocyanine derivatives have been demonstrated as having a high photo-toxic efficiency. These phthalocyanines also show photobleaching kinetics that allow them to be unaltered for a time sufficient for microbial photoinactivation and then undergo subsequent decomposition, avoiding toxicity by systemic absorption and induction of delayed phototoxicity [83, 84]. Due to their solubility in water a good bioavailability and fast metabolism within the organism can be guaranteed for these tetra-substituted phthalocyanine derivatives, that in turn allows them to be valuable for the PDI of Gram-negative bacterial infections [83, 84].

The same applicant mentioned above described a novel series of PS that have advantages over other known compounds. These compounds are actually meso-substituted porphyrins that have an absorption in the region of the visible spectrum, high molar extinction coefficients, and high quantum yield in singlet oxygen production [85]. As previously

mentioned, there are some limitations of porphyrin-based PDT. Among the limitations is the poor selectivity toward eukaryotic cells and the micoorganisms. In tumors, this selectivity can be enhanced by increasing the degree of hydrophobicity of the PS or by imparting amphiphilic properties to its molecule [86]. Alternatively, these meso-substituted porphyrins are conjugated with a bio-organic carrier, ensuring high efficiency and selectivity against the target, i.e. Gram-negative bacteria [85].

There are several problems in using the native chlorophyll (Chl) or bacteriochlorophyll (Bchl) extracts for PDT. For example, under normal light conditions and in the presence of oxygen they are very unstable. In addition, they are highly hydrophobic [87, 88]. To overcome such difficulties, an invention characterized conjugates of Chl and Bchl derivatives with amino acids, peptides and proteins to be used as PS for PDT. Conjugates of bacteriochlorophyll A with a serine and immunoglobulin-G, and of chlorophyll A with a serine were found to be effective against bacteria. In fact, the Gram-negative *E. coli* could be photo-inactivated after being treated with PDT mediated by conjugates of chlorophyll A with serine [87, 88].

The same applicant describes a patent that, among other objectives, aims to inactivate infectious agents using a PS composed of cationic tetracyclic and pentacyclic bacteriochlorophyll derivatives (Bchls) [89]. In this invention [89] Bchls preferably have “an onium group derived from a N-containing aliphatic or heterocyclic radical such as ammonium, guanidinium, imidazolium, pyridinium, and the like or a phosphonium, arsonium, oxonium, sulfonium, selenonium, telluronium, stibonium, or bismuthonium group, or basic groups that are converted to such onium groups under physiological conditions”.

Another synergistic combination could be very important in antimicrobial PDT of bacteria. An invention provides a composition that involves a PS and at least one paraben to provide a synergistic antimicrobial effect against Gram-negative bacteria [90]. This “paraben potentiation”, as described, relates to the fact that the composition has a higher antimicrobial efficacy against a Gram-negative bacteria if compared to the antimicrobial efficacy against the same Gram-negative organism when using the related PS alone plus the antimicrobial activity using the paraben alone [90].

The synergistic effect of paraben can be achieved with any suitable art-disclosed photosensitizer. According to this patent [90] the PS can be a “phenothiazinium salt (e.g., methylene blue, toluidine blue O and their derivatives, etc.), arianor steel blue, crystal violet, azure blue cert, azure B chloride, azure 2, azure A chloride, azure B tetrafluoroborate, thionin, azure A eosinate, azure B eosinate, azure mix sicc., azure II eosinate, haematoporphyrin HCl, haematoporphyrin ester, aluminium disulphonated phthalocyanine,. porphyrins, pyrroles, tetrapyrrolic compounds, expanded pyrrolic macrocycles, and their respective derivatives”.

Still according to the same patent [90] the Gram-negative bacteria susceptible to paraben potentiation include, but are not limited to, “*E. coli, P. aeruginosa, Acinetobacter* sp., and pathogenic Gram-negative organisms residing within the oral cavity (e.g., *Porphyromonas* sp*., Prevotella* sp*., Fusobacterium* sp*., Tannerella* sp*., Actinobacillus* sp*., Selenomonas* sp*., Eikenella* sp*., Campylobacter* sp*., Wolinella* sp., etc.). *P. gingivalis* and *P. intermedia* are examples of such oral pathogenic Gram-negative organisms”.

NONPORPHYRIN-BASED PHOTOSENSITIZERS

It would be fair to say that the use of porphyrin-based PS in PDT is much more common than that of non-porphyrin dyes. Consequently it is much easier to find new patents related

to the use of porphyrinic compounds; however, as found in many of the inventions described above, non-porphyrinoid PS may have some advantages over the classical porphyrins, and some of their characteristics are highlighted below.

The efficacy of porphyrin-based compounds have been investigated and compared to that of other PS. The poly-L-lysine chlorin(e6) conjugate (pL-c_{e6}), for example, was more efficient than toluidine blue O (TBO) in inactivating MRSA and extended-spectrum beta-lactamase (ESBL) *E. coli* [91]. This different response found for TBO versus pL-c_{e6} can be possibly explained by the different cell envelope structures found for Gram-negative and Gram-positive bacteria. Moreover, the outer membrane provides the Gram-negative bacteria strongly negatively charged lipopolysaccharides (LPS) that constitute a very effective permeability barrier to exclude hydrophobic and large hydrophilic solutes [91].

Nonetheless, cationic non-porphyrin photosensitizers, such as chalcogenopyrylium dyes, phenothiazinium and benzo[a]phenothiazinium derivatives, which include methylene blue and toluidine blue are among the PS that may be utilized to perform antimicrobial PDT [92]. A patent describes how TBO can effectively inactivate *B.cereus* spores after a short incubation of 10 minutes followed by irradiation with 635nm light [93]. Actually, this patent describes a killing ratio of more than 99.9% of these spores, which is interesting due to the fact that spores are highly resistant to heat, radiation and are highly impermeable to most molecules [94].

The same patent described above also shows efficient photoinactivation of *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus subtilis* and *Bacillus atrophaeus* spores utilizing TBO dye. Various other photosensitizing dyes were tested for their ability to mediate the photodynamic killing of *Bacillus* spores. “Azure A, Azure B, Azure C, methylene blue, dimethylmethylene blue, methylene green, methylene violet Bernthsen, methylene violet 3RAX, safranine O, neutral red, new methylene blue and malachite green were effective in serving as PS to kill *B. cereus* spores as well” [93].

Other non-porphyrin dyes include hypericin (Hyp), which naturally occurs in the Hypericum species, a genus of flowering plant known as St John’s Wort [95]. Recent results suggest that this dye may be useful for combating infections caused by Gram-positive and Gram-negative bacteria [96]. In fact, hyp-based PDT was able to efficiently inactivate the Gram-positive *Listeria* sp., whereas a comparatively much lower killing of Gram-negative *Salmonella* sp. could only be achieved with higher Hyp concentration, longer incubation time and higher illumination dose. Finally, a high-power pulsed light can enhance the photoinactivation of *Salmonella* sp. mediated by Hyp [96].

As previously found, hypericin may easily serve as a photosensitizing agent in antimicrobial PDT for Gram-positive bacteria. Accordingly, another recent study found efficacious inactivation (>6 log reduction) of Gram-positive methicillin-sensitive and methicillin-resistant *S. aureus* and no effect (<0.2 log reduction) on Gram-negative *E. coli* [97]. Nevertheless, developing cationic hypericin derivatives, as well as water-soluble formulations of hypericin and hypericin micelles have been considered to be relevant [98–100] and may enhance the efficacy of Hyp-mediated PDT of Gram-negative bacteria in the future [97].

Another class of PS that can be utilized for PDT is based on the cyanine dye backbone. Cyanine dyes are able to accumulate much more in the mitochondria of carcinoma and melanoma cells than in normal cells, *in vitro* and *in vivo* [101], that allows them to serve for antitumoral PDT [102–105]. Additionally, there is an interesting study showing the effects of 12 cyanine-derived synthesized compounds upon inactivation of Gram-positive and Gram-negative bacteria, yeast, and fungi. Among these compounds, four were actually

superior at killing Gram-negative cells, and this was attributed to their chemical structure comprising naphthol and cyano groups and pyrazole heterocyclic rings [106].

There are several other “non-porphyrin PS”, including psoralens, anthracyclines, triarylmethanes and acridines. Further investigation of these largely unexplored classes of compounds needs to be addressed [92] and may probably allow advances in antimicrobial PDT, especially for Gram-negative bacteria. Fullerenes, for example, are particularly photostable and manifest little photobleaching when compared to many other traditional tetrapyrrole-backbone based PS found in porphyrins and chlorins [107].

By controlling hydrophobicity, molecular charge, and water solubility of the carbon nanomaterial, C_{60}, C_{70}, C_{74}, C_{76}, C_{78}, C_{80}, C_{82}, C_{84}, higher fullerenes and their functionalized derivatives can be modified in order to be applied in PDT of microorganisms [108]. Consequently, cationic fullerene embodiments functionalized with one, two or three pyrrolidinium groups have a broad-spectrum antimicrobial activity and can rapidly kill more than 99.99% of bacterial and fungal cells [108]. The bacteria utilized in this patent [108] include *S. aureus, E. coli* and *P. aeruginosa*.

Accordingly, deca-cationic fullerenes were used as PS to photoinactivate Gram-positive *S. aureus* and Gram-negative *E. coli* [109]. Due to the higher susceptibility of Gram-positive bacteria to PDT, a lower concentration of fullerene derivatives (up to 10 μM) was used for *S. aureus*, while higher concentrations (up to 100 μM) were demanded to effective kill *E. coli*. Nonetheless, there was an effective antimicrobial PDT for *E. coli* with C70[>M(C3N6+C3)2] compound, achieving a 5 log reduction at a concentration of 60 μM and effectively eradicating the cells at a concentration of 80 μM [109].

ANIMAL MODELS TO TEST PDT OF GRAM-NEGATIVE INFECTIONS

Animal models have become standard tools for the study of a wide array of antimicrobial therapies of wound infections, including antimicrobial PDT. Mice are by far the most frequently used species for wound infection models; however, the principal disadvantages of mouse models relate to the small size of these animals. The number of sequential sampling of blood, other fluids and tissues that can be performed without compromising the mouse is also limited. As a result, *in vivo* studies of PDT utilizing mouse infection models suffer from difficulties in monitoring the development of the infection and its consequent response to the treatment. Standard microbiological techniques used to follow infections in animal models frequently involve the sacrifice of the animals, removal of the infected tissue, homogenization, serial dilution, plating and colony counting. These assays use a large number of animals, are time consuming, and are often not statistically reliable.

In order to facilitate the non-invasive monitoring of animal models of infection, we have developed a procedure that uses bioluminescent genetically-engineered bacteria and a light sensitive imaging system to allow real-time visualization of infections. When these bacteria are treated with PDT either *in vitro* or *in vivo*, the loss of luminescence parallels the loss of colony-forming ability. We have further developed several mouse models of localized infections that can be followed by bioluminescence imaging (BLI) [110].

BLI can be used either to track the course of an infection or to monitor the efficacy of antimicrobial therapies. Interestingly, the bacterial pathogenesis appears to be unaffected by the presence of luciferase genes, and bioluminescence can be detected throughout the study period in the animals. Furthermore, the intensity of the bioluminescence measured from the living animal correlates well with the bacterial burden subsequently determined by standard protocols [111–113]. Transposon-mediated integration of the luciferase operon into the bacterial chromosome to make stable transformants means that reduction of luminescence

from sites of infection in animals can be attributed to reduction of bacterial numbers rather than loss of luciferase-encoding plasmids.

The first mouse model of localized infection using bioluminescent bacteria to be utilized for antimicrobial PDT (aPDT) was a model of simple excisional wounds that were superficially inoculated with bioluminescent Gram-negative *E. coli* bacteria [114]. The same excisional wound mouse model was also used by the same group [115] to test the efficacy of aPDT against the infections induced by a more invasive Gram-negative species, *P. aeruginosa*.

One of the injuries that compromise the protective role of the skin is the burn injury. Not only do the burns breach the cutaneous barrier, but severely burned sites are rendered avascular, immunosuppressed, and rich in bacterial nutrients. Consequently, burns are highly susceptible to infections and large burns that occupied a high percentage of the total body surface area often prove to be fatal due to infectious complications.

PDT of Gram-negative *Acinetobacter baumannii* in a burn wound infection was studied by Dai *et al.* [116] using polyethylenimine chlorine (e6) (PEI-c_{e6}) conjugate and non-coherent red light at 660 nm. Five minutes after the creation of the burns, a suspension of luminescent *A. baumannii* containing 10^8 cells was inoculated onto the eschar of each burn. This led to chronic infections that lasted, on average, 22 days and was characterized by a remarkably stable bacterial bioluminescence. Starting PDT on day 0 was more effective in reducing bacterial luminescence (3-$\log_{10}$ units) than on day 1 or day 2 (approximately 1.7-$\log_{10}$ reduction). (Fig. 4) shows the PDT dose response of bacterial luminescence of a representative mouse burn infected with *A. baumannii* and treated with PDT on day 1 (24 hours) after infection. PDT induced approximately 1.8 logs reduction of bacterial luminescence from the mouse burn that was not seen with the control groups (Fig. 4A). Bacterial re-growth in the treated burn was observed but was generally modest (Fig. 4C). Also the PDT did not lead to inhibition of wound healing.

CONCLUSION

The pervasive growth of antibiotic resistance among the “ESKAPE” pathogens - four of which are Gram-negative- (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumanii*, *P. aeruginosa* and *Enterobacter* sp.), has emerged as one of the most important clinical challenges of the present time [117, 118]. Alternative antimicrobial approaches such as PDI will continue to grow in attractiveness to researchers and will also likely become to be more clinically tested and even obtain regulatory approval as time goes on [119].

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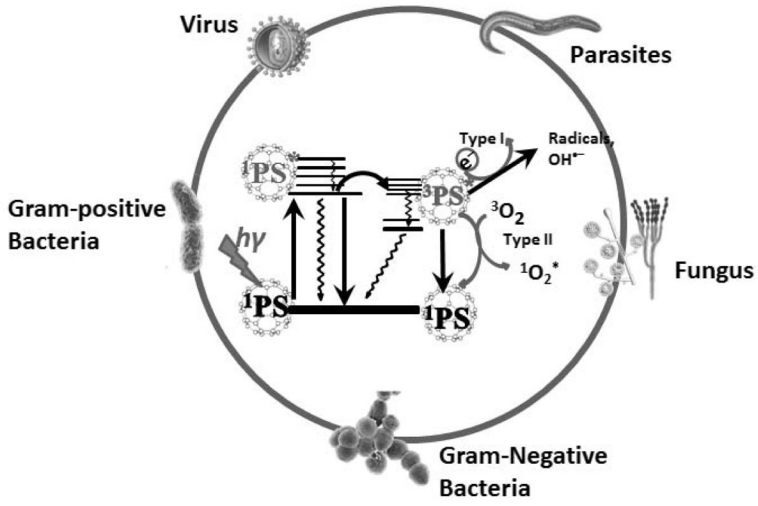

Fig. 1.
Jablonski diagram [14].

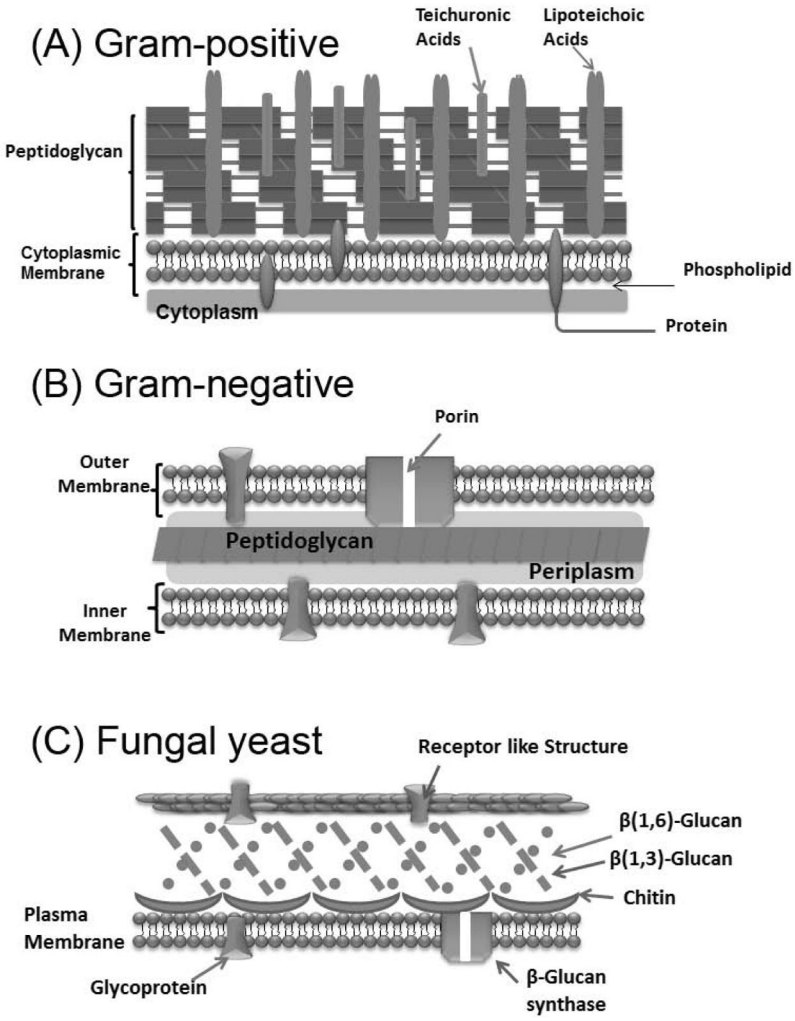

Fig. 2.
Structures of the cell walls of two different classes (Gram-positive and Gram-negative) bacteria and yeast [14].

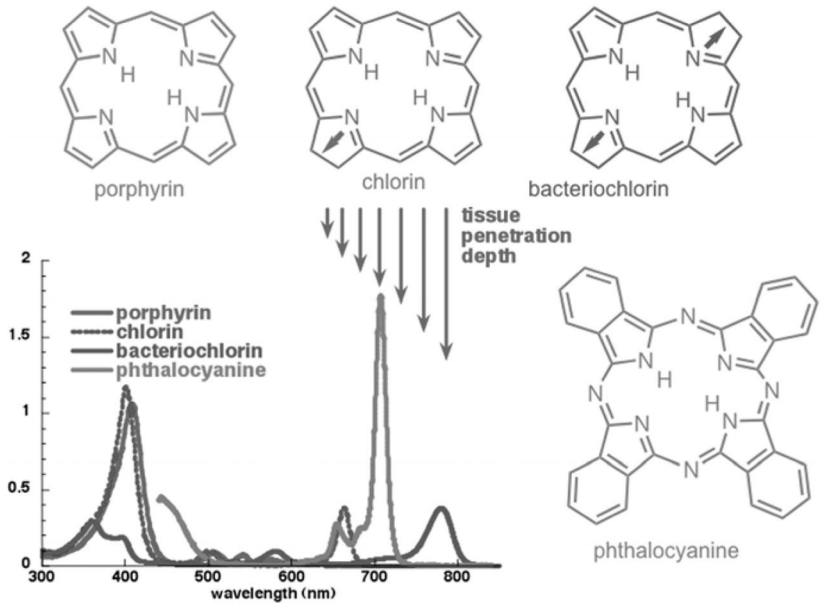

Fig. 3.
Chemical structures and absorption spectra of porphyrin, chlorin bacteriochlorin and phthalocyanine photosensitizers [74].

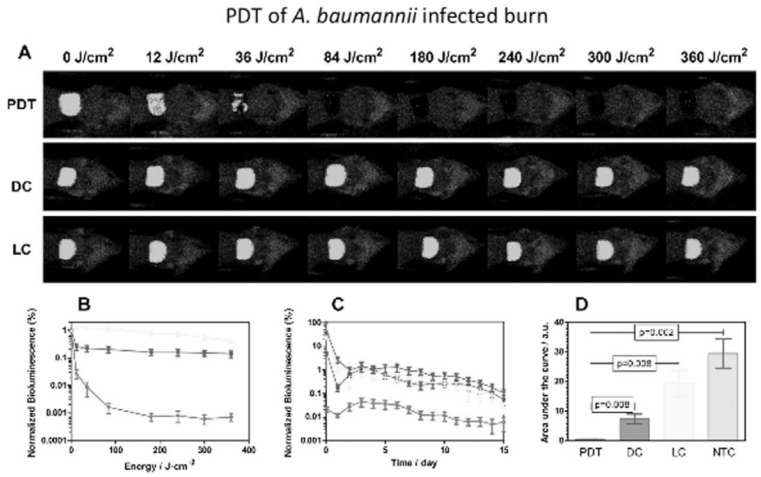

Fig. 4.

PDI of *A. baumannii* infection in a 3rd degree mouse burn mediated by PEI-c_{e6} and 660-nm light [116]. **A**) Dose response of bacterial luminescence from 3 representative mouse burns treated with PDT (PEI-c_{e6} + light), DC (PEI-c_{e6} no light), LC (light + no PS). **B**) Quantification of RLU from images. **C**) Time courses of bacterial luminescence values of the infected burns over 15 days in different groups of mice. **D**) Mean areas under the curves representing the time course of bacterial luminescence of mouse burns in different groups.