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Photodynamic inactivation of biofilm: taking a lightly colored approach to stubborn infection

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Abstract

Microbial biofilms are responsible for a variety of microbial infections in different parts of the body, such as urinary tract infections, catheter infections, middle-ear infections, gingivitis, caries, periodontitis, orthopedic implants, and so on. The microbial biofilm cells have properties and gene expression patterns distinct from planktonic cells, including phenotypic variations in enzymic activity, cell wall composition and surface structure, which increase the resistance to antibiotics and other antimicrobial treatments. There is consequently an urgent need for new approaches to attack biofilm-associated microorganisms, and antimicrobial photodynamic therapy (aPDT) may be a promising candidate. aPDT involves the combination of a nontoxic dye and low-intensity visible light which, in the presence of oxygen, produces cytotoxic reactive oxygen species. It has

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been demonstrated that many biofilms are susceptible to aPDT, particularly in dental disease. This review will focus on aspects of aPDT that are designed to increase efficiency against biofilms modalities to enhance penetration of photosensitizer into biofilm, and a combination of aPDT with biofilm-disrupting agents.

Keywords

antimicrobial photodynamic therapy; biofilm; dental infection; extracellular polysaccharide; microbial resistance; multidrug efflux pump; persister cells; photosensitizers

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It has been discovered in recent years that biofilms are responsible for a wide variety of microbial infections in the body; this could be as high as 80% of all infections [1]. Infectious diseases for which microbial biofilms have been held responsible include urinary tract infections, catheter infections, middle-ear infections, gingivitis, caries, periodontitis, orthopedic implants, some gastrointestinal infections, and so on [2]. Microbial biofilms can be defined as communities of surface-attached microbial cells firmly encased in a layer of extracellular matrix (ECM). These communities have a well-developed communication system that allows them to regulate microbial growth and metabolism. Furthermore, the biofilm lifestyle confers resistance to antimicrobials, and prevents access by host inflammatory cells and can also alter host metabolism [3,4].

Infections by bacterial biofilms are typified by the ability of the bacteria in the biofilms to adapt themselves in undesirable environments with nutrient deficiency, presence of antibiotics and adverse environmental conditions as well as immunological defenses. These microorganisms tend to grow by adhering themselves onto biotic or abiotic surfaces and also onto medical devices implanted into various body parts. These have been a major cause of concern because of the difficulty faced by antimicrobial agents in penetrating the ECM of the biofilm. Owing to the serious nature of these infections and the near-complete death of effective strategies for treating them, novel ways to combat biofilms need to be discovered and developed.

Antimicrobial photodynamic therapy (aPDT), also known as photodynamic inactivation (PDI) or photodynamic antimicrobial chemotherapy, has been reported to be effective in eradicating both planktonic cells and biofilms. aPDT basically involves the synergistic combination of a photosensitizer (PS), molecular oxygen and visible light of appropriate wavelength in order to produce highly reactive oxygen species (ROS), which leads to the oxidation of several cellular components and to rapid cell inactivation. This review will focus on the use of aPDT in the inactivation of biofilms as schematically illustrated in Figure 1.

Biofilms

Biofilm formation

Biofilm is a lifestyle that microorganisms adopt to survive in certain harsh environments. Most biofilms are formed in nature by more than one species that may be attached to a biotic

or an abiotic surface. A mixture of polysaccharides is secreted and assembled into biofilm by a monospecies or by multiple species, so that they can survive and grow [5].

The known types of organisms that can grow in biofilms include various pathogenic bacteria and fungi. Despite these small organisms, the formation of communities in these systems is very complex and involves different factors. The mechanism of formation of this complex appears to be initiated by specific environmental signals such as availability of nutrients. However, even after the cells have established sessile forms, they can return to a planktonic form again if this is more favorable. This dynamic process is directly related to the environment, because environmental changes cause modifications in cell–surface and consequently in cell–cell interactions [6].

Many steps are involved in biofilm formation and these include the presence of a favorable substrate, cell deposition, cell adsorption, desorption of reversible attached cells, irreversible adsorption of other cells, cell-to-cell signaling, exopolymer production, transport of oxygen and nutrient into biofilm, efflux of metabolized substances, cell growth and detachment of parts of the biofilm [7]. Microscopic studies, with Gram-negative (−) bacteria, have identified different stages of biofilm formation: planktonic cells, attachment, microcolonies, macrocolonies and dispersal [8]. Figure 2 graphically illustrates these processes. The biofilm developmental cycle is believed to include the following processes: transport of microbes to a surface; initial attachment; formation of microcolonies; biofilm maturation and biofilm dispersal. Detailed knowledge of the processes involved in the biofilm developmental cycle is essential in order to create strategies to control biofilm development.

Besides the characteristics of the neighborhood and of the substrate, the presence of certain microbial virulence factors such as pili, flagellum, fimbriae and glycocalyx influence the rate of microbial adhesion. Changes in the surface features of these microorganisms are produced by transcription of genes in response to extracellular signals. These new populations will deposit a very heterogeneous hydrated matrix (98% water), after recognizing a favorable generally hydrophobic surface and undergo transcriptional modifications for adherence. The matrix formed promotes a flow of water into the interior that is responsible for bringing nutrients and oxygen to the innermost cells [9].

The biofilm maturation process limits the acquisition of nutrients by the deeply situated cells. Therefore, the concentration of nutrients available is a determinant for the final thickness of the biofilm [10]. The changes in individual cells provide advantages for the survival of the set of cells as a whole. Due to this entire complex interacting network, one can compare the biofilm with a multicellular organism with an ECM between cells.

A quite common practice in biofilm studies is the study of monospecies cultures, whereas nearly all biofilm communities that occur in nature comprise a variety of different species of microorganisms. The interactions between the different species that constitute a mixed biofilm critically influence the development and shape of the community. There are mainly two kinds of interactions occurring within a multispecies biofilm [11,12]: interspecies interactions that involve communication (typically via quorum sensing [QS]) and metabolic cooperation or competition and intraspecies interactions within a biofilm that can be

antagonistic (competition for nutrients and growth inhibition) or synergistic. Synergistic interaction may result in the development of several beneficial phenotypes, including the promotion of biofilm formation by co-aggregation, metabolic cooperation and increased resistance to antimicrobials or synergistic evasion to host immune responses compared with the monospecies biofilms.

The structure and architecture of oral multispecies biofilms have been a favorite subject of many studies since the experimental model development and availability is relatively easy and the subject has high clinical relevance. The extracellular polymeric substances (EPS) matrix was found to modulate the interaction between 3D architecture and virulence of mixed-species oral biofilms [13]. Transcriptomic studies also revealed a constant interplay between all important physiological-, cariogenic- and periodontitis-associated microorganisms (*Streptococcus sanguinis, Streptococcus mutans, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*) [14].

The experimental approaches, although valuable, may be thought to only touch the surface of the organization as well as the mechanisms involved in the formation and assembly of multispecies biofilms. Moreover, the coexistence of such a wide variety of living microorganisms requires advanced computational methodologies and highlights the key observation that multispecies biofilms are socially evolving communities [14,15].

Biofilm architecture & structure—The components of the biofilm are generally referred to as EPS, which in turn are constituted of polysaccharides and proteins. The mechanical stability of biofilms depends on the composition and quantity of EPS secreted by microorganisms. The age of biofilms and several environmental factors influence this stability. Electrostatic and hydrogen bonds are the main forces involved in holding the EPS together. The organic matter exists in a material, that is, 98–99% water, and forms a stable gel. This intriguing composition demonstrates how extensive networks are formed in biofilms [16].

Most EPS are polyanionic by having uronic acids or ketal-linked pyruvate. However, some are neutral macromolecules. The primary conformation of the polysaccharides is determined by their composition and the secondary configuration is usually in helical forms [17]. Three conceptual models of biofilm can be defined: penetrated water-channel biofilm, heterogeneous mosaic biofilm and dense confluent biofilm. Studies have suggested that the structure of biofilm is largely determined by substrate concentration [18,19]. The presence of flagella, pili or fimbriae, pro-thecae and stalks is related with the adherence capacity of many microorganisms and is important in the attachment process. The flagellae and pili must overcome electrostatic repulsive forces between the substratum and microbial cell surface, resulting in a reduction in the energy required for adhesion during the initial stage of attachment [18,19]. EPS is also key for adhesion and cohesion of the biofilm [17,20]. The initial establishment also requires DNA and lipids released from the organisms [21].

Cell–cell signaling of bacteria is responsible for the ability to sense and respond to the environment and this cellular communication promotes the expression of particular genes [18]. Most researchers believe that the biofilm development requires a series of

hierarchically organized genetic elements that control these pathways in response to specific situations. This proposed biofilm formation occurs by a multistage process ordained by transitional form and function that is known as a developmental model [8].

Interactions among species influence both the initial stages such as stages of biofilm development [22]. Molecular studies with *Vibrio cholerae* suggested that pili and flagellae accelerate adhesion and that EPS is involved with the 3D structure of the biofilm [23]. In the maturation phase, this 3D architecture becomes a thick EPS layer with a dense network of organisms [24].

One study on the mechanism of *Staphylococcus epidermidis* biofilm maturation and detachment indicated that there was a relationship between dissemination of biofilm-associated infections and biofilm detachment molecules. This study has shown that low concentration of phenol-soluble modulin-β peptides facilities the formation of channels that increase biofilm and high concentration of these peptides produces detachment and reduction in biofilm mass [25].

In 1995, Costerton *et al*. predicted that with modern tools, we could make direct observations of functional biofilm communities and now we are able to fulfill this prediction [26]. However, we know that molecular studies are difficult to interpret because there are many heterogeneities in biofilms and these communities are poorly understood. In contrast to the developmental model, there is a model based on local ecological and individual adaptation. These two models encapsulate opposing views on the formation and development of biofilms. Some researchers believe that none of the models fully explain the formation of complex biofilms. According to Monds and O'Toole, biofilm formation underscores the ability of a cell to adapt [8], but nevertheless, if we understand all the molecular interactions of the biofilm, it would be a major achievement.

Methodologies to study the biofilm: Varieties of methodologies have been applied to visualize the assembly and development of the biofilm matrix. The majority of these use a diversity of microscopy techniques with a number of limitations identified related to both the lack of resolution and the capability to identify details in biofilm structural elements. Many research efforts have used differential staining and confocal laser scanning microscopy (CLSM). For example, a polysaccharide synthesis locus (Psl) exopolysaccharide identified by a Psl-specific lectin followed by CLSM provided a useful tool to follow the stages of biofilm development in *Pseudomonas aeruginosa* [27]. These included the attachment, the matrix assembly and maturation with Psl accumulation on the periphery of 3D-structured microcolonies. At the dispersion stage, swimming cells appeared in the matrix cavity between the microcolonies. Dead cells and extracellular DNA (eDNA) were also concentrated in the Psl matrix-free area. Deletion of genes that control cell death and autolysis affected the formation of the matrix cavity and microcolony dispersion [27]. A quantitative CLSM approach in *Proteus mirabilis* batch culture biofilms not only verified some of the common trends described for the basic stages of assembly and maturation but also demonstrated a minimal role for the swarmer cells in *P. mirabilis* biofilm formation under the applied conditions [28]. The idea of probing the immune responses to microbial biofilms as a way of detection has been employed for methicillin-resistant *Staphylococcus*

aureus (MRSA), illustrating that immunogenic, cell wall-associated, biofilm-upregulated proteins are promising for *in vitro* visualization of biofilm growth, architecture and space–function relationships [29].

A method for *in situ* visualization of biofilm utilizing CLSM relies on the use of image analysis software that allows quantification of parameters related to architecture. These programs automatically determine threshold values in analysis of 3D CLSM image stacks. Automated threshold calculation can be biased and give erroneous results when a stack contains images that lack pixels with real biological significance. Programs such as Auto PHLIP-ML and techniques such as determination of the optimal percent area covered can be used for image analysis (PACVEIR) by iterative image processing and allow accurate estimation of the area covered by biomass [30].

FISH has been the key methodology to localize the most abundant microbial species participating in the oral biofilm architecture and the spatial distribution of predominant species *in vivo* [31,32]. Many efforts have concentrated on visualization of the architecture of mycobacterial biofilms since the environmental and health impact of these species is of paramount clinical importance [33]. However, the visualization of biofilm architecture has been a challenge, especially because limited exchange of air with the atmosphere occurs during the biofilm formation [34]. Quantum dots (QD) are semiconductor nanocrystals and have emerged as a promising tool for labeling and detection of bacteria. QD conjugate-based and fluorophore conjugate-based immunofluorescence methodologies have been developed and have achieved single-cell resolution of human oral biofilms [35].

Transmission electron microscopy with a set of modifications (replacing Schiff ’s reagent with the osmiophilic amino acid methionine and replacing classical dehydration agents with ethylene glycol and 1,2-pentanediol) has been successfully employed for the artifact-free visualization of the ECM of biofilms on an ultrastructural level and with reproducible contrast for EPS [36]. Electron tomography and 3D visualization provided new insights into the molecular biofilm ultrastructure and community architecture. It was used in combination with the techniques of high-pressure freezing and freeze substitution to reduce the artifacts produced by chemical fixation and sample processing. This technique provided important structural information on how the chromatin body and cytoplasm were organized, and revealed membrane apposition between adjacent cells, and how the pili and vesicles were distributed in the biofilm matrix [37]. The combined techniques of computed x-ray tomography, CLSM and scanning electron microscopy were employed to reveal the spatial architecture as well as the binding motifs of nitrifying bacteria growing in a biofilm immobilized on a 3D matrix of polyurethane foam that allowed efficient water flow in a bioreactor [38]. According to Woznica *et al*., these methods have made it possible to observe large irregular aggregates of bacteria that exist as variable biofilm fragments; spherical aggregates of bacteria localized on the external surface of biofilms and biofilm threads adherent to the surface of polyurethane foam [38].

Although biofilm structure is generally characterized by qualitative methods, however quantification of the biofilm is also important, particularly to monitor the development course and activity of the biofilm. The quantification methods can be classified as: biofilm

biomass assays (based on the quantification of matrix and both living and dead cells); viability assays (based on the quantification of viable cells) and matrix quantification assays (based on the specific staining of matrix components) [39].

Crystal violet is a dye which binds to negatively charged surface molecules and polysaccharides in the ECM [40]. This staining was first described by Christensen *et al*. [41] and has since been modified to increase its accuracy and to allow biofilm biomass quantification in the entire well [42]. Syto9 is another dye that binds to ECM polymers, however, it is a nucleic acid stain that diffuses passively through cellular membranes and binds to DNA, which forms a substantial part of the ECM [21].

The bacterial viability is usually determined by cell metabolic activity. The microbial activity is estimated by the assay of electron transport system, as artificial electron acceptors, redox dyes that can successfully compete with oxygen for electrons [43]. For example, there are stains that involve the use of tetrazolium salts, including 5-cyano-2,3-ditolyl tetrazolium chloride [44] and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide [43]. Alamar Blue is another viability assay based on the reduction of resazurin by metabolically active cells, mainly used for fungi [45]. Bioluminescent assay of total ATP is also an indicator of live biomass. This technique measures light emission produced due to the presence of ATP, which is involved in an enzyme–substrate reaction between luciferin and luciferase (bioluminescence). The quantity of light produced is proportional to the concentration of ATP and, thus, to the number of microorganisms in the original sample [46].

The quantification of the biofilm matrix, an essential component of the biofilm, is also of interest. 1,9-dimethyl methylene blue is originally used to measure levels of sulfated glycosaminoglycans, quantifying biofilm matrices [47]. 1,9-dimethyl methylene blue forms an insoluble complexation product with sulfated polysaccharides in the biofilm matrix. The amount of dye released by adding a decomplexation solution is spectrophotometrically measured and reflects the amount of sulfated polysaccharides present in the biofilm matrix [48].

Extracellular matrix—Biofilms form a stable gel phase where microbial communities grow inside. The biofilm contains an ECM that holds cells together. The molecular mechanisms that regulate different bacteria to form biofilms vary greatly, often depending on environmental signals such as nutrient availability and characteristics of specific bacterial strain. The process of biofilm formation critically depends on various phases such as adherence, proliferation and dispersion [49].

The major biofilm matrix component is water (97%), which can be bound within the microbial capsules or can exist as solvents whose viscoelastic properties are determined by the solutes dissolved in it [50]. The composition of EPS matrix is highly complex, dynamic and primarily consisting of a polysaccharide biopolymer along with other components such as proteins, nucleic acids, phospholipids, lipids, amyloid fibers and humic substances. The EPS matrix constructs the intercellular space of microbial communities and forms the structure of the biofilm [51,52]. The EPS matrix is responsible for the binding of microbial

cells and particulate materials together (via cohesion) and to the surface (via adhesion) and functions as a shield where diffusive transport prevails over convective transport [50]. The main functions of EPS matrix are to facilitate the initial attachment of cells to different surfaces and offer protection against various environmental vagaries and dehydration [3]. The EPS matrix functions as a barrier to protect and allow bacteria to develop resistance to predators such as protozoa, bacteriophages, chemically diverse toxins (biocides), host immune systems and antibiotics [4,52]. The EPS matrix delays/prevents antimicrobial agents from reaching target microbial cells by diffusion limitation and may also do so through chemical interactions with the ECM proteins and polysaccharides [3,20,50]. The structure of the biofilm matrix varies greatly depending on the microbial species and even a single species can produce different EPS matrices depending on prevailing growth conditions [3,50]. Beside structural functions, the EPS matrix also functions as informative, redox-active and nutritive roles. Moreover, the cohesive strength of EPS matrix determines the viscosity of biofilms [51]. In addition, EPS matrix assists in the accumulation of nutrients or other substrates and, also responsible for the establishment of gradients (e.g., oxygen and nutrients diffusing inwards, and waste products as well as signal transduction molecules like nitric oxide and cyclic diguanosine monophosphate diffusing outwards) [53].

Proteins and polysaccharides are responsible for 75–89% of the EPS matrix composition, indicating that these are the key EPS components. Among the polysaccharides, the amounts of hexose, hexosamine and ketose were quite high in EPS-rich strains. It has been demonstrated that amounts of hexose and pentose revealed good correlations with cell adhesion for EPS-rich strains. Furthermore, it has been reported that if the amount of EPS is relatively less, cell adhesion onto a solid surface might be inhibited by electrostatic interactions, however if it is relatively high, cell adhesion might be increased by polymeric interactions [54]. The nature of the ECM exopolysaccharide greatly differs depending on medium, substrates and growth conditions. The exopolysaccharides provide the matrix framework, however there are a wide range of enzyme activities found within the biofilm, which in turn greatly affect the steady state of the biofilm. In Gram (−) bacteria, some of the polysaccharides (homo and heteropolysaccharides) are neutral or polyanionic [50]. The presence of uronic acids or ketal-linked pyruvates increases their anionic properties, and therefore allows the association of divalent cations (calcium and magnesium) to enhance the binding force in biofilm. In some Gram-positive (+) strains, the chemical composition of EPS can be slightly altered owing to their primarily cationic nature [17,52], like some strains of *S. aureus* use a polymer of *N*-acetyl glucosamine to help in biofilm formation [55].

The ECM of biofilms also possesses adhesive proteins, for example the *S. aureus* matrix harbors biofilm-associated proteins (Bap) that are essential for biofilm development [56]. The Bap is present on the cell wall of *S. aureus* and help to hold cells together, probably through interaction with other surface proteins of adjacent cells. However, *Bacillus subtilis* expresses a major ECM protein – TasA – which has been shown to form extracellular filaments and as amyloid-like properties, which may have contributed to the in structural composition of the ECM. On the other hand, in *Escherichia coli*, the curli protein forms amyloid filaments and plays an important role in development of biofilm [3]. Additional surface proteins homologous to Bap have been reported in Gram-positive and Gram (−)

strains like the Esp of *Enterococcus faecalis* and BapA of *Salmonella enterica* Enteritidis [56]. Furthermore, it has been reported that accessory matrix components which help in biofilm development and offer adaptation facilitated by biofilm formation allows for selection of genetic variants with unique colony morphology [49]. The proteinaceous appendage structures, such as pili and fimbriae, are important for biofilm formation and help to adhere cells to each other or to different surfaces. *E. coli* produces type I fimbriae that are vital for adherence to mannose-containing receptors. Mutant *P. aeruginosa*, which were unable to produce type IV pili, or the CupA fimbriae were found to be defective in surface-adhered biofilms [57]. Furthermore, there are other matrix-associated lectin-binding proteins that recognize and bind carbohydrate moieties and facilitate cell–matrix or cell–cell interactions in biofilm formation [58].

In addition to the exopolysaccharides and proteins, eDNA also offers structural integrity to the biofilm, which is produced by cell lysis and subsequent release of genomic DNA [59]. Lipids and nucleic acids might significantly affect the rheological properties and thus offer stability to the biofilms. The eDNA is essential for the initial establishment of biofilms by *P. aeruginosa*, and possibly for other bacteria that specifically release DNA [20]. Moreover, depending on the conditions, different components of the matrix become more or less significant for the stability of the biofilm [3].

QS: the central biofilm regulatory circuit—The widespread chemical communication in response to fluctuation of microbial population density is defined as QS, which has been implicated among other phenomena into biofilm formation, regulation of expression of virulence factors, competence and bioluminescence. In QS, microbial cells produce and detect signal molecules and thereby coordinate their behavior in a cell density-dependent manner [60].

It has been shown that cell–cell signaling is important to the physiology and development of microbial biofilms once they have become slow-growing because of high cell density and limited nutrients. In addition, there are indications that QS may also be a resistance mechanism. However, it is not clear if this is done by upregulation of multidrug efflux systems or by other mechanisms. So, interfering with the QS communication system may increase microbial susceptibility [61].

A variety of signaling molecules are used by microbials in biofilms, meanwhile four main QS systems can be distinguished: the *N*-acyl-L-homoserine lactone (AHL) QS-system in Gram (−) bacteria, the autoinducing peptide QS-system in Gram (+) bacteria, the autoinducer-2 (AI-2) QS-system in both Gram (−) and (+) bacteria and the farnesol (aromatic alcohol) systems in *Candida albicans* and other fungi [60].

The first QS to be described and characterized, used by many Gram (−) bacteria to communicate with each other, was an AHL communication system. The signal molecule from *Vibrio fischeri* was identified as *N*-(3′-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL) and induces luminescence in this species. The *V. fischeri* lux operon responsible for luminescence was found to be under the control of two regulatory elements, LuxR and LuxI, where LuxI synthesizes the AHL, while LuxR functions as a transcriptional regulatory

protein. The LuxR protein encoded in the same lux operon is an AHL detector that upon interaction with AHLs, transcriptionally, activates other genes in order to cause their expression and phenotypic changes [62].

Subsequently, it was found that the *P. aeruginosa* used the LuxRI-type communication system to control the expression of many of its virulence factors. In *P. aeruginosa*, there are two LuxRI-type systems (LasRI and RhlRI) that use the AHL signals *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) and *N*-butyryl homoserine lactone (C4-HSL), respectively [7].

The roles of AHL in biofilm formation and maturation have been demonstrated in a variety of Gram (−) species and the study of the molecular circuits involved is still ongoing. In *Legionella pneumophila*, AHL governs transcriptional regulation of a collagen-like adhesion [63], while in *Burkholderia pseudomallei*, AHL regulates the effect of environmental conditions and decision-making for biofilm formation in clinical isolates [64].

The QS-system of Gram (+) bacteria typically consists of signal peptides such as Agr and RNA-III-activating/inhibiting peptides in *S. aureus*, and a two-component regulatory system made up of a membrane-bound sensor and an intracellular response regulator [65]. Both systems have been key regulators in staphylococcal biofilm formation. Recent studies identified a role for the surfactant secreted peptides peptides (phenol-soluble modulins) controlled by Agr and RNA-III in biofilm maturation and detachment [66].

A third QS-system has been found to be common to many Gram (+) and Gram (−) bacterial species and is mediated by a mixture of interconvertible molecules collectively referred to as autoinducer system 2 (AI-2) [67,68]. A key enzyme in the biosynthesis of AI-2 is LuxS. LuxS enzymatically cleaves S-ribosylhomocysteine to the products homocysteine and 4,5-dihydroxy-2,3-pentanedione. 4,5-dihydroxy-2,3-pentanedione can undergo spontaneous rearrangements and modifications, forming a variety of molecules, collectively termed AI-2. Although LuxS is encoded in more than half of all sequenced bacterial genomes, AI-2 receptors and signal transduction systems have been analytically described in *Vibrio* spp., in *S. enterica* serovar Typhimurium and in *E. coli* [67]. In *Vibrio* spp., binding of AI-2 to LuxP, a periplasmic AI-2 receptor associated with the LuxQ sensor kinase, results in the production of LuxR and ultimately changes gene expression. In *S. enterica* serovar Typhimurium and *E. coli*, AI-2 is first transported into the cell prior to initiating a signaling cascade [67]. Although the elucidation of the molecular circuits affiliated with AI-2 is an ongoing investigation, the role of AI-2 signaling molecules in biofilm formation and maturation has been unequivocally demonstrated in a variety of pathogens. The list includes the nontypeable *Haemophilus influenzae* [69], *Campylobacter jejuni* [70], *A. actinomycetemcomitans* [71], *E. faecalis* [72], *Aeromonas hydrophila* [73], *V. cholerae* [74] and *Klebsiella pneumoniae* [75].

Biofilm resistance

Microorganisms in biofilm can be up to 1000-fold more resistant to antimicrobial agents than cultures grown in suspension [76]. This resistance is more apparent in fungal biofilms due to the complexity of this microorganism that presents structures such as blastoconidia,

pseudohyphae and hyphae [77]. Thus, the antifungal treatment, very often, is prolonged and can promove serious side effects and drug–drug interactions, as well as being ineffective [78]. In addition, it is limited to a very small number of drug substances when compared with antibacterial therapy [79].

Many mechanisms of antimicrobial resistance, such as modifying enzymes and target mutations, are well known [80]. However, these mechanisms particularly apply to biofilms; in this case, resistance seems to depend on multicellular strategies [76]. The possible explanations for the resistance of biofilms that have been introduced in the literature included limited diffusion and limited interaction of antimicrobial agents through the biofilm [81]; altered levels of metabolic activity within the biofilm [80] and genetic adaptation [82].

Mechanisms of resistance—The first hypothesis for the resistance mechanisms was the inability of the antimicrobial agent to penetrate the full depth of the biofilm. Studies *in vitro* that measured the antimicrobial penetration into biofilms have shown that agents can readily permeate microbial biofilms in some cases, while penetrate poorly in others, depending on the particular antimicrobial compound and the microorganisms. This observation suggests that there is no generic barrier to the diffusion of solutes with a molecule weight typical of antimicrobials through the biofilms matrix. However, the antimicrobial compound can be deactivated in the biofilms, and therefore have a retarded penetration. It is thought that the slow penetration is due to a neutralizing reaction between the compound and some components of the biofilms such as glycocalyx matrix. Diffusion of antimicrobial agents through a biofilm may be affected by the polyanionic nature of the glycocalyx that creates a barrier due to charge interactions. This interaction between the cells and the protective matrix increases the time required for the antimicrobial agents to reach the cells that remain attached in deepest portion of the biofilms. To prove this, Hoyle and Costerton evaluated the interaction of tobramycin (Tob) with *P. aeruginosa* mucoid exopolysaccharide [83]. They observed that the bacterial cells were dispersed from biofilms by Tob diffusion across the biofilm-fluid interface and into the biofilms. However, these dispersed cells were 15-times more susceptible to Tob than those in the intact biofilms.

According to Nichols *et al*., the biofilm resistance against Tob is due to the slow growth rates of the microorganisms in this area, which is a result of difficult access of organic nutrients, inorganic ions and oxygen within the depths in the biofilms [84].

Therefore, another possible mechanism to explain biofilms resistance to antimicrobials is the physiological resistance. A number of studies have supported this hypothesis. Experiments using microelectrodes have shown that the oxygen can be fully consumed in the biofilm surface layers and that oxygen penetration decreases with increasing biofilms thickness. Anderl *et al*. showed that glucose was consumed by the *K. pneumoniae* in the biofilm surface, that is, by the bacteria closest to the substratum, and could not penetrate into the biofilms [85]. The growth phase can also contribute to the antimicrobial resistance of the biofilms, when the exponential phase increases this resistance 15-times the stationary phase also exhibits high levels of resistance in both planktonic cells and biofilms [86]. So, the depletion of a substrate or accumulation of an inhibitive waste product might cause some

microorganisms to enter a slow or nongrowing state, constituting an important survival strategy.

Reduced biofilm susceptibility, by genetic adaptation, is still a speculative mechanism that suggests at least some of the cells in a biofilm adopt a distinct and relatively protected biofilm phenotype. This phenotype is a biologically programmed response to growth on a surface. During biofilm formation, the association of the microorganisms with a surface promotes both a repression and an induction of different genes that cause physiological responses. Dagostino *et al*. found that these induced genes did not occur in liquid media since they are specific to the surface–microorganism association [69]. They concluded that there is a tactile receptor in the microorganisms that could have implications for a wide spectrum of phenotypic characteristics in biofilm bacteria.

Specific genes: Since efflux systems are a key resistance mechanism in microbial cells, it has been suggested that their expression contributes to the reduced susceptibility of biofilm to antimicrobials and overall persistence [87–91]. The exact correlation between efflux systems and biofilm resistance to antimicrobials remains a matter of investigation. In *E. coli*, for example, there is evidence for the implication of the putative multidrug efflux system YhcQ in biofilm antibiotic resistance [92]. Under stress conditions or exposure to bile acids, the AcrAB-TolC system has been found to be upregulated [93]. Biofilm formation of emrD, emrE, emrK, acrD, acre and mdtE-deleted mutants was extremely decreased. These results indicate that some multidrug efflux pumps significantly contribute to the biofilm formation of *E. coli* [94]. The interplay between the plasmid-encoded tetracycline resistance efflux system TetA(C) and the ampicillin resistance gene, *bla(TEM-1)*, in biofilms of *E. coli* according to May *et al*., may lead in the induction of chromosomally encoded efflux systems such as EmrY/K and EvgA/S [95].

In a different approach, a transposon-generated random mutant library in *E. coli* EC100 was screened for viability chloramphenicol (Cm). A total of 13 mutants including six resistant as well as seven sensitive clones to Cm were identified. Inverse PCR revealed the genetic identity in 12 out of the 13 clones. Mutation in five genes (*rob, garP, bipA, insK* and *yhhX*) was implicated with higher sensitivity to Cm when compared with the wild-type EC100, whereas mutation in seven genes (*rhaB, yejM, dsdX, nagA, yccE, atpF* and *htrB*) led to increased resistance. Overexpression of Rob, NagA, RhaB and YccE was found to contribute in increased resistance of *E. coli* biofilms to Tob by 2.7-, 2.2-, 2.5- and 2.1-fold, respectively [96].

MexAB-OprM and MexCD-OprJ are clinically significant efflux systems in *P. aeruginosa* implicated in resistance to a variety of antibiotics. There is evidence that both systems are upregulated and involved in biofilm-specific mechanisms of resistance to the macrolide azithromycin [97], but there is no indication for upregulation for both pumps in a developing biofilm [98]. A concept for the correlation of efflux pumps with antimicrobial resistance in the biofilm phenotype may be attributed in multiple factors related with the physiology of efflux and the genetic determinants for efflux phenotypes [99]. The *ndvB* gene, coding for a glucosyltransferase, is the only solid genetic determinant related with biofilm antibiotic resistant in *P. aeruginosa* [100]. Exposure of *P. aeruginosa* biofilms in model antimicrobials

(colistin, chlorhexidine) as well as detergents (SDS and ethylenediaminetetraacetic acid [EDTA]) and development of tolerant subpopulations to these agents highlighted the predominant, but also complex, role of efflux systems in tolerance. In the case of colistin, tolerant subpopulations were found to depend on mexAB-oprM, mexCD-oprJ and muxABC-opmB as well as the *pmr* genes which encode for lipopolysaccharide (LPS)-modifying enzymes, but not *mexPQ-opmE* genes. In the case of chlorhexidine-tolerant subpopulations, the *mexCD-oprJ* genes play an important role but none of the above genes were found to be implicated. Tolerance to the detergents, SDS and EDTA, in *P. aeruginosa* biofilms is not linked with any of the efflux systems and is rather connected with metabolically active cells [101].

Persisters: a new dogma in biofilm-associated infections: The term 'Persister' is used to identify a special bacterial subpopulation, which occupies only <0.1% of the whole population with different features from the majority of the bacterial cells and without harboring resistant mutants [82]. The mechanism of their formation is complex, and they are difficult to isolate and culture. It was discovered that these cells were resistant to further large increases in the antimicrobial concentration. Persisters share the capability of adapting to an adverse environment via 'dormancy-growth-proliferation' to maintain their survival and cell structure stability, and play a vital role in the microbial biofilm multidrug tolerance.

Persisters were initially discovered in 1944, but the molecular mechanism of dormancy began to emerge in the last decade. The production of the persister is regulated by the growth stage of the microorganism population. Spoering and Lewis showed that *E. coli, S. aureus* or *P. aeruginosa* cultures at the early logarithmic phase produce insignificant persister numbers, but the stationary phase population makes more persisters as well as being more tolerant to antimicrobial challenge when compared with biofilm cultures [102].

The persister cell populations have been implicated with the elevated biofilm antibiotic resistance in many microbial systems [102–106]. This surviving ability has been attributed to the overexpression of genes that promote a dormant 'growth' stage for the persistent population. For example, overexpression of the chromosomal toxin–antitoxin modules will shut down cellular functions and subsequently inactivate the antibiotic targets, resulting in a dormant cell that is tolerant to the lethal action of antibiotics [107].

While resistance and tolerance are mechanistically distinct, there is sufficient information to believe that tolerance may lead to development of resistance. Indeed, the probability of resistance development is proportional to the size of the pathogen population, and a lingering chronic infection that cannot be eradicated due to tolerance will go on to produce resistant mutants and strains acquiring resistant determinants by transmission from other bacteria [108]. Combating tolerance then becomes a major component in preventing resistance.

Antibiofilm approaches

A number of factors in biofilm formation and biofilm maturation have been explored as targets for development of antibiofilm strategies. These include material or surface modification of medical devices to prevent the colonization and formation of biofilm.

Electrical approaches also have emerged as an important approach that enhances the antimicrobial activity against established biofilm. This effect consists of concurrent application of an antimicrobial and a weak electric field (between 1.5 and 20 Vcm^{-1}) to kill microorganisms [109]. Robinson *et al*. showed that the concentrations of antimicrobials against biofilm decreased 5000 times when electric field was applied, probably because this bioelectric effect is due to the electrolytic generation of oxygen [110].

Another antibiofilm strategy is ultrasound that enhances the transport of antimicrobial agents targeted onto the surface of the biofilms. Carmen *et al*. investigated this hypothesis and found that *E. coli* bacterial colonies in biofilms decreased when subjected to ultrasound [111].

Liposomes and polymer-based drug delivery vehicles are other approaches that can increase penetration through the surface of the biofilms and towards deeper cells. Liposomal delivery showed 10,000-fold greater activity than free antimicrobial agents. There are two types of liposomes (cationic or anionic) that can be used in this technique, however it was observed by Robinson *et al*. in different species of biofilms that the effect of anionic liposomes was the smallest [110]. The biodegradable polyesters, as effective drug carriers, tend not to suffer from the same difficulties of low encapsulation efficiency and poor stability on storage typically exhibited by liposomal formulations; however, the encapsulation of the drugs depends on the physicochemical properties of the polymer and antimicrobial as well as the encapsulation method [39,110,112].

In addition, attack against EPS can be an initial step of biofilm disruption, once this matrix becomes a natural barrier which keeps the microorganisms protected. Strategies to break this barrier are being increasingly studied in order to destroy this protection and consequently the microorganisms [44]. So, aPDT can improve the efficiency of other inactivation strategies used in a combined protocol.

Antimicrobial photodynamic therapy

aPDT is a minimally invasive antimicrobial approach that has been proposed as an alternative treatment for localized infections in response to the problem of antimicrobial resistance. The main advantages of this therapy is that PDT is a multitarget process [113], there is no development of microbial resistance [114,115]; microorganisms are killed equally regardless of their intrinsic or acquired antibiotic resistance [116]; the PS can be designed to be localized preferentially in microorganisms and not in tissue or human cells to give selective killing of microbial cells with minimal damage to the host tissues [116]; the microbial killing is rapid (within seconds); the very broad-spectrum action removes the need to identify the particular pathogen before choosing a therapeutic [117,118] and because PDT is topically applied to the infected area, it is effective against infections in damaged tissue with compromised blood perfusion such as burns [119].

aPDT utilizes a combination of a PS, visible light and oxygen to produce cytotoxic ROS that cause destruction of pathogenic microorganism. The PS has a stable electronic configuration, which is a singlet state in its lowest ground energy level (^{1}PS) Following absorption of a light-photon, the molecule is promoted to an excited singlet state, ^{1}PS* and

may then convert to the triplet state, $^3PS^*$, that occurs via intersystem crossing involving a change in the spin of the excited electron. The triplet PS has a sufficiently long lifetime to allow it to undergo chemical reactions. The photochemical reactions of the triplet state can occur in two different pathways. The type I mechanism involves electron transfer reactions from one molecule to another, resulting in the formation of toxic oxygen species such as superoxide ($O_2^{\cdot -}$), H_2O_2 and hydroxyl radicals ($^{\cdot}OH$). The type II mechanism involves energy transfer to molecular oxygen, producing excited state singlet oxygen ($^1O_2$) that is a strong oxidant compared with ground state triplet oxygen. These reactions can occur at the same time but the relative proportions may depend on the PS structure and also on the microenvironment [120].

A wide range of compounds of remarkably different structures can behave as PS and new ones are regularly synthesized for the first time; however, very few have made it to clinical trials, and even fewer are readily commercially available and widely applied to patients.

Properties of PSs

PSs are planar unsaturated organic molecules with extensive electron delocalization [121]. This electronic structure means that PSs tend to be deeply colored because the energy required to excite the electron in the highest occupied molecular orbital to the lowest unoccupied molecular orbital is low compared with less delocalized molecules and, therefore, the visible absorption bands tend to be in the longer wavelength (red) spectral region and are large in magnitude (reflecting the high probability of electronic excitation). Acridine orange (Figure 3B) was the first PS agent used for microbial photoinactivation [122]. Most of the PS that have been employed over the last 100 years for the treatment of cancer and other tissue diseases are based on the tetrapyrrole nucleus with a heavy prevalence on the use of porphyrins [123]. Other tetrapyrroles such as chlorins [124], bacteriochlorins [120] and phthalocyanines [125,126] have been frequently proposed as antimicrobial PS (Figure 4) and the long-wavelength absorption band moves to the red and increases in magnitude as the number of saturated rings in the tetrapyrrole nucleus increases. A number of non-tetrapyrrole dyes and natural products have also been tested, including halogenated xanthenes such as rose bengal (RB) (Figure 3C) [127], phenothiazinium salts such as toluidine blue O (TBO) (Figure 3D) and methylene blue (MB) (Figure 3E) [128], perylenequinones such as hypericin (Figure 3F) [129] cationic buckminsterfullerenes (e.g., C_{60}) (Figure 3G) [130,131], cationic BODIPY dyes (Figure 3H) [132] and psoralens (e.g., 4´aminomethyl, 4,5´,8 trimethylpsoralen) (Figure 3I) [133].

Differences in susceptibility to aPDT among different microorganisms

The permeability barrier in Gram (−) bacteria is responsible for the susceptibility differences in aPDT compared with Gram (+) bacteria [134]. Many neutral or anionic PS are more effective against Gram (+) bacteria than against Gram (−) bacteria [135]. The high susceptibility of Gram (+) species can be explained by the fact that PS (both cationic and noncationic) can penetrate through the relatively porous layer of peptidoglycan and lipoteichoic acid and reach the cytoplasmic membrane. However, the cell wall of Gram (−) bacteria consists of a many-layered outer structure that acts as a permeability barrier. Outside the peptidoglycan layer, there is an additional membrane layer with an asymmetric

lipid structure containing strongly negatively charged LPS, lipoproteins and proteins with porin function. This structural organization dramatically reduces the permeability and enhances the resistance.

Photosensitivity of Gram (−) bacteria can be achieved by pretreatment with agents such as Tris-EDTA or nonapeptide polymyxin B [135]. The Tris-EDTA removes the divalent cations (e.g., Ca^{2+}, Mg^{2+} ions), neutralizing negative charged LPS molecules present on the outer membrane. Thus, the onset of electrostatic repulsion allows the penetration of molecules with high molecular weight to the inner cytoplasmic membrane or inner cellular compartment. On the other hand, the incubation with cationic compounds, such as polymyxin B, tends to undergo an electrostatic binding with the negatively charged cell surface molecules that alters the physical arrangements of the lipid layer with less densely packed hydrocarbon lipid chains. Polymyxin B nonapeptide does not release LPS from the cells, but ‘expands’ the outer leaflet of the membrane, allowing PS to penetrate.

These observations led to a series of investigations for the development of cationic PS with broader spectrum PDI activity as both Gram (+) and (−) bacteria have anionic cell envelopes [136]. A possible explanation for the superiority of cationic PS for Gram (−) species is because these compounds are taken up through the so called ‘self-promoted uptake pathway’. These cationic compounds have two to four orders of magnitude higher affinity to anionic binding sites associated with surface LPS molecules than the normally bound divalent Ca^{2+} and Mg^{2+} cations. Consequently, these metal ions are displaced by the cationic PS leading to the reorganization of the outer membrane structure and permeabilization of the outer membrane to allow even more cationic PS to penetrate. It should be mentioned that there are some reports of Gram (−) species being killed by noncationic PS such as RB [137,138].

Fungi present much more complex targets than bacteria since they are surrounded by a rigid cell wall composed of a mixture of glucan, mannan, chitin and lipoproteins and which is separated from the plasma membrane by a periplasmic space. However, available evidence suggests that the fungal cell wall makes the cells inherently more permeable to PS than Gram (−) bacteria [139–142]. The photodynamic mechanism of fungal cell destruction is by perforation of the cell wall and membrane with PDT-induced singlet oxygen and oxygen radicals, thereby allowing the dye to be further translocated into the cell [143]. The activated PS induces some initial functional alterations of the cytoplasmic membrane that promote damage to inner organelles such as lysosomes, mitochondria and nucleus, inducing cell death [144].

Overall, the best structures for antimicrobial PS have one or more cationic charges, and a large number of papers have described the synthesis and testing of different cationic PS to give broad-spectrum photobactericidal activity [119,145–148].

PDT against microbial biofilms

Recently, there have been numerous studies on the PDI approach to combat microbial biofilms. It has been proven through various studies that aPDT can be effective against biofilm-dwelling bacteria and fungi.

Gram (+) bacteria—Many of the aPDT studies to date have focused on Gram (+) bacteria (Table 1), particularly those regarded as extracellular pathogens such as *Staphylococcus* spp. and *Streptococcus* spp. aPDT mediated by MB promoted an average reduction of 2.81 log(10) CFU in *S. mutans* biofilms, as well as an average reduction of 3.29 log(10) CFU in *S. aureus* biofilms [149]. Cationic PS such as pL-ce6- and MB-induced aPDT could overcome the protective effect of extracellular slime and stationary bacterial growth that applied aPDT to inactive wild-type and transposon mutants of *S. epidermidis* and *S. aureus* that were lack of capsular polysaccharide and slime production [150]. TBO-induced aPDT reduced 4.5 log(10) CFU of MRSA biofilms [151] or 2.10–3.11 log(10) CFU of *S. mutans* biofilms [152]. It also made an impact on staphylococcal biofilms which damaged biofilm biological architecture and disrupted bacterial cell membranes [126]. TBO-induced aPDT also had an effect on *S. mutans* biofilms in different maturity stages [127], as well as mature *Streptococcus sobrinus* and *S. sanguinis* biofilms [128].

Hypericin-mediated aPDT induced significant killing of *S. aureus* cells (>4 log[10] CFU reduction) [153]. aPDT with merocyanine 540 illuminated by 400 J/cm^2 green light had an antimicrobial effect on viability of biofilms from Gram (+) pathogens [154]. Cationic porphyrin, tetra-substituted (*N*-methyl-pyridyl) porphine-induced aPDT made impact on both biofilm models when combined with antibiotics or host defense mechanisms [155]. Tri-meso (*N*-methyl-pyridyl), meso (*N*-tetradecyl-pyridyl) porphine (C14)-mediated aPDT had a significantly greater potential inactive effect on *S. epidermidis* biofilms [156]. Erythrosine (ERY)-induced aPDT was found to have potential effects on inactivation *S. mutans* biofilms [157].

Soukos *et al.* used MB (25 μg/ml) PDT, illuminated by 665 nm red light with a fluence of 30 J/cm^2, to fully eliminate all bacterial species with the exception of *E. faecalis* (53% killed) in infected root canals of extracted teeth [158]. *E. faecalis* biofilm bacteria in root canals were reduced by 97% after aPDT with the same concentration of MB and an increased fluence (222 J/cm^2) of red light.

Our laboratory reported on the use of PDT to treat a chronic biofilm infection caused by MRSA in skin abrasion wounds in mice [159]. The authors employed stable bioluminescent genetically engineered bacteria and a low-light imaging camera that allows noninvasive real-time monitoring of the extent of infection and its change over time in living mice [160]. The authors used a skin abrasion caused by an array of orthogonal needle scratches and topically applied bioluminescent MRSA. The resulting infection lasted more than 10 days demonstrating the chronic nature of a biofilm infection as seen in Figure 5A, the actual biofilm in the tissue is shown by histology in Figure 5D. When the PS polyethylenimine chlorine (e6) (PEI-ce6) was topically applied to the infection followed by illumination with red light (up to 240 J cm^{-2}), the bioluminescence signal disappeared in the PDT group (but not the PEI-ce6 dark control) in Figure 5B, and this could be quantified to give a semi-log dose response (Figure 5C). The loss of bioluminescence is observed immediately after PDT and does not return unless the bacteria regrow, which takes at least 24 h. There was no reduction of bioluminescence in light alone controls.

Gram (–) bacteria—Several *in vitro* and *in vivo* studies have been carried out to verify the efficiency of PDT in eradication of Gram (–) biofilms (Table 2), particularly in oral infections and chronic wound infection. Lee *et al.* described the use of aPDT using δ-aminolevulinic acid (ALA) to eradicate *P. aeruginosa* planktonic forms and biofilms. Results showed that planktonic *Pseudomonas* was eradicated with 10-mM ALA at a light dose of 240 J/cm^2, while the biofilm was also eradicated using ALA 20 mM at the same dose of light 240 J/cm^2, but it required two separate treatments to achieve complete eradication of the biofilm [156]. In another *in vitro* study, Wood *et al*. collected 7-day oral plaque biofilms *in vivo*. The cells were then incubated with a cationic Zn(II) phthalocyanine PS and irradiated with white light. The results demonstrated that the PS was taken up into the biomass of the biofilm and PDT significantly killed the bacteria. Moreover, after PDT treatment, severe damage to bacteria was observed by electron microscopy, including vacuolation of the cytoplasm and membrane damage [157].

Fimple *et al*. studied the use of aPDT mediated by MB and red light on multispecies root canal biofilms composed of *Actinomyces israelii, F. nucleatum, P. gingivalis* and *Prevotella intermedia*. These biofilms were grown in root canals of extracted human teeth *in vitro*. PDT reduced the bacterial numbers by 80% in a CFU assay [161]. Moreover, aPDT using TBO was investigated against periodontopathogens (*A. actinomycetemcomitans, P. gingivalis, F. nucleatum* and *P. intermedia*) to verify its efficacy for periodontitis [162]. The study suggested that the TBO and diode laser light (630 nm) was effective for the killing of biofilm-forming bacterial strains. In one clinical study performed in implants contaminated with *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans*, it was found that the combination of TBO and irradiation with a diode soft laser (660 nm) was effective to reduce the bacterial counts by 2 log [163]. Garcez *et al.* compared the efficiency of PDT, standard chemomechanical endodontic treatment and the combination of both using bioluminescent bacterial biofilms of *P. mirabilis* and *P. aeruginosa* in extracted teeth. PDT reduced bioluminescence by 95%. Endodontic therapy reduced bacterial bioluminescence by 90%. The combination of both reduced bioluminescence by >98% [164].

Recently, nanotechnology has been investigated to improve the treatment of an oral infection caused by polyspecies biofilm [165]. Klepac-Ceraj *et al*. [166] reported that PDT mediated by MB-loaded poly(lactic-co-glycolic) nanoparticles (NP) with a positive or negative charge could be used to reduce biofilms of human dental plaque bacteria *in vitro* [166]. All these studies demonstrated that PDT could be considered a good alternative method to manage Gram (–) biofilms.

Several studies have been focused on biofilm infection in chronic wounds and infections of the dermis, including burns and surgical site infections that are a big problem in healthcare. One of the major problems in the management of biofilms is that usually biofilms consist of multiple bacterial species. Much advancement has been achieved in molecular diagnosis to provide methods of identifying microbes present in wounds [167]. Recently, several studies have focused on mono- and poly-species biofilms to better understand the interaction between different bacteria stains and the environment [158,163].

Our laboratory reported a study in mouse third-degree skin burns infected by bioluminescent *Acinetobacter baumannii* [168]. The chronic nature of the infection is illustrated by its continuance for well over 8 days as shown in Figure 6A and the actual biofilm is shown by Gram stain in Figure 6B. When PDT was mediated by PEI-ce6 and 660-nm light, topically applied to the burn, a light dose-dependent reduction in bioluminescence signal was seen in the PDT group, which is not seen in either the light alone control or the PEI-ce6 dark control (Figure 6C).

Fungal biofilms—*Candida* biofilm is one of the main causes of clinical problems affecting implanted biomaterials such as intravascular catheters, pacemakers, prostheses, stents, shunts, urinary catheters and orthopedic implants [169]. Its treatment with current antifungal agents is considered challenging and aPDT alone or in conjunction with another therapy has been suggested as an alternative approach [170]. Yeast biofilm is composed of a heterogeneous mixture of blastoconidia, pseudohyphae and hyphae embedded in EPS that form channels and pores and display different phenotypic features compared with planktonic yeasts [24]. The ECM is composed of polysaccharides, proteins, hexosamine, uronic acid and DNA, which are responsible for promoting biofilm formation and adhesion, protecting the cells from phagocytosis, which in return maintains and limitd the diffusion of substances, such as antifungal agents, and also restrictd penetration of PS and light during aPDT [24,171]. The high density and larger size of cells in yeast biofilms, the presence of nuclear membrane in the cells, the reduced number of targets for singlet oxygen per unit of cell volume, existence of multiple species (bacteria and yeasts together), enzymatic alteration of active agent, presence of persister cells – a subpopulation of cells that spontaneously go into a dormant, non-dividing state and which in turn remain alive following anti-microbial treatments and drug-efflux pumps further increase the resistance to both antifungal agents and PDT [24,124,169,172,173].

Several studies have been focused to use aPDT against fungi (Table 3) – Chabrier-Rosello *et al.* investigated the effects of Photofrin-mediated PDT (Hg arc lamp, 400–700 nm, 15 $mWcm^{-2}$, 0.9–18 J cm^{-2}) against *C. albicans* biofilms and germ tubes [174]. Biofilm submitted to PDT at 18 J cm^{-2} demonstrated significant reduction of metabolic activity compared with the biofilm treated with amphotericin B (10 μg ml^{-1}) alone. Following treatment, the authors observed that the germ tube, which is important for the formation of hyphae and constitute the mature biofilm, lost its membrane integrity, leading to cellular death. Moreover, phototoxic responses to biofilm were fluence and concentration dependent with significant differences seen in organism metabolic activities in irradiated cultures. Chabrier-Rosello *et al*. reported that photosensitivity was retained in the absence of serum and the gradual loss of photosensitivity occurred in the presence of serum [174]. Based on this observation, the group have suggested that *C. albicans* germ tubes do not significantly alter the photosensitizing properties of Photofrin, nor do they pump the PS from the cell in a rapid manner. Instead the presence of serum within the incubation medium may lead to leaching of PS to the external milieu, which is also commonly observed in tumor cells [175,176].

The same authors also obtained interesting results against biofilms of *C. albicans* and *Candida dubliniensis* (this species produces a complex mature biofilm with the same fungal

morphologies expressed by *C. albicans* and isolated predominantly from the oral cavities of patients with AIDS) using ERY (400 mM) and green light-emitting diode (LED) light (532 ± 10 nm, 90 mW, 237 mW cm^2, 42.63 J cm^2). Significant reductions in CFU/ml of 0.74 log_{10} and 0.21 log_{10} have been observed, respectively [177].

Mucocutaneous oropharyngeal candidiasis, known informally as oral thrush, is predominately caused by *C. albicans*, and it is frequently isolated from denture wearers and from immunosuppressed patients [178]. PDT can be an alternative treatment option however highly colored nature of PS causes staining of teeth, and oral mucosa, and therefore using it as a liquid mouthwash becomes undesirable [179]. Donnelly *et al.* suggested using a mucoadhesive patch containing TBO that is capable of resisting dissolution when immersed in artificial saliva [179]. When released directly into an aqueous sink, patches were able to eradicate both planktonic and biofilm-grown *C. albicans* upon illumination (635 nm, 100 $mWcm^{-2}$, 200 J cm^{-2}). However, it is worthwhile to note that sterilization of biofilm structures required higher concentrations of TBO (5.0 mg ml^{-1}) and longer times of application of the patches (3 h) as opposed to planktonic grown *C. albicans* (2.0 mg ml^{-1} and 30 min) [179].

Costa *et al.* investigated the antifungal activity of PDT with two different PS – RB and ERY – that have light absorption at wavelengths of 450–600 and 500–550 nm, respectively [124]. *C. albicans* biofilms subjected to PDT (455 ± 20 nm, 200 mW, 95 J cm^{-2}) exhibited a reduction in the number of cells in relation to the control group, which received neither dye nor light irradiation. The highest mean reductions in the number of cells, in relation to the control group, was 0.15 in log_{10} when submitted to ERY-mediated PDT and 0.12 log_{10} when submitted to RB-mediated PDT. Furthermore, reduction in number of hyphae and deformed cells was observed with PDT treatment [124].

In an *in vitro* study, water-soluble phthalocyanine complexes of silicon (SiPc1) and germanium (GePc1) were tested as potential PS for PDT of *C. albicans* biofilm cultures [180]. Results demonstrated that fungal suspension was completely inactivated at low concentration of SiPc1 (1.8 mM) using 635 nm, 50 J cm^{-2}, 60 mW cm^{-2} PDT, however it was not as effective on mature fungal biofilms grown on denture acrylic resin [180]. Moreover, the effect of GePc1 was negligible (1 log) even at strong light treatment [180].

A recent study reported that gold NP enhanced aPDT of MB against recalcitrant *C. albicans* biofilm [169]. Following application of 38.2 J/cm^2 of 660-nm diode laser for activation of gold nanoparticle–MB conjugate and against *C. albicans* biofilm and cells, significant reduction of biofilm and killing of *Candida* cells was observed [169]. Based on the results, the authors of this study suggested that gold nanoparticle conjugate-mediated aPDT may be used against nosocomially acquired refractory *C. albicans* infections [169].

The effects of aPDT using a green LED (532 ± 10 nm, 90 mW, 14.34 J cm^{-2}) with erythrosin (400 μmol l^{-1}) on buccal candidiasis in mice was evaluated [178]. Reduction of 0.73 log_{10} was observed in *C. albicans* biofilm [167]. However, the authors noted that the reduction could have been greater if the number of PDT sessions was increased. Mima *et al.* treated oral candidiasis lesions of 71, 6-week-old female Swiss immunosuppressed mice

with Photogem (a hematoporphyrin derivative) at concentrations from 400 to 1000 μg/ml and irradiated them with a mixed blue LED and red LED (305 J cm^{-2}, 200 mW, 455 nm and 630 nm) [181]. Results showed reduction of 1.04 to 1.59 $\log_{10}$ of *C. albicans* cells concluding that treatment method used in this study promoted significant reduction in the viability of *C. albicans* biofilm without harming the tongue tissue [181].

Our laboratory used our bioluminescence technology to demonstrate the effectiveness of PDT of a chronic biofilm-form *Candida* infection in mouse 3rd degree cutaneous burns (Figure 7A & B) [182]. When the NMB was combined with 660 nm light at 78 J cm^{-2}, the bioluminescence signal did not occur in the days following this.

Delivery systems

The biomedical applications of NP are endless. Many experimental approaches employing NP have been carried out with a great emphasis on imaging and drug delivery. NP has the potential to revolutionize the diagnosis and treatment of various diseases while allowing targeted delivery of a drug to specific sites within cells. NP possess proprieties such as a high surface-to-volume ratio, unique shapes and optical characteristics, and NP can be associated with other materials and further improve these proprieties. Multifunctional nanostructured platforms with well-defined combinations of reporter and effector molecules are a possibility that exemplify the versatility of NP. A strategic advantage of these platforms is that they retain efficient solubility and colloidal property for use in more complex environments (blood, tissue, among others) [183,184].

Different sizes and shapes of NP affect uptake rates. Spherical shaped nanostructures seem to be better than rod-shaped ones due to their higher uptake and lower exocytosis. The accumulation and rate of uptake of the NP in cells is determinate by their geometric features. The selection of the correct NP can result to improvements in biomedical applications [185].

NP plays an important role in PDT and can be classified into drug delivery vehicles or the actual PS. The molecular encapsulation by NP provides an inert environment, which protects the drug (or PS) from recognition or removal until it has reached the target [183]. NP allows delivery of a hydrophobic drug to its site of PDT action. This process of drug delivery is highly efficient and reduces the time of PS uptake into cells [186]. NP themselves can be used as PS, for example, including the following classes of molecules: fullerenes, ZnO, TiO_2 and QD [187]. These NP-PS produce ROS to kill cells because of their intrinsic optical and photochemical proprieties [188,189]. Nano-TiO_2 and ZnO combined with visible light is termed photocatalytic disinfection when used for sterilization and may also have a great potential to treat infections [190]. NP can also be ideal for PDT as a result of improved $^1O_2$ production by efficient energy transfer [191].

Delivery systems such as lipid and detergent nanostructures have been frequently used in aPDT. Comparisons between the free PS and encapsulated PS have demonstrated that liposomes and micelles increased the efficiency of aPDT [192,193]. In antimicrobial endodontic PDT, the utilization of NP encapsulated with PS resulted in a promising adjunct treatment [194]. The ability of gold NP to enhance the photoantimicrobial activity of TBO was shown in a study with *S. aureus*. The nanogold increased the light capture and the

formation of reactive species by the PS [195]. Many different types of interactions between NP and PS have been reported (PS embedded in polymeric NP, PS bound to the surface of NP, PS alongside NP, NP as the PS themselves) and all of them have been shown to photodestroy microorganisms [196].

The usefulness of encapsulating PS in the matrix of hydrogel NP was demonstrated by the light-induced reduction of microbial and biofilm growth. These nanoplatforms diminished the dark toxicity of free dyes and their photobactericidal activity was higher for Gram (+) bacteria than for Gram (−) [186]. Multifunctional platforms can be designed to provide both diagnosis and treatment of recalcitrant bacterial and biofilm infections. Viral protein cage structures, liposomes and dendrimers have been used to produce the architecture of these platforms. Bacterial pathogens express several surface proteins that can be specific molecular targets to bind ligands on nanoplatforms and to deliver substances to biofilms [197].

Drug delivery systems represent an important way to permeate biofilms and kill microorganisms. Nanoemulsions (NE) could be used as a topical cream to treat superficial fungal infections. NE present a promising strategy to penetrate PS into biofilms and NE offers greater stability to the light-sensitive drug [187].

Cationic fullerenes and other derivatives have also been described to have an antimicrobial effect via aPDT [130,198]. These fullerenes have a clinical potential as antimicrobial PS and have the advantage of high photostability [199]. The combination of diagnostics, imaging and therapeutic agents on the same platform is now known as theranostics [187]. Multifunctional NP fit perfectly in this context because of their properties [200].

Combined approaches

The low yield of ROS, the poor accumulation of PS in biofilms and the difficulty that PS faces in penetrating to the bottom of EPS layers are the principal limitations to the effectiveness of aPDT in biofilms [150]. According to Zanin *et al*., absolute uptake of dye by biofilm is tenfold higher when a cationic PS is used compared with anionic PS [152]. They suggested that this may be explained by the EPS ‘trapping’, the PS on the outside of the cell owing to ionic or hydrophobic interactions and therefore reducing the amount of PS that is able to penetrate to the plasma membrane, which is thought to be one of the important sites of PDT-mediated damage [152].

To enhance PDT, new strategies have been tested, for example, the use of aerosol NP, surfactants, nanosized crystals and microbubble emulsion. Vatansever *et al*. loaded the PS MB with aerosol OT alginate, which promoted enhanced intracellular ROS production leading to killing of cells [200]. Another method for delivering hydrophobic drugs for PDT is the use of nanocrystals of the drug. This method proposes that the dispersion of the PS is realized by reprecipitation. Results of a study showed *in vivo* and *in vitro* efficacy [201]. Microbubble emulsion combined with sonic or ultrasonic agitation reduced biofilms significantly in root canal models [202].

Biel has proposed a variety of methods to potentiate the penetration and photoactivity of the phenothiazinium dyes, MB, TBO and analogues in bacterial biofilms. These additives have included polymyxin B [301], SDS [302] and EDTA [303].

Doukas *et al*. and Lee *et al*. developed a method of using laser-induced shock waves or photomechanical waves to permeabilize various biological membranes including cell membranes and stratum corneum for enhancing drug delivery [203–205]. The method relies on ablation of a black plastic target by a picosecond laser pulse. They also went on to study this effect in bacterial biofilms [206]. Biofilms of *Actinomyces viscosus* were formed on bovine enamel surfaces. The photomechanical wave was generated by ablation of a target with a Q-switched ruby laser and launched into the biofilm in the presence of 50 μg/ml MB. How well the MB penetrated into the biofilm was measured by CLSM. After the biofilms had been treated with MB and shock waves, they were illuminated with 666-nm light. Surviving cells were quantified by disaggregation and CFU enumeration on blood agar plates. CLSM showed that a single laser-induced photomechanical wave was able to increase the penetration depth of MB into the biofilm by 75%. The shock wave significantly increased the concentration of MB in the biofilm and allowed greater bacterial killing.

PDT in dental biofilms

Periodontal diseases are among the most common oral diseases in the world, leading to tooth loss. Upon poor oral hygiene, constituents of the diet interact with oral bacteria leading to changes in the local microflora resulting in an upsurge of oral biofilms that adhere in teeth and soft tissues of the mouth. Thus, bleeding of the gingiva (gingivitis) can advance and involve supporting tissues of the teeth such as bone, creating the periodontal pocket (periodontitis). An adequate treatment must be done in order to stop the advance of the disease. However, due to the complex biofilm composition, systemic therapy may be associated with conventional therapy of periodontal diseases.

Chronic use of antibiotics has led to the resistance of to many bacterial strains. Thus, the development of alternative therapies is necessary in order to control anaerobic Gram (−) microorganisms in periodontal infections. The large number of review articles on the combination of low power laser with PS to carry out PDT in periodontal disease conditions reflects the increasing use of PDT in dentistry, which represents a novel therapeutic approach in the management of oral biofilms [157,207,208].

Early in 1996, Wilson *et al.* quantitatively investigated the lethal photosensitization of *Streptococcus sanguis* which is a predominant organism in dental plaque [209]. The *S. sanguis* grown as biofilms under similar conditions to those existing in the oral cavity can be killed by using a light-activated antimicrobial agent – aluminium-di-sulfonated phthalocyanine ($AlPcS_2$) – with GaAlAs laser light [209].

In 2002, O'Neill *et al.* performed a study where a multispecies biofilm of oral bacteria was irradiated with helium/neon laser with 31.5 J in the presence of 25-mg ml^{-1} TBO [210]. They observed that 97.4% were killed concluding that this may be useful in the treatment of dental plaque-related diseases [210].

Fontana *et al.* investigated the PDT effect of MB (25 μg ml^{-1}) in reducing the number of dental bacteria in the planktonic phase and in biofilms after exposed to red light for 5 min [211]. They observed 63% reduction of bacteria in the suspension form and only 32% in the biofilms. Thus, the conclusion was that PDT is less effective in biofilm conditions than in bacteria planktonic phase [211].

A. actinomycetemcomitans is a microorganism responsible for causing aggressive periodontitis. Goulart Rde *et al.* evaluated the photoinactivation of this microorganism by RB in planktonic and biofilm cultures. The PDT caused 45% of reduction in the biofilm without damage to gingival fibroblast cells. The reduction of bacteria was dependent on RB concentration and irradiation time [212]. The MB and ERY have been also effective against *A. actinomycetemcomitans*. However, ERY is more efficient at killing these bacterial cells in planktonic (75%) and biofilm (77%) than MB (50 and 54%, respectively). In summary, RB, ERY or MB could be an efficient option for pocket decontamination in aggressive periodontal diseases [161].

An *in vivo* study evaluated PDT as an adjunctive treatment for induced periodontitis in diabetic rats. In addition to the scaling and root planning (SRP) as conventional treatment of periodontitis, PDT was applied once with TBO 100 μg ml^{-1} and a red source laser for 133 s. Results showed that PDT groups had less bone loss than groups given only the SRP treatment. Even though the animals had a systemic disease, the adjunctive therapy was able to decrease the signals of periodontal destruction [213].

A clinical study conducted in ten patients with a diagnosis of aggressive periodontitis were treated with SRP or MB-PDT and evaluated in terms of clinical outcomes. After 3 months, both treatments led to improvements in reducing the signs of disease, but with no significant statistical differences between them [214].

Another clinical study evaluated 58 individuals submitted to SRP alone, SRP + MB-PDT and MB-PDT alone. The two most significant outcomes measured (probing depth, PB and clinical attachment level) had no statistical difference among the groups. However, bleeding on probing decreased in the group with the combined therapies [215].

In 2011, a statement released by the American Academy of Periodontology reported that there was a lack of evidence of the effectiveness of PDT based on clinical trials, where only two trials observed great bacterial reduction from ten already published. Therefore, the American Academy of Periodontology stated that PDT was unpredictable and inconsistent in the ability to reduce bacterial loads compared with SRP alone [203].

Other clinical applications of PDT in biofilms

PDT was tested as a novel antimicrobial treatment in bacterially colonized chronic leg ulcers and chronic diabetic foot ulcers [216]. The PS used was cationic 3,7-bis(di-n-butylamino) phenothiazin-5-ium bromide (PPA904), which has been shown to kill a broad spectrum of bacteria *in vitro* and in biofilms. The trial included 16 patients with chronic leg ulcers and 16 patients with diabetic foot ulcers (each eight active treatment/eight placebo) in a blinded, randomized, placebo-controlled, single treatment, Phase IIa trial. All patients had an ulcer

duration of >3 months, bacterially colonized with >10(4) CFU ml^{-1}. After quantitatively assessing pre-treatment bacterial load via swabbing, PPA904 or placebo was applied topically to wounds for 15 min, followed immediately by 50 J cm^{-2} of red light and the wound was again sampled for quantitative microbiology. Wound area was measured for up to 3 months following treatment. The treatment was well tolerated with no reports of pain or other safety issues. In contrast to placebo, patients on active treatment showed a reduction in bacterial load immediately post-treatment ($p < 0.001$). After 3 months, 50% (four out of eight) of actively treated chronic leg ulcer patients showed complete healing compared with 12.5% (one out of eight) placebo patients.

There are other emerging clinical applications of PDT in this arena of infectious disease, and we can highlight three of these. Chronic sinusitis is caused by bacterial biofilms building up in the cavity of the maxillary sinuses that can be reached by fiber optic to deliver light after MB in the correct formulation has been applied into the cavity [217]. Furthermore, care must be taken that this PDT procedure does not damage the relatively sensitive ciliated mucosa that lines the sinus cavity [303]. Endotracheal tubes are common in intensive care units where respirators are employed, and can rapidly become contaminated by bacterial biofilms. This contamination dramatically raises the incidence of hospital-acquired pneumonia and its associated mortality and morbidity. A PS like MB could be periodically applied into the tube followed by red light illumination to reduce the biofilm buildup [218]. The last emerging application is in the problem of decontamination of the nose from MRSA before surgery. The anterior nares are frequently contaminated with MRSA biofilms and it has been shown that use of mupirocin cream up the nose before surgery known as ‘decolonization’ decreases the incidence of postsurgical MRSA infection [219]. However, the problem of mupirocin resistance is rising and could soon become problematic [220]. Again MB and red light applied up the nose has been shown to decolonize the MRSA, and trials are underway to test whether PDT can reduce the incidence of postsurgical MRSA infections [401].

Expert commentary

Even though PDT still has a long way to progress in clinical applications of biofilm based infections, it is already clear that this therapy can be effective in many clinical situations due to the unique characteristics of this approach [13,14]. In dentistry PDT applications related to caries, endodontic infections and dental implants have been evaluated and promising results have been achieved [17]. Several studies have showen that clinical infections related to biofilms are heavily involved in the chronic nature of the disease. The human trials in non-healing leg ulcers, chronic sinusitis, decontamination of endotracheal tubes and decolonization of the nose from MRSA all show that clinical use of PDT in biofilm infections will continue to progress.

Five-year view

Fifty years ago, infectious disease was pronounced ‘cured’ because of the amazing success of antibiotics. It was thought that progress would continue in the same vein until the problem ceased to exist. Instead the exact opposite happened. Due to overuse of antibiotics

for viral infections, in livestock feedstuff, and inappropriate prescribing in many countries, multiantibiotic resistance has spread around the world. Combining the problem of antibiotic resistance with the realization that a large proportion of human microbial infections are caused by biofilm-dwelling species has led to an international search for more effective antimicrobial therapies to overcome existing resistance and to avoid inducing new resistance. aPDT and PDI are promising approaches to kill microbial cells that otherwise would hide in biofilms and escape from conventional antimicrobial agents. Because aPDT involves *in situ* generation of highly reactive species such as singlet oxygen and hydroxyl radical, there is a good likelihood that structurally important elements of the biofilm will be oxidatively damaged resulting in weakening of its integrity of the biofilm and its eventual dispersal. Nevertheless, it is likely that combination modalities will be needed to realize the full potential of aPDT to destroy biofilms, and in this context, nanotechnology and physical energy delivery methods will have major roles to play.

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Key issues

- Biofilms are complex multicellular communities of microorganisms that develop due to environmental stress.
- Extracellular polysaccharide forms a matrix in which microbial cells are embedded and nutrients reach the lower regions via water channels.
- Although many laboratories study single-species biofilms, in nature, multispecies biofilms are much more common.
- Biofilms are up to 10,000-times more resistant to antimicrobial compounds than free growing cells.
- Photodynamic therapy (PDT) or photo inactivation combines nontoxic dyes and harmless visible light to produce toxic reactive oxygen species that kill microbial cells.
- A multitude of compounds can act as photosensitizer but possession of one or more cationic charges is necessary to be a broad-spectrum photoantimicrobial.
- Biofilms are susceptible to PDT depending on conditions, but biofilm cells are more resistant compared with planktonic cells.
- Various combination strategies can be used to potentiate PDT of microbial biofilms.
- Clinical trials of PDT have been successfully conducted for many human infections characterized by microbial biofilms.

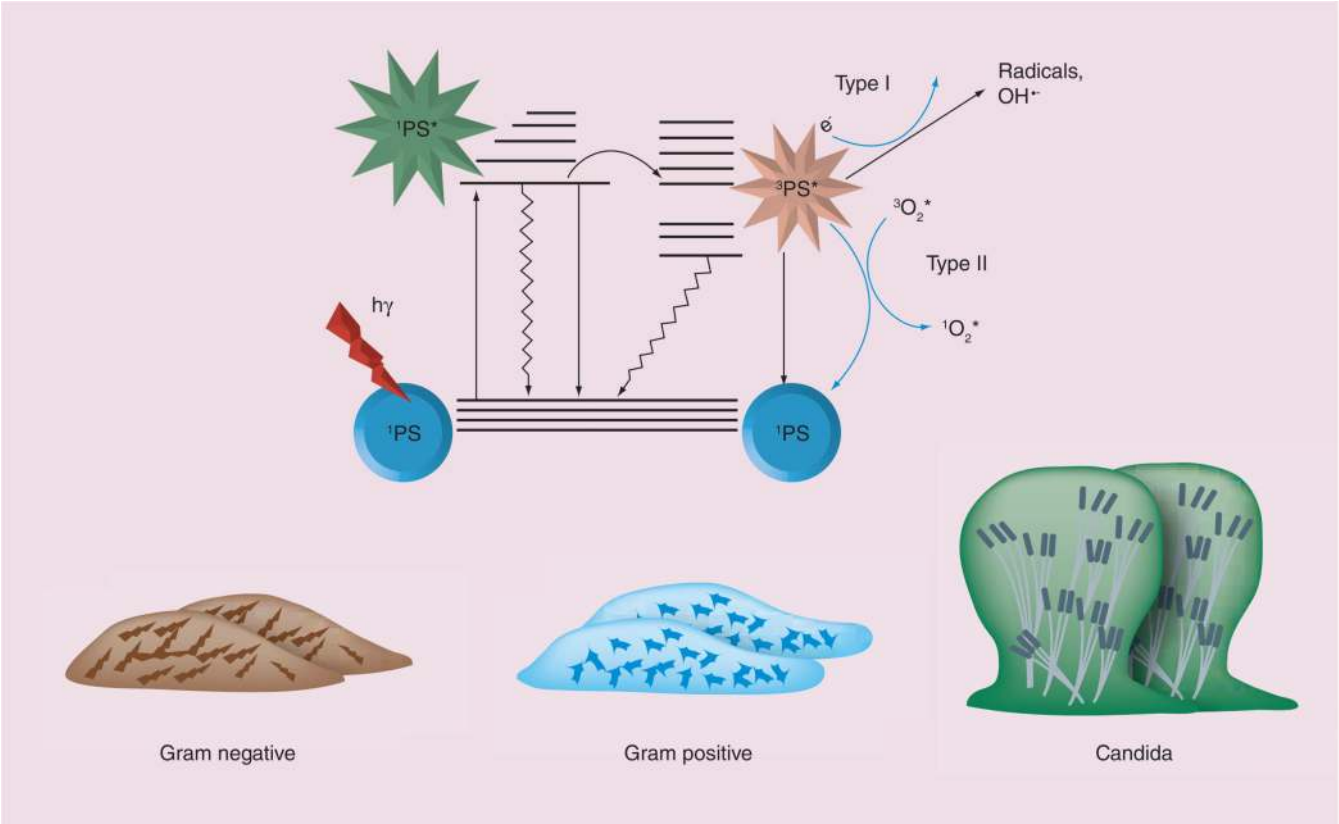

Figure 1. The photosensitizer absorbs photons from light and causes excitation to the singlet excited state (1PS*)

The singlet excited PS* can decay back to the ground state with the release of energy in the form of fluorescence. It is possible for the singlet to be converted into the long-lived triplet excited state (^{3}PS*), which is able to transfer energy to another triplet (ground state oxygen) or alternatively carry out electron transfer to oxygen producing hydroxyl and other radicals via superoxide. These reactive oxygen species can disrupt biofilms and kill both bacterial and fungal cells.

PS: Photosensitizer.

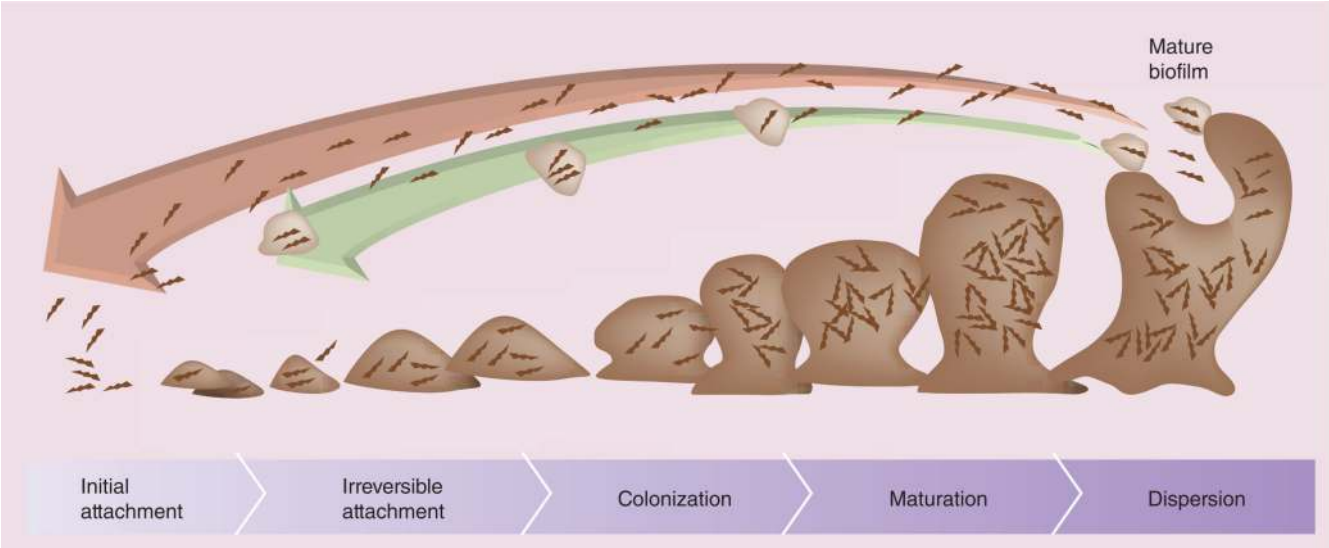

Figure 2. The five stages of biofilm development

Initial attachment, irreversible attachment, colonization, maturation and dispersion.

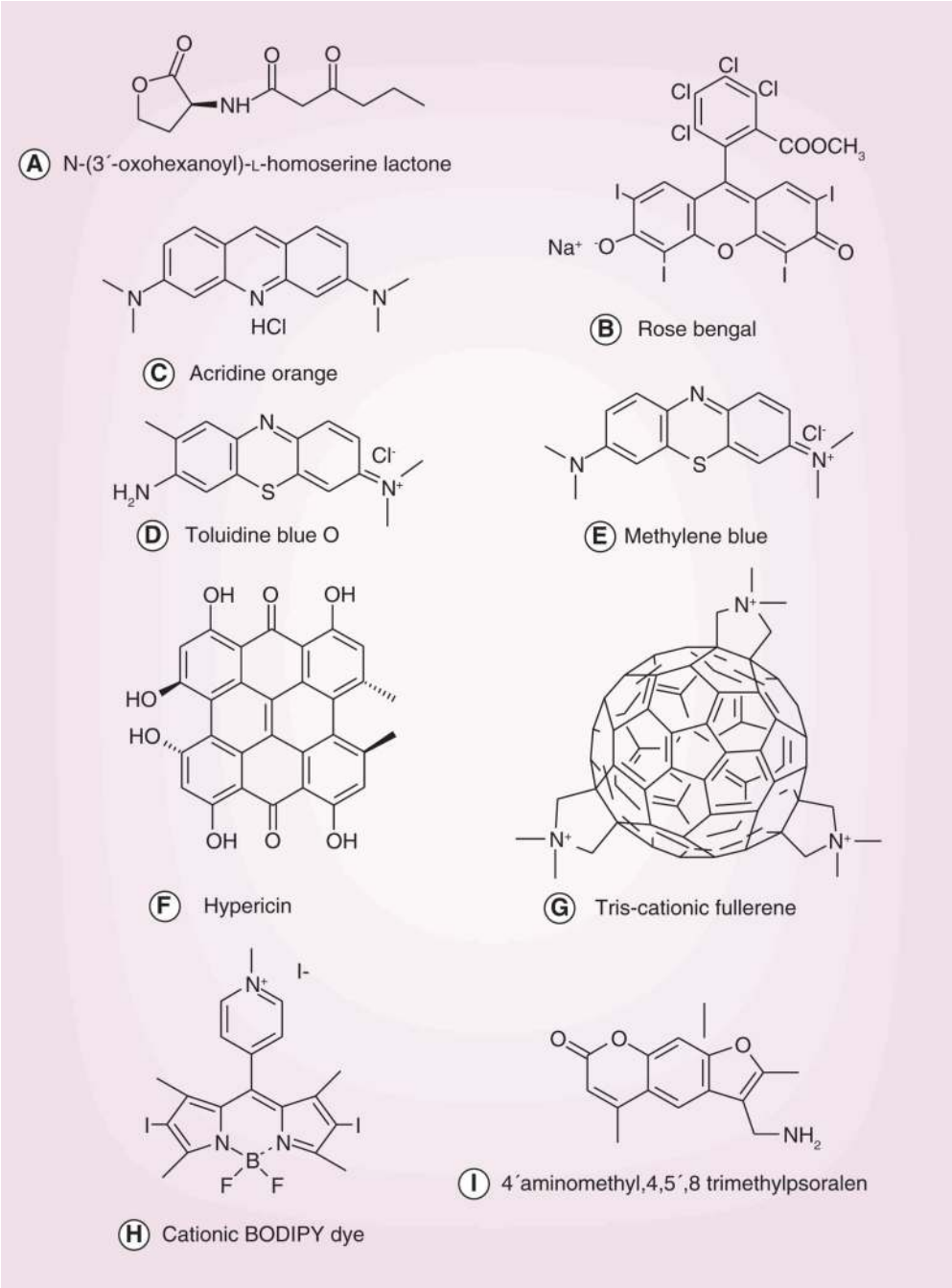

Figure 3.

(A) Homoserine lactone and eight antimicrobial photosensitizers, **(B)** rose bengal, **(C)** acridine orange, **(D)** toluidine blue O, **(E)** methylene blue, **(F)** hypericin, **(G)** a Tris-cationic fullerene, **(H)** a cationic BODIPY dye and **(I)** 4´aminomethyl,4,5´,8 trimethylpsoralen.

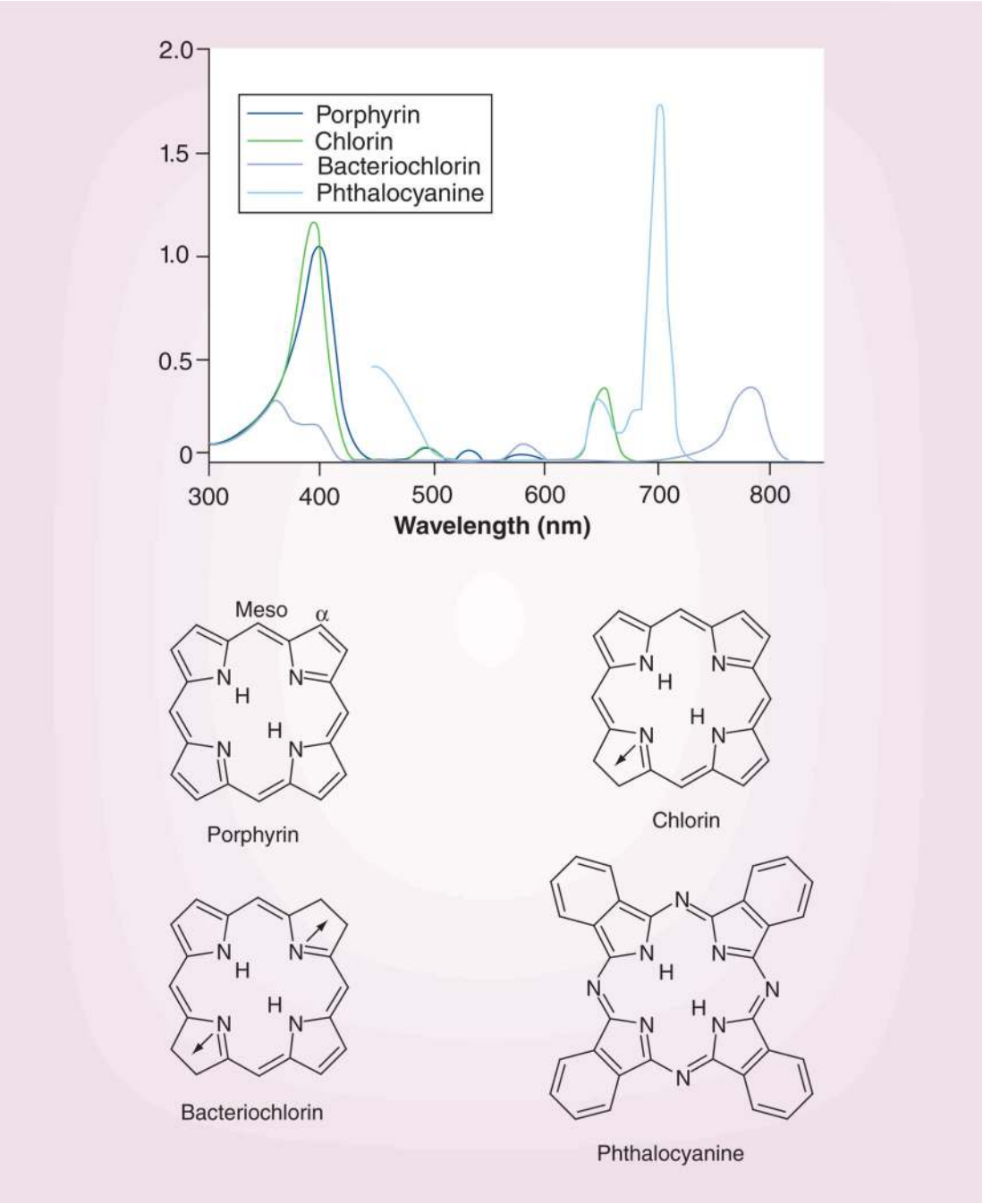

Figure 4. Absorption spectra and chemical structures of the four main classes of tetrapyrrole photosensitizers

(A) Absorption spectra and **(B)** chemical structure of the four main classes of tetrapyrrole photosensitizers. As the number of saturated rings in the macrocycle increases, the long-wavelength absorption band is red-shifted and also increases in magnitude.

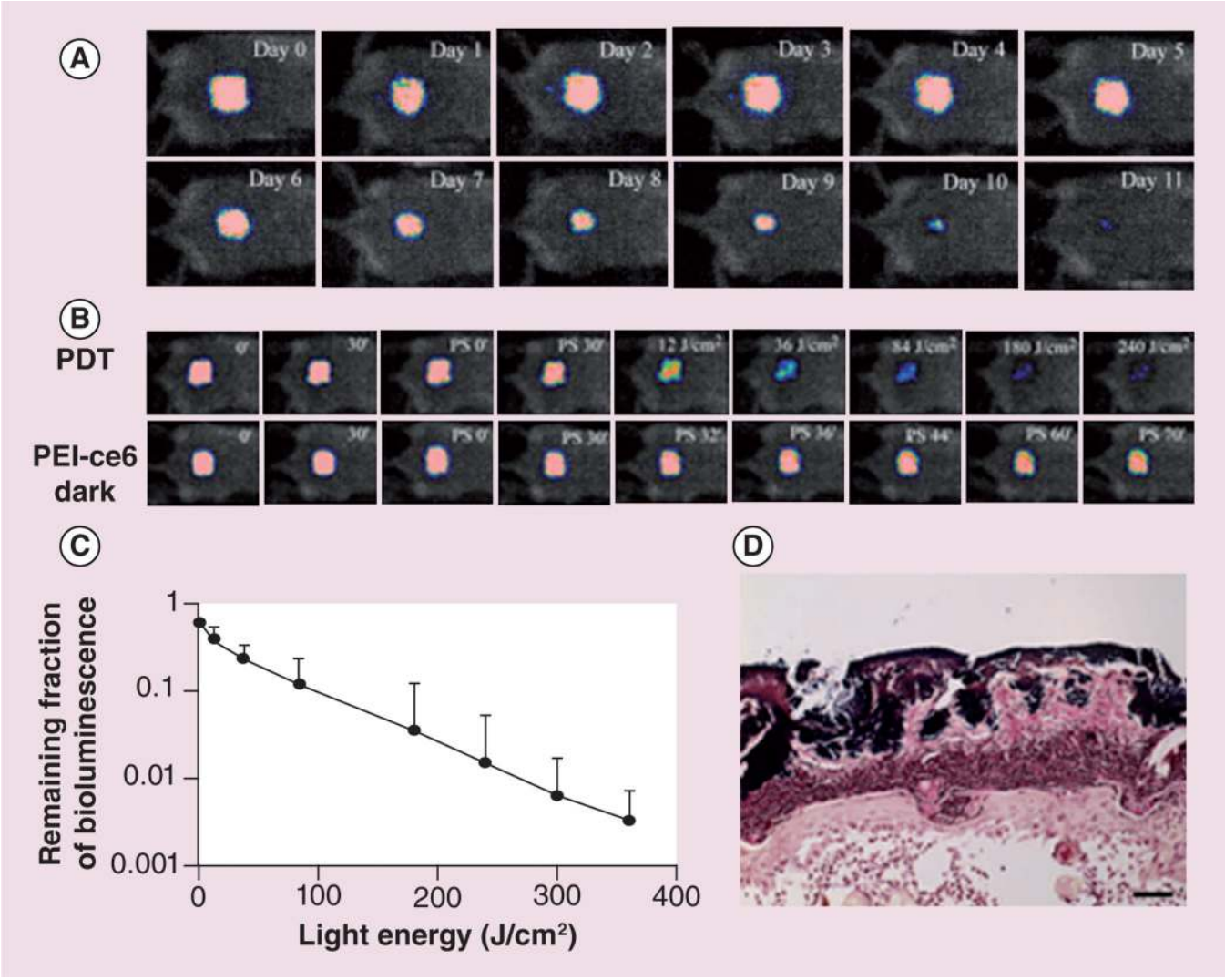

Figure 5. Photodynamic therapy for methicillin-resistant *Staphylococcus aureus* infection in a mouse skin abrasion model

(A) Successive bioluminescence images over 11 days of a representative mouse skin abrasion wound infected with bioluminescent methicillin-resistant *Staphylococcus aureus* (MRSA). **(B)** Successive images of a PDT-treated wound receiving aliquots of light or the PEI-ce6 dark controltreated wound left for equivalent periods of time. **(C)** Quantification of luminescence signal from PDT-treated wound. **(D)** Gram-stained section of a mouse skin abrasion specimen showing the biofilms formed by Gram-positive MRSA near the skin surface. Scale bars: 20 μm. Dark blue area: biofilms of MRSA. The mouse skin abrasion specimen was harvested at day 3 postinfection.

PDT: Photodynamic therapy; PEI-ce6: Polyethylenimine chlorine (e6); PS: Photosensitizer. Reproduced with permission from [159].

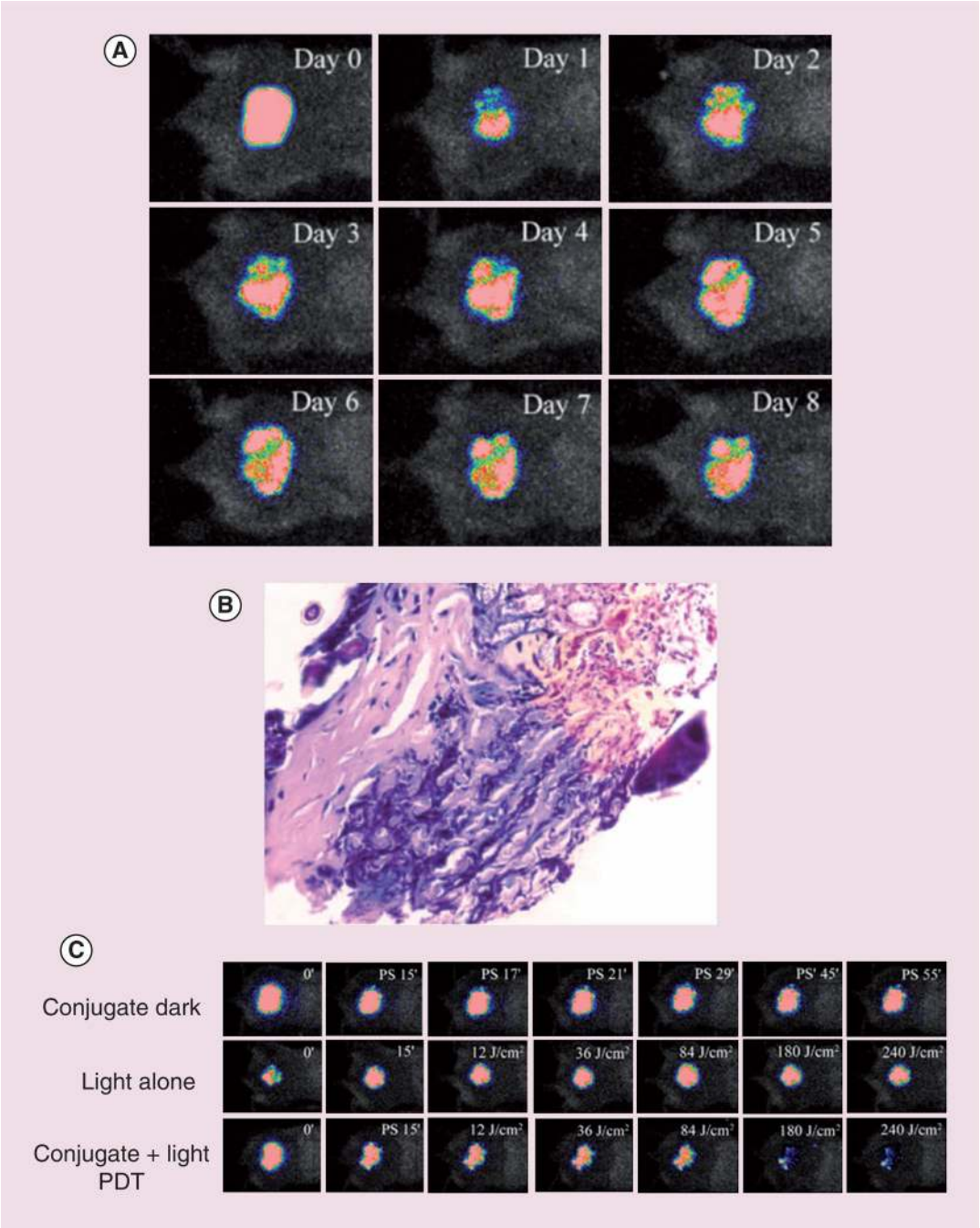

Figure 6. Photodynamic therapy for *Acinetobacter baumannii* burn infections in mice

(A) Successive bioluminescence images of a representative mouse burn infected with bioluminescent *Acinetobacter baumannii*. **(B)** Gram-stained histology sections of the mouse burns at day 5 after bacterial inoculation. **(C)** Successive bioluminescence images from representative mice treated with polyethylenimine chlorine (e6) in dark; increasing fluences of light with no photosensitizer; PDT with polyethylenimine chlorine (e6) plus red light. PDT: Photodynamic therapy; PS: Photosensitizer.

Reproduced with permission from [168].

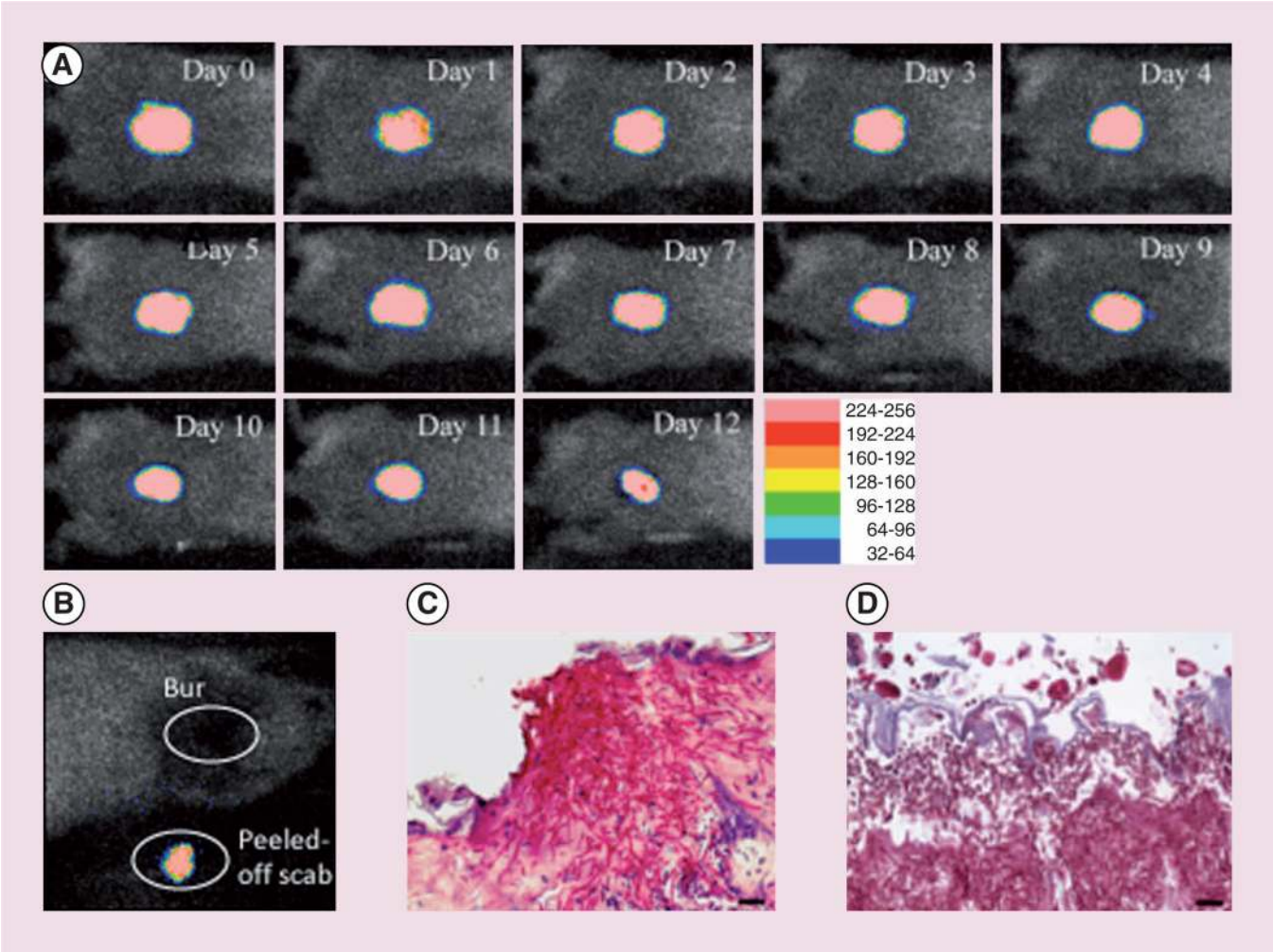

Figure 7. Photodynamic therapy for *Candida albicans* cutaneous infections in mice

(A) Successive bioluminescence images captured daily for 12 days representative of a mouse burn infected with bioluminescent *Candida albicans*. **(B)** A representative bioluminescence image of a mouse burn immediately after the peel-off of its scab as well as the detached scab from the same mouse. **(C & D)** Representative periodic acid Schiff-stained histological sections of *C. albicans*-infected mouse burns biopsied on day 1 and day 4 postinfection, respectively. Scale bars: 20 μm.

Reproduced with permission from [182].

Table 1

Antimicrobial photodynamic therapy studies in Gram-positive bacterial biofilms.

Gram-positive bacteria	PS	PS concentration	Light source (wavelength [nm])	Light dose (J/cm^{-2})	Reductions microbial biofilms CFU log_{10}	Ref.
Staphylococcus Aureus	MB	0.1 mg ml^{-1}	InGaAlP (660)	350	3.29	[149]
	MB	50 μM	Diode laser (665)	40	2.00	[150]
	pL-ce6	1 μM	Diode laser (665)	40	1.00	[150]
	TBO	40 μM	Diode laser (640)	200	5.00	[151]
	Hypericin	100 nM	Halogen lamp (690)	135	5.00	[153]
	mTHPC	100 nM	Halogen lamp (690)	135	5.50	[153]
	Merocyanine	20 μg ml^{-1}	Green light (510–570)	600	7.00	[154]
	TMP	10 μM	White light (400–800)	200	2.00	[155]
	PEI-ce6	400 μM	Noncoherent light (660)	360	2.70	[159]
Staphylococcus mutans	MB	0.1 mg ml^{-1}	InGaAlP (660)	350	2.81	[149]
	TBO	0.1 mg ml^{-1}	HeNe laser (632)	294	4.50	[152]
	TBO	0.1 mg ml^{-1}	Diode laser (638)	294	3.00	[152]
	TBO	0.1 mg ml^{-1}	Diode laser (638.8)	85.7	1.00	[221]
	Erythrosine	22 μM	Tungsten lamp (500–550)	20.5	3.00	[157]
	MB	22 μM	Tungsten lamp (600–650)	20.5	2.60	[157]
	Photofrin	22 μM	Tungsten lamp (600–650)	20.5	1.10	[157]
S. aureus/S. mutans	MB	0.1 mg ml^{-1}	InGaAlP (660)	350	2.22 /2.10	[149]
Staphylococcus epidermidis	MB	50 μM	Diode laser (665)	40	1.20	[150]
	pL-ce6	1 μM	Diode laser (665)	40	1.50	[150]
	TBO	40 μM	Diode laser (640)	200	6.00	[151]
Streptococcus sobrinus	TBO	0.1 mg ml^{-1}	Diode laser (638.8)	85.7	1.50	[221]
Streptococcus sanguinis	TBO	0.1 mg ml^{-1}	Diode laser (638.8)	85.7	4.00	[221]
Enterococcus faecalis	MB	25 μg/ml	Diode laser (665)	222	4.00	[158]

MB: Methylene blue; mTHPC: m-tetrahydroxyphenylchlorin; pL-ce6: Poly-L-lysine chlorine (e6); PEI-ce6: Polyethylenimine chlorine (e6); PS: Photosensitizer; TBO: Toluidine blue O; TMP: Tetra-substituted (*N*-methyl-pyridyl) porphine.

Table 2

Antimicrobial photodynamic therapy studies in Gram-negative bacterial biofilms.

Gram-negative bacteria	PS	PS concentration (μm)	Light source (wavelength [nm])	Light dose (J/cm^{-2})	Reductions microbial biofilms CFU log_{10}	Ref.
Pseudomonas aeruginosa	TMPP	225	Mercury vapor lamp (400)	240.0	4.20	[222]
	pL-ce6	200	Diode laser (660)	240.0	>6	[160]
	δ-ALA	20	Diode laser (630)	120.0	9.00	[162]
	PEI-ce6	10	Diode laser (660)	9.6	>6	[164]
	HB:La^{+3}	10	Diode laser (660)	24	2.00	[165]
Escherichia coli	pL-ce6	200	Diode laser (660)	165.0	>6	[160]
Aggregatibacter	Erythrosine	1	HHP (400–500)	2.0	6.75	[161]
actinomycetemcomitans	MB	1	HHP (400–500)	2.0	4.50	[161]
Proteus mirabilis	PEI-ce6	10	Diode laser (660)	9.6	>6	[164]
Acinetobacter baumannii	PEI-ce6	900	Noncoherent light (660)	240.0	3.50	[168]

ALA: Aminolevulinic acid; HB:La^{+3}: Hypocrellin B with lanthanide ions; HHP: Hand-held photopolymerizer; MB: Methylene blue; PEI-ce6: Polyethylenimine chlorine (e6); pL-ce6: Poly-L-lysine chlorine (e6); PS: Photosensitizer; TMPP: 5,10,15,20-tetrakis(1-methyl-pyridino)-21H,23H-porphine.

Table 3

Antimicrobial photodynamic therapy studies in fungal biofilms.

Fungus	PS	PS concentration	Light source (wavelength [nm])	Light dose (J/cm^{-2})	Reductions microbial biofilms CFU log_{10}	Ref.
Candida albicans	Erythrosine	400 μM	Diode laser (532)	14.34	0.73	[178]
	Photofrin	10 μg ml^{-1}	Hg arc lamp (400–700)	18.00	1	[174]
	Erythrosine	400 μM	Diode laser (532)	42.63	0.74	[177]
	TBO	5 mg ml^{-1}	Paterson Lamp (635)	200	5.00	[179]
	SiPc1/GePc1	5.8 μM	Diode laser (635)	50	3.00/1.00	[180]
	Photogem	1000 mg l^{-1}	Diode laser (455)	305	1.41	[181]
	Photogem	1000 mg l^{-1}	Diode laser (630)	305	1.41	[181]
	TBO	20 μm	Noncoherent light (400–800)	120	0.8	[182]
	MB	20 μm	Noncoherent light (400–800)	120	0.6	[182]
	NMB	20 μm	Noncoherent light (400–800)	120	4.5	[182]
Candida dubliniensis	Erythrosine	400 μM	Diode laser (532)	42.63	0.21	[177]

GePc1: Germanium phthalocyanine 1; MB: Methylene blue; NMB: New methylene blue; SiPc1: Silicon phthalocyanine 1; TBO: Toluidine blue O.

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