

Antimicrobial photodynamic therapy (aPDT) for biofilm treatments. Possible synergy between aPDT and pulsed electric fields

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ABSTRACT

Currently, microbial biofilms have been the cause of a wide variety of infections in the human body, reaching 80% of all bacterial and fungal infections. The biofilms present specific properties that increase the resistance to antimicrobial treatments. Thus, the development of new approaches is urgent, and antimicrobial photodynamic therapy (aPDT) has been shown as a promising candidate. aPDT involves a synergic association of a photosensitizer (PS), molecular oxygen and visible light, producing highly reactive oxygen species (ROS) that cause the oxidation of several cellular components. This therapy attacks many components of the biofilm, including proteins, lipids, and nucleic acids present within the biofilm matrix; causing inhibition even in the cells that are inside the extracellular polymeric substance (EPS). Recent advances in designing new PSs to increase the production of ROS and the combination of aPDT with other therapies, especially pulsed electric fields (PEF), have contributed to enhanced biofilm inhibition. The PEF has proven to have antimicrobial effect once it is known that extensive chemical reactions occur when electric fields are applied. This type of treatment kills microorganisms not only due to membrane rupture but also due to the formation of reactive compounds including free oxygen, hydrogen, hydroxyl and hydroperoxyl radicals. So, this review aims to show the progress of aPDT and PEF against the biofilms, suggesting that the association of both methods can potentiate their effects and overcome biofilm infections.

ARTICLE HISTORY

Received 12 November 2020
Revised 20 July 2021
Accepted 22 July 2021

KEYWORDS

Antimicrobial resistance₁; biofilms₂; EPS₃; ROS₄; aPDT₅; PEF₆; photosensitizer₇ and electroporation₈

Introduction

The over-growing of antimicrobial resistance has been one of the major global health concerns. World Health Organization (WHO) reported that we might return to a time where common infections and minor injuries can kill, causing disastrous consequences over life spans and across generations (1). The hard-won victories against infectious diseases over the last fifty years will be compromised, increasing the hospital stays and public health care costs [2]. Statistics studies from the British Society for Antimicrobial Chemotherapy presume that in 2050 deaths attributable to antimicrobial resistance could be as high as 10 million a year killing more people worldwide than cancer and diabetes combined. This may cost £1 billion to hospital treatment and societal costs in Europe and £66 trillion in lost productivity to the global economy [3].

Antimicrobial resistance is generally associated with the microorganism's ability to form a biofilm. This microbial community presents several aspects that contribute to biofilm resistance, including reduced

metabolic and growth rates, protection by extracellular polymeric substances (EPS), and specific resistance mechanisms conferred by the altered physiology of biofilm compared with planktonic cells [4]. Thus, the current treatments applied to overcome biofilm infections are often inadequate [5]. If the biofilm infection is associated with a medical device, removal of the implant is the best therapeutic alternative (when is possible). However, this strategy can increase patient morbidity and hospital costs [6]. For tissue or sputum-associated biofilms, the only available therapy nowadays is a long-term treatment with high doses of antimicrobials and sometimes a combination of these drugs with different killing mechanisms [6]. Nevertheless, biofilm-growing persists and destroys the infected tissue due to long-term inflammatory response, causing a chronic infection that may lead to sepsis and patient death [7].

As commented above, biofilms show multi-factorial aspects that decrease the efficacy of current antimicrobial treatments. Highlighting the main ones we can

include 1) **components of the biofilm matrix**, mainly EPS, that bind with antimicrobials and/or inactivate them by enzymes (e.g. beta-lactamases), restricting and hampering the penetration of antimicrobial through biofilms [8]. 2) **Differential physiological activity**, caused mainly by limited oxygen and nutrient penetration through the biofilm. This promotes a low metabolic activity of the microorganisms and consequently decreases the antimicrobial effects, once many of these drugs target processes that occur in microbial growing (e.g. replication and cell wall synthesis) [8]. 3) **Persister cells** that are microorganisms in a dormant (non-dividing) state, expressing a low metabolic activity that diminishes the susceptibility to all known antimicrobials [9].

The factors described above might lead to the prediction of the “end of the antimicrobial era” [10,11], confirming the urgency to develop new strategies against biofilm resistance. Therapies applying reactive oxygen species (ROS) have been successfully used in chronic and medical device infections caused by biofilm, e.g. antimicrobial photodynamic therapy (aPDT) and pulsed electric field (PEF). ROS presents versatility to be delivered at many clinical sites and a great potential for bio-burden control [12].

aPDT have been shown as a promisor anti-biofilm therapy because the method has multiple targets while presenting a low effect against host cells [13]. Basically, aPDT involves the synergistic combination of a photosensitizer (PS), molecular oxygen and visible light of an appropriate wavelength in order to produce highly reactive oxygen species, which leads to the oxidation of several cellular components and rapid cell inactivation [14]. On the other hand, the PEF is not considered as a powerful approach to produce ROS but can potentiate the aPDT effect in two ways: (I) increasing the PS permeabilization through the cell membrane and biofilm matrix [15], as well as (II) presenting phenomena as electrodisassociation of molecules and electrolysis, possibly including ROS formation [16].

This review will be followed by a discussion about the aPDT and PEF effect against biofilms, suggesting the synergism between them to potentiate the production of ROS and overcome the main defense mechanisms of biofilm.

Biofilms

Biofilms have been one of the most significant problems faced for public health, estimating to be responsible for around 80% of all infections, causing many deaths and high health costs worldwide [17]. These problems are directly connected to the biofilm being

significantly less susceptible to antimicrobials and host defenses than their planktonic forms [18], tolerating up to 1000 times higher levels of antimicrobials [19].

It has been described that biofilm formation can be found on tissue surfaces (biotic) as well as on medical devices (abiotic) [20]. A variety of indwelling medical devices have been associated with biofilm infection, including urinary/vascular catheters, implants, heart valves and prostheses [21–24]; the intravascular devices followed by urinary catheters and orthopedic implants, are the main causes to nosocomial bloodstream infections [20,25]. Generally, the biofilm infections lead to device malfunction or chemical degradation of biomaterials and consequently require surgical intervention for the implant removal compromising the patient’s quality of life [21].

In addition, biofilm infections commonly manifest as chronic and recurrent diseases [18], and these may not be associated with implanted devices such as chronic airway infections (e.g. pulmonary diseases, wound infections, dental diseases, and so on) and soft tissues infections (e.g. the intestines or lungs) [20].

Regardless of the infection site, a competition between the host cells and pathogenic microorganisms occurs to gain the tissue or biomaterial surface area. Since the pathogen reaches the surface and adheres successfully, they initiate biofilm formation, which alters the microorganism’s virulence properties and protects them against the antimicrobials [26]. These virulence properties, also, allow the microbial cells to survive and grow in adverse conditions, including limited nutrient availability, desiccation, low pH, and so on [27,28].

Thus, biofilm-associated cells present specific mechanisms that lead them to be tolerant or resistant against antimicrobial drugs. Among these mechanisms, we can include the presence of the extracellular matrix (ECM), high cell density that changes the microbial physiological state, and the presence of quorum sensing molecules (QS) and *persister* cells [29,30].

This multifactorial complex phenomenon, which is the biofilm, shows five stages of the development cycle: reversible and irreversible attachment, maturation, mature biofilm, and dispersion (Figure 1). During the whole biofilm formation process, the microbial cells express specific phenotype traits that contribute to their virulence and mechanism factors described above [31,32].

The initial contact of the microbial cells to the surfaces (reversible attachment) occurs through the presence of a few virulence factors like cell membrane adhesins, pili, flagellum, fimbriae, and glycocalyx that directly influences the rate of microbial adhesion. In

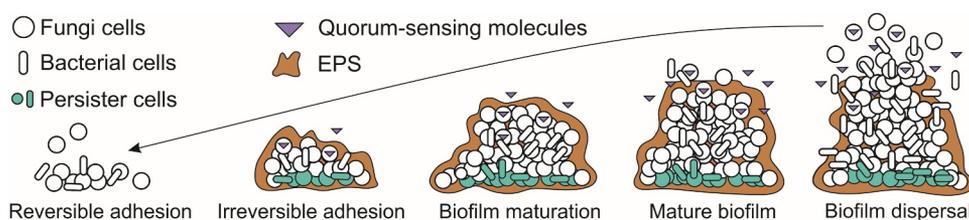


Figure 1. Adapted from [36]: Biofilm formation stages: 1) **Reversible adhesion** occurs when the planktonic cells adhere to a surface area (biotic or non-biotic) through the presence of a few virulence factors (adhesins, pili, flagellum, fimbriae, and glycocalyx) and chemical reactions (van der Waals forces, electrostatic forces, hydrophobic effects), starting the biofilm formation. At this stage, the microbial cells are susceptible to antimicrobials drugs. 2) **Irreversible adhesion**, at this step microorganisms start to grow and replicate, forming colonies that undergo transcriptional modifications for adherence, promoting an exchange of substrate, distribution of important metabolic products, and excretion of metabolic end-products; as well as secrete EPS making the biofilm cells less susceptible to host defense and antimicrobial drugs. 3) **Biofilm maturation**, at this phase the amount of ECM increases around the microcolonies due to continued secretion of EPS and beginning the intracellular communication system through the quorum-sensing molecules (QS), both are important factors of resistance. 4) **Mature biofilm** contains a high concentration of EPS and cavities between it that serve as transport channels of water, nutrients and planktonic cells throughout the biofilm community. 5) **Biofilm dispersal** involves the biofilms detachment due to the restriction of nutrients for the cells. This fact can occur by erosion and sloughing, and the cells search for another surface area to continue surviving.*EPS: extracellular polymeric substrate.

addition, chemical reactions may govern the cells-surface interactions, including van der Waals forces, electrostatic forces, hydrophobic effects, and so on [17,33]. It is important to highlight that microorganisms have more attraction to hydrophobic and nonpolar surfaces, once it reduces the force of repulsion between the cells and the surface [34]. Subsequently, this attachment becomes stable and the microorganisms start to multiply and secrete EPS, forming microcolonies (irreversible attachment). These colonies undergo transcriptional modifications for adherence, promoting an exchange of substrate, distribution of important metabolic products, and excretion of metabolic end-products [35]. In addition, these new populations will produce a heterogeneous matrix containing 98% of water and a mix of polysaccharides, proteins, nucleic acids, phospholipids, lipids, amyloid fibers, humic substances, and in some cases, surprising amounts of extracellular DNA (e-DNA) [36].

According to Donlan [37], inside the ECM, a channel of water is responsible for bringing nutrients and oxygen to the innermost cells, except for the deep cells named as “dormant cells”. These cells, also called *persister* cells, can be defined as a state in which cells are metabolically inactive and comprise only to <0.1% of the biofilm population [38]. The *persisters* cells are tolerant to the large increases of the antimicrobial concentration having the ability to adapt in adverse environments via “dormancy-growth-proliferation”, and reach this state without undergoing genetic change [39]. This mechanism contributed to a vital role in the microbial biofilm multidrug tolerance, once maintains their survival and cell structure stability [27]. Thus, the *persister* cells can be associated as one of

the responsible for the recalcitrance of chronic infections, once it remains viable and regrow the biofilm population after the level of the antimicrobial drops [38,39].

During the biofilm maturation, the concentration of nutrients available determines the final biofilm size [34]. This stage of biofilm formation presents important factors of resistance such as the intracellular communication system that occurs through the quorum-sensing molecules (QS) and the increased secretion of EPS [40].

QS molecules are responsible for a process of cell-cell communication that allow microorganisms to share information about cell density and adjust gene expression [41]. The QS molecules respond to extracellular signaling molecules called auto-inducers (AIs) [42], which accumulate in the environment with the increase of microbial population density [41]. This phenomenon enables the microorganism to track changes in their cell density and gene expression [43,44]. Independently of the microorganism species, the QS systems depend on three basic principles: (I) the production of AIs by microbial community; (II) AIs detection by receptors present in the cytoplasm or cells membrane; and (III) the gene expression activation, as a result of AIs detection and consequently the production of AI [45,46]. Thus, at low cell density, the AIs diffuse away to increase the microbial population density and at high cell density, the cumulative production of AIs leads to molecular and genetic responses, synchronizing the microbial communication [47].

Currently, there are four well-identified and characterized groups of QS molecules: N-acyl-L-homoserine lactone (AHL) QS-system in Gram (-) bacteria, the

autoinducing peptide (AIP) QS-system in Gram (+) bacteria, the autoinducer-2 (AI-2) QS-system in both Gram (-) and Gram (+) bacteria and the farnesol, (aromatic alcohol) systems in fungi [48]. These molecules induce signals that control gene expression involved with microbial process including, bioluminescence, sporulation, resistance or tolerance to antimicrobials and evasion to host immune reactions, biofilm formation, and virulence factor secretion (e.g. EPS secretion) [9,49–51]. It is important to emphasize that the inhibition of this QS mechanism may interrupt the biofilm formation, as well as reduce the microbial virulence and resistance [52].

As mentioned above the secretion of EPS is an important virulence factor that confers to the microorganisms both increased antimicrobial resistance and defense from host immune responses [53]. The EPS acts as an adsorbent or reactant reducing the antimicrobials penetration by diffusion limitation and through chemical interactions with the ECM proteins and polysaccharides [54]. In addition, the aggregation of microorganisms into EPS-coated biofilms turns them less susceptible to phagocytosis [35]. The proteins and polysaccharides are the key components of EPS, representing 75–89% of the EPS matrix composition. These components contribute to some functions of EPS matrix, including facilitation of the initial attachment of the cells to different surfaces and protection against several environmental [55].

Biofilm development is a cyclic process because once the cells have established sessile forms they can return to a planktonic form, in case it is more favorable [56]. This process corresponds to the last phase of biofilm formation, named detachment/dispersion (Figure 1). In this stage the disruption of the biofilm occurs, the debris of the biofilm spreads and may cause septicemia. The detachment happens with the decrease of available nutrients promoting biofilm erosion (continual

dispersal of a single cell or small portion of the biofilms) or sloughing (large pieces of the biofilms are significantly lost) [57]. According to [34], during the step of dispersion, the biofilm cells produce different saccharolytic enzymes that help to release the surface of the microbes into a new area for colonization.

Important to highlight that the resistance and persistence of the biofilms increase when it is polymicrobial, commonly been associated with chronic infection and occurring in the lung, inner ear, urinary tract, oral cavity, in wounds, and those that are device or foreign body-related [58–60]. Polymicrobial biofilms have a genetic diversity of microorganisms due to an expanded gene pool, which can be more easily shared within the confines of a biofilm community. This may increase the fitness of the residing community, making them more equipped to survive environmental stresses [61]. In addition, several are the advantages that a polymicrobial community (bacterial and/or fungal species) show, including QS system, metabolic cooperation, passive resistance, and many other synergies [58,60]. In this manner, biofilm-community composition and interactions have huge influences on microbial behavior, i.e. the comportment of microorganisms in single species versus multispecies systems is dramatically different [59].

Microbial-biofilm interactions are complex and highly dependent on community structure, including the composition and spatial distribution of members within the biofilm population [59] (Figure 2). These interactions can be antagonistic (defined as the suppression of one microbial species by another and/or synergistic (defined as a cooperative interaction between two or more species of microbes that produces an effect not achieved by an individual species alone) [62]. In polymicrobial biofilm infections, the synergism “effects” between microorganisms may include increased microbial growth, enhanced production of EPS, and antimicrobial tolerance, virulence and persistence [63,64]. Metabolic cross-feeding is

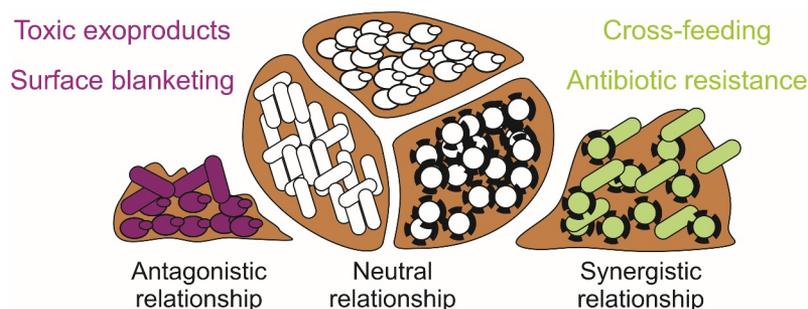


Figure 2. Adapted from [59]: Polymicrobial biofilm interactions. Multiple-species biofilm can be found between the same species of microorganisms (neutral relationship) and between two or more species of microbes such as bacteria and fungi (antagonistic and synergistic relationship). Microbial antagonism produces toxic exoproducts and surface blanketing, while microbial synergy promotes antimicrobial resistance and cross-feeding.

Table 1. Effect of different approaches against microbial biofilms.

| Approaches | Microorganisms | CFU (log ₁₀) reduction | References |
|------------------|-------------------------------|------------------------------------|---------------------------|
| Nanoparticles | <i>Staphylococcus aureus</i> | 4.13 | (84) |
| | <i>Escherichia coli</i> | 5.32 | (84) |
| Photo-grafting | <i>Candida albicans</i> | 6.44 | (85) |
| | <i>E. coli</i> | 5.00 | (86) |
| Furanones | <i>Pseudomonas aeruginosa</i> | 2.55 | (87) |
| RNA III | <i>S. aureus</i> MRSA | 4.58 | (88) |
| | <i>S. aureus</i> MSSA | 4.64 | |
| | <i>S. aureus</i> | 4.00 | (89) |
| Ultrasound | <i>S. aureus</i> | 4.00 | (90) |
| | <i>E. coli</i> | 4.85 | (91) |
| DNAse | <i>P. aeruginosa</i> | 3.20 | (92) |
| | <i>Enterococcus faecalis</i> | 6.40 | |
| | <i>Salmonella typhimurium</i> | 5.60 | |
| | <i>S. aureus</i> | 7.20 | |
| | <i>S. aureus</i> | 5.00 | (93) |
| Plant extraction | <i>Klebsiella pneumoniae</i> | 3.08 | (94) Sánchez et al., 2016 |
| | <i>E. faecalis</i> | 2.7 | (Sánchez et al., 2016) |
| | <i>E. coli</i> | 3.00 | (Sánchez et al., 2016) |

* Table shows few studies that have been realized to find an alternative approach to overcome the microbial biofilm.

also a classic cooperative interaction that makes a metabolic “byproduct” enhancing the growth of a neighbor [65]. On the other hand, the antagonistic mechanism is a fierce competition for nutrients and niches between the microorganisms, making them produce virulence factors and chemical signals that can interfere in the behavior or physiology of microbial neighbors [66–68]. This may promote “Surface blanketing” where one species occupies all the attachment sites on a surface, preventing the attachment of another [68,69].

In the literature, have been reported some polymicrobial biofilm interactions between different microorganisms species. *Pseudomonas aeruginosa* and *Candida albicans* are known to form dual-species biofilms that can play extensive roles in nosocomial infections and infection in immunocompromised individuals [70,71]. This occurs cause *P. aeruginosa* attached to *C. albicans* hyphae surface and the Interspecies competition enhances the production of virulence factors and increases mutability, altering the course of host-pathogen interactions infections [70,72,73]. *P. aeruginosa* and *Staphylococcus aureus* coexist in multiple infection settings, being the most prevalent respiratory pathogens in patients with cystic fibrosis and often co-isolated from chronic wounds, including difficult-to-treat diabetic foot ulcers [74–76]. Their interactions mechanisms still are poorly understood, but some studies showed that secreted products of *P. aeruginosa* could enhance biofilm tolerance of *S. aureus* to vancomycin by 100-fold, through the function of the electron transport chain and slowing growth of this gram-positive bacteria [77].

[69] reproduced mixed-species biofilm comprising *P. aeruginosa*, *Pseudomonas protegens* and *Klebsiella pneumoniae* to study how interspecies interactions affect

biofilm development, structure and stress responses. The authors reported that the mixed-species biofilm exhibited distinct structures, presenting a delay of 1–2 days compared with the single-species biofilms. The composition and spatial organization of polymicrobial biofilm changed along the flow cell channel, where nutrient conditions and growth rate of each species could have a part in community assembly. In addition, the microorganisms in a polymicrobial biofilm showed to be more resistant to antimicrobials sodium dodecyl sulfate and tobramycin than the single-species biofilms. Suggesting, community-level interactions are unique to the structured biofilm community, where the members are closely associated with each other.

Thus, biofilms mechanisms of resistance, multiple or single species ones, limit the effectiveness of conventional antimicrobial drugs, making urgent the development of new strategies a target of scientific attention [34].

Nowadays, several anti-biofilm approaches have been proposed, focusing on the inhibition of biofilm adhesion and growth (Table 1). The alterations of the medical device materials are one of the most studied by researchers in order to prevent biofilm formation. Several are the physical-chemistry techniques [36] (e.g. ion-beam, plasma-technique, surface photo-grafting) and the materials used [78] (e.g. polyvinylchloride, polyester, chitosan, polystyrene) to produce these new devices. In addition, electrical approaches [79], ultrasound [80], nanoparticles [81], liposomes [82], and polymer-based drug delivery vehicles [83] have been applied against the biofilm, aiming to increase the penetration through the surface of this microbial community and toward deeper cells.

Despite the large number of therapies being practiced to achieve biofilm killing, generally, they focus on a specific microorganism or aims at one specific target

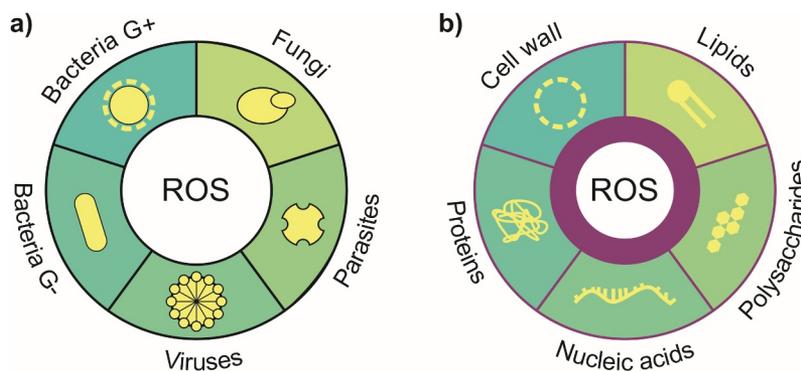


Figure 3. Adapted from [97]: ROS presents (a) a wide therapeutic window that affects different species of microorganisms (bacteria, fungi, virus and parasites) and (b) it is a nonselective multiple target oxidizing various biomolecules, promoting substantial cell damage.

of the biofilm structure [95]. ROS can be a novel solution to overcome these factors once shows a wide therapeutic window that affects different species of microorganisms (bacteria, fungi, virus and parasites), are nonselective with multiple targets in focus and known to oxidize various biomolecules, promoting substantial cell damage [96,97] (Figure 3). Therefore, therapies that produce ROS can be a great potential to reduce biofilm antimicrobial resistance.

Bio-effects of aPDT and PEF

Antimicrobial photodynamic therapy

aPDT is a minimally invasive method that has been reported as an alternative treatment of infections caused by several pathogens [36]. This therapy presents some advantages that can overcome antimicrobial resistance problems, including a broad spectrum of action, the efficient inactivation of multi-antibiotic-resistant strains, the low mutagenic potential, and the lack of selection of photo-resistant microbial cells [98].

aPDT has been further studied, for the past several decades, showing to be effective against biofilm of Gram-negative and Gram-positive bacteria and fungi, that can be multiple or single-species biofilm [99]. Several authors have been reported that aPDT-generated ROS has many possible targets on biofilms, mainly against EPS matrix [99] that includes the DNA [100], lipids [101], proteins [102,103] and extracellular polysaccharides [104]. In addition [105], showed the efficacy of the aPDT against several stages of biofilm development. Thus, they designed experiments to analyze the effect of different light doses (4.23; 8.46; 12.70; 16.93 and 21.16 J/cm²) against several ages of *S. aureus*

biofilms (0; 6; 11; 17; 24; 32; 40 and 48 hours). As result, optical density analysis showed the most optimum biofilm reduction happened when biofilm age is perfectly constructed (about 17 hours) and with 91% reduction; as well as the longer biofilm age lived among those biofilms, the greater the reduction.

[106] studied the aPDT effect combined with usual endodontic therapy, against the Gram-negative bacteria, *Proteus mirabilis* and *P. aeruginosa* formed at root canals. Bioluminescence imaging was used to periodically quantify bacterial burdens and the treatment effect. The aPDT study was performed using a conjugate between polyethylenimine and the PS chlorin (e6), as a light source was used a 660-nm diode laser. They realized that this combination reduced the bioluminescence by >98% and the bacterial regrowth after 24 hours of the treatment [107], also combined the endodontic therapy with aPDT using Zn(II)chlorin e6 methyl ester (Zn(II)e₆Me) activated by red light against monospecies against mixed biofilms of *Enterococcus faecalis* and *Candida albicans*. The results showed that Zn(II)e₆Me once activated was able to remove around 60% of the biofilm biomass.

The chlorin (e6) have been used as PS for several aPDT application, as pediatric otitis media caused by *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and nontypeable *Haemophilus influenza* [108]. demonstrated that the chlorin (e6) elicits significant bactericidal activity against both planktonic cultures and established biofilms formed by these three major pathogens (with an efficacy of ≥99.9% loss of viability) [109]. performed a study applying Photodithazine® (commercially available cationic chlorin-e6) against multispecies biofilms from *Streptococcus mutans*, *C. albicans* and *Candida glabrata*. After being exposed to red light from a LED light source aPDT-treatment showed

reductions of 1.0 or 2.0 log₁₀ for *Candida* spp. or *S. mutans*, respectively. Concerning porphyrins, other tetrapyrrolic PS, upon irradiation have been reported to inactivate about 4.0 log₁₀ of the *P. aeruginosa* biofilm [110], reduced 5.0 log₁₀ of *C. albicans* biofilm [111] and up to 2.0 log₁₀ of *Staphylococcus aureus* biofilm [112].

[113] evaluated the efficacy of aPDT mediated by chloro-aluminum phthalocyanine encapsulated in cationic nanoemulsions (CIAIP-NE) to treat oral candidiasis *in vivo* and its effect to prevent the *C. albicans* biofilm formation. aPDT was as effective as Nystatin reducing 1.4 and 2.0 log₁₀ of the cell viability, respectively, meaning that both treatments reduced the ability of *C. albicans* to adhere on a surface and form biofilm. Later [114], showed that the oxidative stress caused by aPDT affects the expression of *C. albicans* genes related to adhesion and biofilm formation (ALS1 and HPW1) and oxidative stress response (CAP1, CAT1, and SOD1). In addition, [115] reported the potential of chlorophyll extract of papaya leaf (0.5 mg/L) as an exogenous photosensitizer against *C. albicans* biofilm. Thus, upon irradiation by diode laser at 445 nm and 650 nm the *C. albicans* biofilm was reduced to 25% and 32%, respectively.

The phenothiazinium has been, extensively, applied in aPDT studies, mainly the dyes methylene blue and toluidine blue (TBO). Both are designated to be potential efflux pump substrates in a variety of microbial species [116,117], enhancing the effect of the photodynamic inactivation on Gram positive species [118,119]. showed the aPDT effect against polymicrobial biofilms of *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA). They mimic, *in vitro*, chronic recurrent sinusitis and applied the methylene blue as photosensitizer and 670 nm non-thermal-activating light, which results in 99.99% of biofilm reduction [120]. showed the aPDT action against antibiotic-resistant polymicrobial biofilms of *P. aeruginosa* and MRSA grew in endotracheal tube. With a methylene blue photosensitizer and 664 nm non-thermal activating light they obtained biofilm reduction around >99.9% ($P < 0.05\%$) after a single treatment.

It was evaluated by [121] the synergisms effect of laser diodes 830 nm (as the light source) and Toluidine blue O (TBO) on the following periopathogenic bacteria: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Prevotella intermedia*. After the irradiation, the microorganism suffered aPDT effect, mainly the *A. actinomycetemcomitans* and *P. intermedia* that were 100% reduced. The photo-effect of toluidine blue was also evaluated against several strains of MRSA and methicillin-sensitive *S. aureus* (MSSA), isolated from

burns patients. Upon irradiation by red LED (630 ± 10 nm) the TBO significantly reduced the mean cell survival in the MRSA (2.5–3 log₁₀) and MSSA (2.75–3.1 log₁₀) isolates [122,123]. verified the effect of two phenothiazinium dyes (TBO and new methylene blue (NMB)) against *S. mutans* biofilm. TBO was found to have a better antibacterial as well as an anti-biofilm effect than NMB. They correlated this result with the highest production of singlet oxygen production by TBO than NMB, even the NBO can produce free radical (HO·).

Another PS that showed aPDT effect against *S. mutans* biofilm was the curcumin that even in low concentrations (above 3 g/L), after irradiation by LED (central wavelength: 450 nm; light dose: 5.7 J/cm²) caused a 3 log₁₀ of bacteria reduction [124]. In addition [125], compared the effect of rose bengal and erythrosine against *S. mutans* and *Streptococcus sanguinis* biofilms. These PSs at a concentration of 5 μM and after irradiation of blue LED (455 ± 20 nm) for 180 s, caused a reduction of 0.62 and 0.52 log₁₀ CFU mL⁻¹ for *S. mutans* biofilms ($p = 0.001$), and 0.95 and 0.88 log₁₀ CFU mL⁻¹ for *S. sanguinis* biofilms ($p = 0.001$), respectively.

An interesting PS is the perinaphthenones (phenalenones) which is a BODIPY dye used as a reference standard for the generation of ¹O₂ [126,127]. modified this PS to SAPYR [2-((4-pyridinyl)methyl)-1 H-phenalen-1-one chloride], introducing a batch of positively charged derivatives based on a 7-perinaphthenone-structure to increase the aPDT effect. They verified the SAPYR anti-biofilm properties against *E. faecalis* and *Actinomyces naeslundii* after the irradiation of blue LED. The authors concluded that this PS has two mechanisms of action against biofilms, including the disruption of biofilm structure without illumination; and after irradiation, it inhibits the polymicrobial biofilm after one single treatment with efficacy of ≥99.99%. Later, [128] showed that the effect of SAPYR against the same bacteria-biofilms (*E. faecalis* and *Actinomyces naeslundii*) can be increased by the application of a formula specific to adjust the number of photons absorbed by PS after the irradiation. In addition, the authors exhibited that aPDT effect of MB is smaller or none compared to SAPYR. Recently, it was compared the aPDT effect of two phenalen-1-one derivatives (SAPYR and SAGUA) against a polymicrobial biofilm commonly found in periodontal disease. As result, SAPYR reduced up to 6.1 log₁₀ of CFUs while SAGUA was less effective inhibiting up to 2.8 log₁₀. In addition, the flow cytometric analysis revealed no damage of cytoplasmic membranes after aPDT with both phenalen-1-one derivatives.

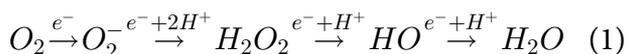
Besides that this experiment is the first step to get understandings of the mechanism and damage patterns of this PS class in aPDT [96].

As commented above, polymicrobial biofilms can be inhibited by aPDT [129]. applied the following conditions, MB (500 µg/ml), red light (664 nm) of 150 mW/cm² with a light dose of 216 J/cm², against the biofilm of multidrug-resistant *P. aeruginosa* (clinical isolate) and MRSA clinical isolate that was 99% reduced [130]., reported the effect of Photodithazine (125 mg/L) and red light (660 nm, 25 mW/cm², 37.5 J/cm²) against the multiple-species biofilm of *C. albicans*, *C. tropicalis* and *C. glabrata*, inhibiting to 0.9, 1.4 and 1.5 log₁₀, respectively [104]. incubated the polymicrobial biofilm of *S. aureus* and *C. albicans* with the PS Tetra-Py⁺-Me (20 µM) and after irradiation by white light with (64.8 J/cm²), inhibited 6.5 log₁₀ and 4.6 log₁₀, respectively. Duo-species biofilm of MRSA and methicillin-resistant *S. epidermidis* was inhibited up to 80% to 90% by PS ALA (40 mM) after irradiation by red light (635 nm) at 300 J/cm⁻² [131].

Fundamental mechanisms

Basically, aPDT involves the synergistic combination of a photosensitizer (PS), molecular oxygen and visible light of an appropriate wavelength in order to produce highly reactive oxygen species (ROS), which leads to the oxidation of several cellular components and rapid cell inactivation [36].

ROS are radical or molecular species of oxygen that are in a more reactive state than molecular oxygen and can be reduced. Molecular oxygen contains two unpaired electrons with parallel spin configurations in its outer shell. Because of this spin restriction, one-electron redox reaction takes place with other atoms or molecules, which results in several high-reactive intermediates, such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (HO[•]):



Due to their high reactivity, free radicals can abstract electrons from other compounds to attain stability. The attacked molecule thus loses its electron and becomes a free radical itself, beginning a chain reaction cascade, which could finally affect the living cell. Cellular ROS are generated endogenously mainly during the process of mitochondrial oxidative phosphorylation, in which molecular oxygen is reduced to water in the electron transport chain. The superoxide radical O₂^{•-} is produced at several sites in the mitochondria, then it is converted to H₂O₂ or HO[•] by various enzymes. The

production of various ROS also appears in other organelles, such as peroxisomes or endoplasmic reticulum, also because of the normal metabolism of various cellular enzymes, such as NADPH oxidase and others. ROS may arise from exogenous sources, such as xenobiotic compounds, ultraviolet light, ionizing radiation, pollutants, electrical pulses as well.

ROS are thought to play a dual role in cells. They are required for maintenance of physiological cell functions, including proliferation, of the stem cells via stimulation of specific target proteins, signal transduction, hydrogen peroxide is the most important signaling molecule of redox metabolism, host defense (phagocytosis), but are also often associated with the oxidative stress [132,133]. Human tissues have a substantial ability to tolerate ROS under normal conditions. Nevertheless, when the production of ROS exceeds the capacity of antioxidant defenses, oxidative stress is inflicted, which leads to harmful effects on the function and structural integrity of lipids, proteins, and DNA [132–134].

(Figure 4) shows Jablonski diagram portraying the aPDT mechanism: after irradiation, the PS transfers from its ground state singlet (lowest energy level, ¹PS) to a short-lived excited singlet state (¹PS*). The ¹PS* fastly loses energy by the following process: (I) radiative by fluorescence or (II) non-radiative decay pathways by internal conversion-heat. The vibrational relaxation (VR) is also an important process involved in aPDT mechanism, in which an electron in a high vibrational level of an excited state can fall to the energetically lowest level of that state (S1) and the energy will be dissipated as heat [135]. If an electron has been boosted to a higher energetic state, after VR, it will fall for the first excited singlet state. Fluorescence emission promotes molecular relaxation to S0 and always starts from the lowest level of S1 [136].

On the other hand, a fraction of ¹PS* molecules can be converted to a longer-living excited triplet state PS (³PS*), where two electrons are unpaired and have the same spin, through a non-radiative process called intersystem crossing (ISC). This process promotes a “spin-forbidden transition” that violates the rule of no spin change during a change of an electronic state. As a result of spin-orbit coupling, such transitions take place with a certain probability. In addition, occurs the phosphorescence process (emission of an electromagnetic quantum) after a rapid VR within ³PS* energetic level and a radiative relaxation to S0 [135,137]. In other words, ³PS* can decay back to the ground state by emitting a phosphorescent photon or by mechanisms generating ROS (Type I and II) [10,96,138].

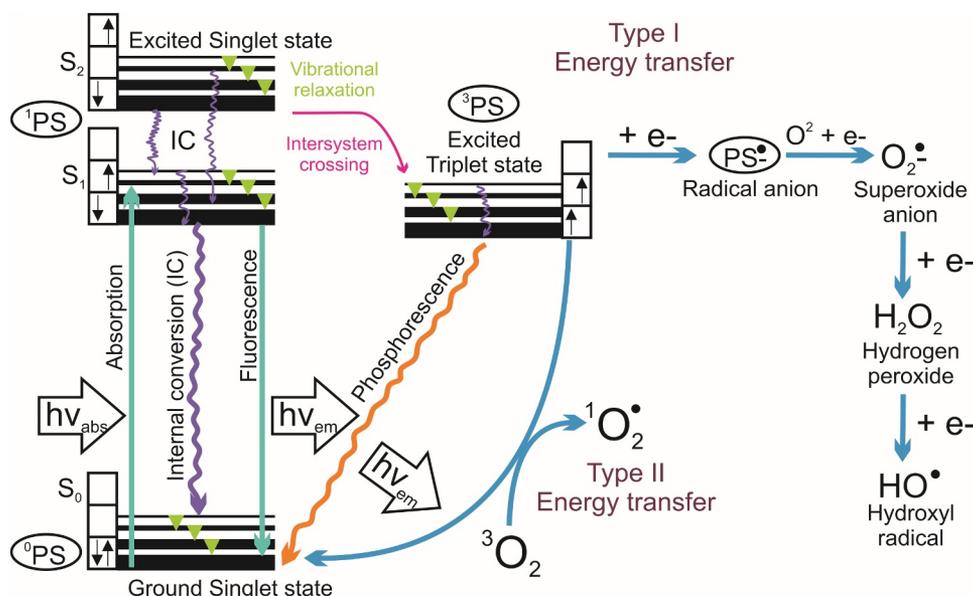


Figure 4. Adapted from [10]: Jablonski diagram showing the photochemical and photophysical mechanism of aPDT.

*PS: photosensitizer; O₂: molecule oxygen; ³O₂: ground state oxygen; superoxide anion: O₂^{•-}; hydrogen peroxide: H₂O₂; hydroxyl radicals HO[•] and ¹O₂ singlet oxygen

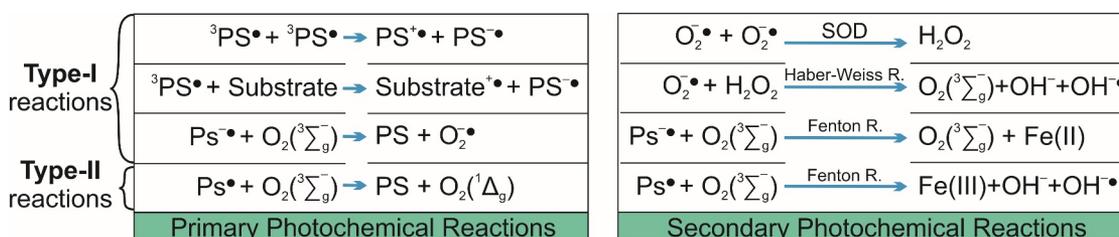


Figure 5. Adapted from [135]: Overview of photochemical reactions during aPDT represented by primary and secondary photochemistry reactions that produce ROS, promoting cytotoxic reaction that causes cellular damage.

* H₂O₂: hydrogen peroxide; O₂ (¹Δ_g): singlet oxygen (excited state); O₂ ³Σ_g⁻: triplet oxygen (ground state); O₂^{•-}: superoxide anion; OH⁻: hydroxyl radical; SOD: superoxide dismutase; X^{-/+}: anion/cation species; X[•]: radical species.

The ³PS* has a much longer lifetime (μsec) than ¹PS* (nsec), providing it a satisfactory time to interact, specifically, with a molecule in its electronic triplet ground (as dioxygen, ³O₂) once interactions between triplets and singlets are spin-forbidden [10]. This long lifetime of 3PS* enables it to transfer its energy by colliding to molecular oxygen (O₂), leading to the produce of ¹PS and oxygen singlet (¹O₂) molecules, this process is named Type II photochemical process [138]. In addition, the 3PS* can undergo a chemical process, named as Type I pathway, occurring electron transfer reactions that form ROS (superoxide O₂^{•-}, hydrogen peroxide H₂O₂ and hydroxyl radicals HO[•]) [14]. The superoxide is produced through a reaction between a radical anion (PS^{•-}) and ground state oxygen. The O₂^{•-} undergoes a dismutation or one electron reduction forming H₂O₂, which is the precursor of HO[•] formed via Fenton-like reaction (Figure 4) [96].

The PS returning to its ground state (¹PS) may permit that a new photochemical cycle happens since it is ready to absorb a new photon and generate more ROS. According to [96], one molecule of PS has enough lifetime to produce thousands of oxygen singlet molecules. The ¹O₂ is the most reactive ROS and, consequently, it has a short lifetime of 3–4 ms that promotes a no longer diffusion of 0.3 mm. Its short life can be associated with a physical and chemical quenching that occurs by collision or reactions with surrounding molecules, respectively [10,96,139,140]. The ¹O₂ lifetimes depend on the surrounding medium, and it has been described that is much longer in lipophilic environments than an aqueous medium [10].

Summarizing (Figure 5), aPDT photochemistry reactions to promote cellular damage (cytotoxic reactions) are defined as primary (type I and II mechanisms) and secondary (SOD, Haber Weiss and Fenton reaction)

[135]. Type-I photochemical reaction may form radical anion or cation by transfer of electrons (or protons) to molecular oxygen or other adjacent molecules, respectively [137]. Superoxide anions are formed by the transfer of an electron from the PS to molecular oxygen [141]. Despite these anions being not very reactive in biological systems, they easily react with H_2O_2 that in turn is quite relevant in promoting cellular damage, mainly because is not restricted to one cellular compartment [142].

During the energy transfer to molecular oxygen from a type-II photochemical reaction, the very reactive $^1\text{O}_2$ is formed [135,141]. This oxygen species is an uncharged molecule that diffuses through the cytoplasm and biological membranes [142]. The lifetime of $^1\text{O}_2$ modifies according to the localization and medium, in the cytoplasm due to the presence of reacting molecules it is reduced by more than one order of magnitude, max $1 \times 10^{-7}\text{sec}$ [143]. On the other hand, the diffusion constant for singlet oxygen in cells is $1.4 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ [144].

Both type-I and type II photochemical reactions occur in parallel, and at higher concentrations of hydrogen peroxide, occurs Haber Weiss reaction, where it reacts with superoxide anions to form the very reactive hydroxyl radical. In addition, hydrogen peroxide in a medium with a redox potential of E_0 equals 1.35 V shows very low activation energy and can attack and oxidize any molecule within a cell [135].

Thus, ROS's short lifetime diffusion distance hinder reaches pathogens localized far from where the ROS are produced [10,145]. For most photosensitizers employed in aPDT, the type-II photochemical reaction represents the dominant process [146]. As a consequence, the intracellular localization of the PS greatly determines the site of cellular damage set by aPDT (Figure 3 shows some of the ROS targets). These factors highlight the importance of PS diffusion through the EPS to reach the deeper layer of the biofilm and bind to the microbial cells, mainly the *persister* cells. Another important challenge of aPDT is that bacteria may upregulate the expression of some enzymes, contributing to their adaption toward ROS generated from the type I mechanism. On the other hand, if the distance between the oxidative burst and these defense enzymes is too long, it will not help the bacteria to overcome the aPDT [147,148].

Recently, a Type III photochemical pathway has been proposed, which implies an oxygen-independent photoinactivation of the microorganism, although the "photodynamic" term by definition involves oxygen [10]. This process involves photoinduced electron transfer that produces reactive inorganic radicals. The

authors assume that this mechanism may occur in anaerobic or hypoxic tissue infections, where the O_2 concentration is below 0.5% [10]. They justify this hypothesis once some studies have been shown the increase of the aPDT efficacy in the absence of oxygen. The PSs psolarens and tetracyclines are examples of that and the addition of organic salts, mainly potassium iodide and sodium azide, have potentiated the bacterial killing of aPDT [10].

Photosensitizers

The aPDT effect depends on PS localization and accumulation in diseased tissue as well as the amount and time of ROS produced after the light delivery [138]. The efficiency of aPDT strongly depends on PS concentration and its Physico-chemical properties as well as the irradiation time (depends on the light source) and the microorganism morphology [149,150].

In addition, an ideal PS would be a chemically pure drug with specific uptake by the target tissue, should have a substantial triplet quantum yield to promote a high production of ROS, and do not present dark toxicity (i.e., activated only upon irradiation) [151,152]. Another important point is the rapid clearance of the PS from non-infected tissues, minimizing the unique side effect of the aPDT, phototoxicity [138,153]. The chemical stability on shelves and in solvents commonly used in pharmaceutical preparations (e.g. saline solution) is an important factor for PS activity; thus, amphiphilic PS could show the requirements for aqueous dissolution and hydrophobic moieties to enable it to traverse cell membranes [151]. The low cost and easy production of the PS on large scales are also very important.

The "optical window" for biological tissue is a relevant aspect of the PS properties, meaning high photonic absorption of the PS in the far red/near-infrared portion of the electromagnetic spectrum. This range (550–700 nm) is ideal once water absorbs infrared energy (>750 nm) and ultraviolet light (100–400 nm) is deleterious to cell homeostasis, melanin, heme, and other endogenous biological pigments absorb shorter wavelengths [151].

Although it is already known the properties of an ideal PS, to date no one presents all. On the other hand, several of them are available to be applied in aPDT and they are classified by generations. The first generation of PSs includes Photofrin, which is a mixture of porphyrin oligomers. The second generation of PSs includes improved and purified synthetic tetrapyrrole derivatives, including meso-tetra-hydroxyphenylchlorin, 5-aminolevulinic acid. The third generation, which is a combination of second-generation PSs and

a drug delivery carrier, e.g., antibodies or liposomes [154].

According to several authors, small PS with cationic charges and an asymmetric structure can reach a broad spectrum of microbial inactivation [10,155]. In general, Gram-positive bacteria and yeasts are easily affected by neutral and anionic PS, while Gram bacteria were not [99]. Gram-negative bacteria have a complex structure that is many-layered containing strongly negatively charged lipopolysaccharides (LPS), lipo-proteins, and proteins with porin function, making this pathogen more susceptible to cationic PS [13]. This fact has been explained due to cationic PS “self-promoted uptake pathway”, where it shows higher affinity to anionic binding sites associated with surface LPS molecules than the normally bound divalent Ca^{2+} and Mg^{2+} cations [156, 157, 158]. Thus, the metal ions reorganize the bacteria membrane allowing the diffusion of the PS and consequently increasing the concentration of PS inner the cytoplasm [36,156,157].

Gram-positive species are easily killed by any type of PS, once has a porous cell wall (peptidoglycan and lipoteichoic acid) allowing the PS to reach the cytoplasmic membrane [159,160].

Despite the fungi confer more specialized organelles than Gram-negative bacteria and a rigid cell wall, have been evidenced that they are more susceptible to aPDT [161]. Activated PS induces alterations on cytoplasmic membrane of fungi, causing damage to their organelles as lysosomes, mitochondria and nucleus that lead to fungal death [162,163].

Fungi and bacteria organized as a biofilm, normally, hinder the PS permeabilization through EPS to reach microbial cells, decreasing the aPDT effect. Some studies, however, showed that the PS can be totally sequestered by EPS and bind to biofilm matrix [164,165]. Between the EPS components, the polysaccharide, proteins and DNA are the most abundant, attracting more attention to be photodamaged. A study showed that

only the PS Tetra-Py⁺-Me promoted an EPS reduction of around 30%, once its interaction with the matrix caused a polysaccharide detachment. Upon irradiation the polysaccharide level was further reduced up to 80%, reducing 2.8 log₁₀ of the microbial cells [104].

Damage caused to protein and DNA by aPDT significantly reduces the biofilm cellular metabolism and may disrupt the biofilm structure [103]. Hematoporphyrin has been known to break single-stranded and double-stranded DNA and to promote the disappearance of the super-coiled fraction of plasmid DNA in bacteria species [166]. Other powerful PS as Pc 4, promoted microbial nuclear damages, leading the *C. albicans* to exhibit characteristics of apoptosis [167]. While some less powerful PS caused non-lethal damage to DNA that may be repaired by DNA repair systems [168].

On the other hand, these less powerful PSs can easily photodamage the proteins once they are an abundant target and shows a rapid reaction rate with ROS. The sulfur-containing amino acids, cysteine, and methionine are more susceptible to ROS. The ROS reaction on amino acid residues may cause the production of dityrosine moieties, form the protein-centered and ROO[•] radical, as well as cleave peptide bonds. Thus, affects the metabolic activities of the proteins, interfering directly in the biofilm and cells development [99,102,169].

In addition, some authors affirmed that cationic PS presents more effectiveness do bind and inhibit the biofilm than anionic PS. This may be explained by the EPS “trapping”, i.e. outside of the cell occurs ionic and hydrophobic interaction with the anionic PS decreasing the PS able to site the plasma membrane, considerate as one of the most important aPDT-target [36,170].

The chemical structure of the PS also has an influence on the photochemical mechanisms pathways and the ratio of ROS produced [171]. (Table 2). According to [172], Gram-positive bacteria and fungi are more

Table 2. Photosensitizers and reactive oxygen species (ROS) produced by them.

| Photosensitizer | Type of ROS produced, predominantly | References |
|----------------------------------|---|------------|
| Phthalocyanines | $^1\text{O}_2$ | [138] |
| Porphyrins | $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ | (173) |
| Chlorins | $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ | [96] |
| Bacteriochlorins | $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ | [138] |
| Phenothiazinium (MB, TB and NMB) | $^1\text{O}_2$ | [174] |
| Rose Bengal | $^1\text{O}_2$ | (175) |
| Eosin and Erythrosine | $^1\text{O}_2$ | (176) |
| Hypericin | $^1\text{O}_2$ and $\text{O}_2^{\cdot-}$ | (177) |
| Curcumin and it derivatives | $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ | [192,193] |
| Riboflavin and it derivatives | $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ | [191,194] |
| Fullerenes | Hydrophobic environments: $^1\text{O}_2$ Hydrophilic environments: $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ | [205] |
| Phenalenones | $^1\text{O}_2$ | [127] |

* Table shows the main ROS (superoxide anion: $\text{O}_2^{\cdot-}$; hydrogen peroxide: H_2O_2 ; hydroxyl radicals HO^{\cdot} and $^1\text{O}_2$ singlet oxygen) produced by each PS, however is important to emphasize that the PS are capable to produce all type of ROS. MB: methylene blue; TB: toluidine blue; NMB: new methylene blue.

susceptible to $^1\text{O}_2$, while Gram-negative bacteria are more sensitive to HO^\cdot . Suggesting that Type II mechanism have more effect against Gram-positive bacteria and fungi while Type I shows more influence on Gram-negative. Important to highlight that both mechanisms happen during the aPDT, but depending on PS and microorganism involved one of the mechanisms may be more effective than another one.

In biofilm cases, HO^\cdot and $^1\text{O}_2$ are the two most commonly found to attack diverse components of biofilm matrix, cell surface and inside the cells. Large amounts of ROS may oppress the antioxidant defenses of microbial cells, resulting in the collapse of the biofilm matrix and disintegration of microbial cells [165].

Given the above, oxidative targets of aPDT mostly depend on the respective localization of the PS and its chemical structure as well as on the ROS diffusion lifetime. For a better understand, in the following, the main classes of PS are described shortly (Table 2).

The PS can be classified accordingly to some properties and characteristics that they similarly present, for example, be synthetic or natural dye. Among the synthetic PS, phenothiazinium salts and the rose Bengal have shown better results against microbial infections. Phenothiazinium as methylene blue (MB) and toluidine blue (TB) is effective PS that presents at least a single positive charge and present a low $^1\text{O}_2$ quantum yield (0.4), acting mainly as stated by Type I process [96,174]. In addition, they present an excellent light absorption property (600–690 nm) to treat local microbial disease and exhibit low toxicity levels in mammalian cells [174,178]. Besides MB and TB, new phenothiazinium structures have been improved and applied against dental caries, in oral candidiasis, stomach and leg ulcers [179,180].

Rose Bengal is an anionic water-soluble xanthene dye that absorbs in a visible light range of 480–800 nm [181]. This PS exhibits a good quantum yield of singlet oxygen production (0.6 to 0.8) [182] and increases its triplet yield with the addition of heavy atoms into the ring structure [138]. Studies have shown the effect of RB in aPDT against biofilm of different bacteria species, such as *S. aureus*, *Listeria innocua*, *Enterococcus hirae* and *E. coli* [183], and fungi as *C. albicans* [184]. Eosin Y and erythrosine are also xanthene dyes that show intensive absorption between 480 and 800 nm (green spectrum) and act mainly according to type II mechanism; however, their negative charges decrease the aPDT efficiency [96,126].

Natural products have been extensively applied in several medical therapies and this fact is not different for aPDT. Hypericin, riboflavin and curcumin are

natural PS, commonly, used in aPDT to disrupt microbial biofilm [185–187]. Hypericin, a polycyclic quinone extracted from *Hyperforin perforatum* (St. John's wort) that has a maximum absorption peak of ~599 nm [188]. It shows a substantial quantum yield, intense absorption spectrum in the visible region, low photo bleaching, short half-life and a wide excitation range; all significant advantages for aPDT applications [189,190]. Riboflavin also called vitamin B12, is a very poor hydrophilic PS that shows two absorption peaks 360 nm (UVA) and 440–470 nm (blue spectrum) [96]. This PS derivatives with positive charges exhibit a considerable improvement of aPDT effect, mainly due to the increase of oxygen singlet quantum yields up to 0.8 [191]. Curcumin is a hydrophobic polyphenol found in the rhizome of turmeric (*Curcuma longa* L.) that presents an absorption spectrum between 300 and 500 nm with a relatively high extinction coefficient, contributing to increase the $^1\text{O}_2$ generation [192]. Hydrogen peroxide and hydroxyl radical are the others main ROS that curcumin-derived photolytically produces [192], acting accordingly to Type I mechanism [96]. Important to highlight that have been reported chemical modification in curcumin structure, as the addition of positive charges, to overcome its hydrophobicity [193].

Bacteriochlorins can present as natural or synthetic tetrapyrrolic derivatives, which have two reduced pyrrole rings and these reduced centers are diagonally opposite [195]. This PS can be effective at lower concentrations, once has high molar absorption coefficients in the therapeutic window (600–800 nm) [196]. Moreover, have been shown photostability, long-lived triplet states, and high quantum yields in the generation of ROS [197].

Porphyrins, chlorins, and phthalocyanines are also heterocyclic-macrocyclic compounds that consist of four pyrrole cycles (porphyrins, phthalocyanines) or three pyrrole and one pyrroline subunits (chlorins) [96]. Chlorins and porphyrins are most efficiently activated at Soret band (~405 nm) and present small peaks at Q bands (500 nm) [138]. These PSs exhibit single oxygen quantum yields between 0.5 and 0.8 structure, acting predominantly according to Type II mechanism [96]. In addition to their eight positive charges, has been reported its chemical modification adding more cations to increase the aPDT efficacy [110,198].

On the other hand, phthalocyanines are hydrophobic and uncharged photosensitizers that have generally been applied to photodynamic field, being necessary to attach different substituents at its peripheral position, as cationic charges [199]. These positive charges affect amphiphilic character of this PS, increasing the effect against microbes [126]. The phthalocyanine shows

strong absorption in far red-light in the wavelength 600–800 nm and photo-physical properties such as quantum yield of singlet oxygen that can be enhanced by conjugating the phthalocyanine with metal nanoparticles such as Ag, Zn, Co and Fe [200,201]. Zinc phthalocyanine has been extensively applied against biofilm due to its high molar extinction coefficient and singlet oxygen quantum yield of 0.67, as well as exhibit photochemical properties more stable than other phthalocyanines [202].

Nanoparticles also have been used as an alternative not only to increase the solubility of some PSs but to deliver them through microbial cells [203]. Liposomes, micelles, nanoemulsions are all used to self-assemble, mainly, the hydrophobic PSs once in an aqueous medium it can aggregate and decrease significantly the ROS produced [138,204].

Interestingly, the fullerenes (C60) are nanostructures that present PS properties such as a large molar absorption coefficient in visible light and high triplet yields. These PS-nanoparticles may switch the photochemical mechanism according to the medium, in aqueous environments the fullerenes are very efficient to produce HO* (Type I) while in hydrophobic medium produce singlet oxygen (Type II) [138,205]. Generally, the C60 are derivatives with functional groups to be used as PS and to exhibit biological compatibility. According to the literature cationic fullerenes, are mostly aPDT efficient against various classes of microbial cells [206]. The di-serinol-functionalized C₆₀ presents to be more promisor once presents no dark toxicity, but shows a typical light dose-dependent loss of colony-forming ability [207].

As commented above the phenalenones are also an important PS used in aPDT studies. Despite this BODIPY dyes can be negatively or positively charged PS, and exhibit high singlet oxygen quantum yields, acting almost always accord to Type II mechanism [126].

Within the background, it is possible to assume that at this moment there is not an ideal PS developed to successfully disrupt 100% of microbial biofilms. Therefore, it is important to combine the aPDT with an approach/technique that enables, at the same time, the bind of the PS to EPS and its permeabilization through the extracellular matrix to reach deeper cells of the biofilm, enabling efficiently the ROS production and action. The PEF is an applicable example of that once can form porous in the cells membrane and through biofilm [208], can also stimulate the ROS production [16].

Light sources

The light source is essential for aPDT once is the one that activates the PS to produce ROS. Some basics

requirements have been made for aPDT light source, including their electronic absorption spectrum that should match with PS activation spectrum (usually the longest wavelength peak), they need to be deliverable to the target tissue ergonomically and with high efficiency, must be reliable in the clinical environment and be cost-effective [209].

In addition, it is necessary to consider body tissues as a bulk medium, where the light propagation can suffer the processes of refraction, reflection, absorption and scattering [135]. Refraction that is governed by Snell's law [210] and reflection from the interface between two media governed by Fresnel's law [211] show processes that are proportional to the angle of incidence; i.e one can minimize them by applying the light beam perpendicular to the interface between the two media [135,212]. According to [213], reflection and refraction process impacts on the loss of intensity are determined by the relative values of their refractive indices. Light scattering in tissue has a pronounced effect on light intensity and directionality. When scattering occurs together with refraction, results in a loss of fluence rate (given as power per unit area of light in [W.m⁻²]) as well as a change in the directionality of the light beam, due a widening of the light beam [214].

Absorption depends on tissue composition, it is determined by the aPDT "optical window" and can be minimized by activation in the far-red wavelength region [135,212]. In tissue, the main chromophores are water, oxyhemoglobin (HbO₂) and deoxyhemoglobin, melanin and cytochromes [215], and their absorption spectra define the optical window for aPDT [137]. It is known that hemoglobin (Hb) and HbO₂ presents absorption in the range of 600–800 nm, and in some cases (e.g. in dental caries) hemoglobin chromophore may be absent and consequently, blue and green light would present no interference-effect [135].

Thus, most PS's are activated by red light between 630 and 700 nm, corresponding to a light penetration depth from 0.5 cm to 1.5 cm, limiting to reach the depth of necrosis [216]. The light source, total light dose, dose rates and aPDT effect depend on the localization of the tissue treated and the PS applied [217].

The first light source used in aPDT was the conventional bulb, which did not yield good results once presented properties as polychromaticity, strong thermal component, and incoherency [218]. Nowadays, three main classes of aPDT light sources are being applied: lasers (e.g. argon, diode, or neodymium doped: yttrium, aluminum, and garnet [Nd:YAG] lasers), light-emitting diodes (LEDs) and gas-discharge lamps (e.g. quartz-tungsten-halogen or xenon-discharge lamps) [96].

Lasers are very convenient and reliable, due to their monochromaticity, high efficiency (>90%) of coupling into single optical fibers and high potency, as well as they can be interstitial light delivery devices. However, they do have a high cost (being Diode laser one of the lowest-priced among them), has a single wavelength requiring a separate unit according to PS used [219].

On the other hand, LED presents advantages over lasers such as low cost and ease configuration that arrays into different irradiation geometries, they are more compact and portable [209,219,220]. The output power used is no longer a limitation for LED, once it arrays with hundreds of mWcm^{-2} are available spanning most of the visible–NIR spectrum. Thus, linear arrays of LEDs can be used endoscopically or even interstitially, but they continue presenting relatively poor electrical-to-light conversion efficiency of conventional LEDs [209]. LED output spectrum typically has a bandwidth of around 25 to 30 nm and an efficiency factor for the typical PS spectrum of about 50%, the same can be noted with filtered halogen lamps [209]. The main characteristic of filtered halogen lamps is that they can be spectrally filtered to match any PS, but they can cause heating and cannot be efficiently coupled into optical fiber bundles or liquid light guides [219]. In addition, with broadband sources, their effective output potency is lesser than lasers' source but proportional to the integrated product of the source output spectrum and the PS activation spectrum [221].

An important point is about the irradiation period and the respective light source used, that depending on the energy dose applied, can cause a temperature increase, leading to tissue damages [96,222]. Have been reported that the dose of energy is a parameter that generally shows a great variation, correlated to different potencies and exposure times. The highest potencies are related to lasers sources [170], once they can concentrate great energy in a small area, as tungsten lamps that adopted longer irradiation time once present a broad spectrum and low intensity requiring longer time interval to achieve an efficient energy emission [219,223].

Concerning continuous light and pulsed light sources, there are still controversial points of view and no conclusions. However, for biofilms infection, we may highlight pulsed light for two special reasons: (i) it allows recovery of the level of regular oxygen; (ii) allows the temperature of tissue to be kept at acceptable levels [224].

Therefore [209], suggested that for high aPDT efficiency, it is required: the presence of more regular oxygen close to the PS and the target tissue, multi-

wavelength laser diode systems and LED arrays with user-configurable geometry to match with the treatment area, development of disposable packages with an integrated light source and delivery components, ultrafast (fs) pulsed laser sources for two-photon; and all that with low-cost systems, mainly for treating localized infections.

Pulsed electric field

Fundamental mechanism

The plasma membrane of the cell could be imagined as an insulator layer with an aqueous solution of electrolyte at both sides. When cells are exposed to high power external pulsed field, cell membrane permeability to various impermeable molecules increases suddenly, this process is called electroporation or electropermeabilization. It is the most revealed bioelectric effect on the cells. Then, the pulsed electric field polarizes (rotate) the water and other dipolar molecules, e.g., lipid head groups, this can modulate the transmembrane potential and locally can reach a critical permeabilizing threshold (0.2–0.6 V) by inducing some local disorders (pores) in a lipid membrane [225]. There are many excellent reviews devoted to electroporation and membrane permeabilization in general [226,227]. If the electric field parameters are above some certain level, cells are not able to return to an intact state and the process thus leads to cell death. However, membranes of living cells can spontaneously return to their primal state if the electric field is not too intense. In classical cell membrane electropermeabilization the electric field pulse lasts from submicro- to several milliseconds and does not exceed 10 kV/cm electric field strength for eukaryotic cells [228]. In reference to the pulse length, they could be divided into three main categories: mili-, micro- and nano-second length pulses each of them having a different bioelectric effect on living cells. Basically, the characteristics of electric field parameters for various targets differ depending on the final aim of the PEF treatment, experimental configuration, evaluation methods, the characteristics of the electroporation buffer, cell culture or tissue. In case the cells *in vitro* must be destroyed, electric field parameters are chosen above some certain level, where the cells can't return to the intact state and die. Meanwhile, there is no evidence of PEF application on biofilms in clinics. The second bioelectric effect on the cells is the generation of ROS after exposure to PEF. It can be (i) direct when free radicals are formed at the surface of the electrodes and (ii) indirect when free radicals are generated as a cell reaction to the PEF as abiotic stress. Both types are further discussed.

PEF effect to the cells and biofilm

Interestingly, the generation of oxygen reactive species comparing the cells exposed to micro- and milliseconds range electric field strength was more significant in the samples treated with milliseconds pulsed electric field (mPEF). After such treatment, the mammalian cell membrane was permeabilized reversibly. It was concluded, that increased production of ROS was directly associated with a permeabilized state of the plasma membrane [229]. Such ROS generation might be induced by the oxidative stress associated with the local nonspecific permeabilization of the plasma membrane causing the peroxidation of the lipids (Table 3) [230]. However, there is some contradictory experimental evidence with the yeast and bacteria cells, where ROS generation was not associated with plasma membrane permeability. The data support the idea that intracellular ROS generation by the mPEF is determined by the metabolic activity of the cells [230,231]. There is no correlation between permeabilization and ROS generation when short (microsecond) pulsed electric field (μ PEF) was applied [232]. Furthermore, experimental studies have shown that even shorter sub-nanosecond and nanosecond pulsed electric field (nsPEF) can generate intracellular and extracellular ROS and it might be involved in lipids oxidation, but do not associate with plasma membrane permeabilization [233,234]. These experimental evidence support the idea that the mechanisms of ROS generation by the short electrical pulses (nsPEF) are fundamentally different from which causes an increase in ROS generation by the longer (mPEF) pulses.

Therefore, the increased ROS production in permeabilized cell by μ PEF remain unclear. One of the possible

explanations could be that the source of ROS generation might be a membrane NADPH-dependent oxidase (NOX) [235]. The calcium depended NOX activation is very important for cellular defense against viruses and other biotic and abiotic stresses [236]. Moreover, μ PEF, as abiotic stress, involved in intracellular calcium release might be an indirect activator of NOX. It is hard to speculate because there is indirect evidence that NADPH could be released from the yeast or mammalian cells after permeabilization too [237–239]. Unfortunately, there is no direct evidence that activation of NOX depends on pulsed electric field treatment of eukaryotic cells.

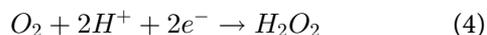
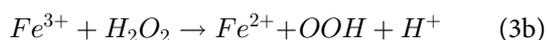
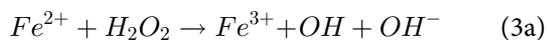
Electrical pulses of durations between 10 and 1000 ns (nsPEF) usually affect intracellular structures and create pores in the cell plasma membranes (Table 3) [240,241]. Electroporeabilizing pulses of 100 ns long and 30 kV/cm strength triggered an increase in reactive oxygen species (ROS) generation in human pancreatic carcinoma cells and such a generation of ROS can be downregulated by modulating an intracellular Ca^{2+} concentration by adding an antioxidant [Nuccitel242]. It was concluded, that Ca^{2+} is an important effector for ROS generation by nsPEF. Moreover, it was shown, that nsPEF pulses with moderate duration ($\tau = 10\text{--}90$ ns) induce caspase-dependent apoptosis in yeast cells and most likely such a programmed cell death process could be triggered by cytochrome C release from nsPEF permeabilized mitochondria in the presence of Ca^{2+} [243,244]. Such hypothesis can be justified considering that ROS is derived from mitochondria after mitochondrial permeability transition pore formation. Finally, ROS is an important player which facilitates apoptosome formation in eukaryotic cells [245]. Furthermore, present evidence shows that nsPEFs generate mitochondrial ROS that activate heme-regulated inhibitors, leading to eIF2 α phosphorylation by modulating eukaryotic integrated stress response [246].

The second way of ROS formation is the direct burst of ROS at the surface of the electrodes during the PEF treatment [247]. The extent of ROS formation also depends on the electrode material [248,249]. During PEF treatment cell suspension is exposed to a strong electric current that passes the solution. Various electrochemical reactions occur at the interface of electrode–solutions, electrons are transferred from or to an electrode and the charge is thus allowed to flow completely around the circuit. At each electrode–solution interface, electrochemical half-reactions of the species with the most favorable redox potential take place, namely reduction on the cathode and oxidation on the anode. Cathodic

Table 3. The ROS type generated after treatment with PEF in various types of cells.

| Electric field parameters | ROS type | Cells | References |
|--|--------------------------|--|------------|
| 0.5 kV/cm; 1 Hz; 6 ms 0.9 kV/cm; 1 Hz; 10– 100 μ s | H_2O_2^- | Chinese hamster ovary (CHO) | [229] |
| 5 kv/cm; 25 μ F, 200 Ω ; 600 μ s | Hydroperoxides | Erythroleukemia K562 | [230] |
| 1.75 to 3.25 kV/cm; pulse number up to 9; up to 1 ms | General ROS | Saccharomyces cerevisiae | [231] |
| 0.44 kV/cm; 1 Hz; 1 ms 0.44 kV/cm; 1 Hz; 100 μ s | General ROS- | CHO | [232] |
| 1–13 kV/cm; 1 Hz; 300 ns | H_2O_2 | Jurkat | [234] |
| 30 kV/cm; pulse number up to 10; 100 ns | General ROS | Homo sapiens pancreas BxPC-3 cells | [242] |

reactions include the reduction of metal cations, reduction of water. Anodic reactions include oxidation of water, anions and metals [247]. Besides primary electrochemical reactions happening at the electrode–solution interface, the secondary chemical reactions might also appear including those, generating ROS formation:



Longer, microsecond range electrical pulses lead to electrolysis and changes in pH and thus the generation of free radicals [248,249]. The electrode material also matters for the quantity of ROS generated at the electrode surface. Higher electroporation efficacy comes with higher ROS formation, especially when copper electrodes are used in comparison to aluminum and steel electrodes applying both nanosecond and microsecond pulses range. However, not only the pH changes but also metal ion release proved to play a significant role in ROS formation [248,249].

For example, the formation of H_2O_2 was proposed to happen through nsPEF electrochemical effect on the electrodes. Very high intensity is associated to a short-lived current. The increased oxidation of Amplex Red (N-Acetyl-3,7-dihydroxyphenoxazine) occurred due to the formation of H_2O_2 in nsPEF-treated media, specifically, H_2O_2 in the presence of horseradish peroxidase (HRP) promotes high fluorescent resorufin (fluorescence). On the other hand, the ROS generation by the buffer was not reported (e.g. longer pulses as usual used in electroporation. nsPEFs generated both extracellular (in part electrochemical) and intracellular ROS, among which H_2O_2 [250].

One of the conclusions summarized in this part of the review was that nsPEFs were the most likely to cause most intracellular effects leading the ROS production. It is clear, that most of these evidence were observed in mammalian cells. More experimental results on nsPEF-induced ROS production in eukaryotic microbes, like yeast, would be very profitable for developing fused technologies in the future.

Several studies have been reported the effect of PEF against several microbial biofilms [16,251]. applied 200 Hz pulsed electric field of 18 V/cm for 2.5 μs and then sonicated per 5 minutes, reducing 95% of *S. aureus* attachment. On the other hand [252], applied low-voltage (0.5–5 V) pulsed electric fields using an interdigitated electrodes with 29 μm spacing between 22- μm -wide electrodes to prevent *P. aeruginosa* biofilm development [253]. also investigated the effect of PEF against *P. aeruginosa*, applying 1500 V through a central electrode, with pulse duration of 50 ms, and pulse delivery frequency of 2 Hz. Bioluminescent imaging and Scanning Electron Microscopy showed that the area at which 100–80% of bacteria were eradicated was $50.5 \pm 9.9 \text{ mm}^2$ for 300 pulses, and $13.4 \pm 0.65 \text{ mm}^2$ for 150 pulses. In addition [254], proposed a new methodology centered on nanosecond high frequency electric field, which can successfully eradicate bacteria in *in vivo* studies models. They applied a high frequency 15kV/cm, with up to 900 ns pulsing bursts in combination with acetic acid (0.1–1%) to treat *P. aeruginosa* in a murine model. With the results, they suggested a direct application of PEF for treatment of wounds and ulcers when chemical treatment is no longer effective.

These studies have contributed to the discussion about the mechanism of action of the bioelectric effect and some hypotheses were formulated as (I) reduction of the biofilm capacity for binding to the antimicrobial agent [255]; (II) electrophoretic augmentation of the antimicrobial agent transport [256]; (III) membrane permeabilization [256]; (IV) electrolytic generation of oxygen [257]; (V) electrochemical generation of potentiating oxidants [258]; (VI) cell wall and membrane permeabilization [259].

Future research directions for the combination of aPDT and PEF, against microbial biofilm

aPDT system should present characteristics to be effective to disrupt and kill the biofilm cells, without any side-effects for human body [96]. It has been reported that these characteristics are, mainly, head to PS permeabilization and its ROS production. Thus, an ideal PS must present characteristics as aqueous medium affinity and presence of positive charges [260], low molecular weight [96], high photostability [261] and singlet oxygen yield [140], and no toxicity or mutagenicity [262]. However, within the background, it is possible to verify that until now there is not any class of the dye showing these all characteristics.

Thus, alternatives have been proposed to enhance one or more of the PS properties. In this review, we are

suggesting that a merge of aPDT to PEF may promote the increase of PS permeabilization, even hydrophobic ones as well as enhance the amount of ROS produce, to be applied against biofilm. The PEF is not a powerful generator of ROS as aPDT, but [16] reported that this technique presents mechanisms as electrochemical generation of potentiating oxidants and an indirect mechanism to produce ROS as electrolytic generation of oxygen.

Currently, the combination of light with PEF to prevent or kill microorganisms has been shown [263]. conducted a study combining ultraviolet light (UV) irradiation, PEF, and ozone for the inactivation of *E. coli* O157:H7 in poultry chiller water. As result after the application of 200 pulses and an electric field at 30 kV/cm, occurred a reduction of 4.1 log₁₀ of *E. coli*. Most of the studies about PEF and light are focusing on food preservation or decontamination. According to [264] the merge of non-thermal hurdles such as ultraviolet light (UV) (5.3 J/cm²), high-intensity light pulses (HILP) (3.3 J/cm²), PEF (34 kV/cm, 18 Hz, 93 μs) decreased 6.0 log of *E. coli* [265], studied the effect of PEF and UV against six microorganisms (*S. aureus*, *L. monocytogenes*, *E. coli*, *Salmonella* spp., *Cronobacter sakazakii* and *Campylobacter jejuni*) and realized that only PEF was able to induce any damage to the microorganism, mainly in neutral pH. However, by changing the pH and applying light, the PEF microorganism resistance decreased.

Although the association of PEF and light demonstrated promising results against microorganisms, the idea was not very explored in aPDT field but applied in PDT for cancer treatment [266,267]. At this moment, only one paper reported the effect of aPDT and PEF association, against microorganisms. In this study, they

evaluated the effect of aPDT with PEF on the planktonic cells *S. aureus* and *E. coli*. After irradiation (590 nm) of hydrophobic PS, hypericin, the microorganisms were inactivated in 2.65 logs and 3.67 logs more than aPDT alone, respectively. The authors concluded that the PEF improves the delivery of PS into the cells, enhancing the aPDT effect [15].

So, the necessity for more studies to evaluate the influence of the PEF in the aPDT effect against microbial biofilm still stands. Despite that, we hypothesize how the association of aPDT with PEF can act over the biofilm and increase microbial death (Figure 6). The PEF would allow the PS diffusion through the extracellular matrix, increasing the PS concentration to bind with the EPS and biofilm cells. In addition, the PS may be allocated in all biofilm layers and reach the deeper cells (*persister* cells), preventing the biofilm re-grow. ROS production by PS will be not affected by PEF; on the contrary, it will contribute to increase their yield.

Authors opinion

Given the many mechanisms of biofilm resistance, the permeability of the EPS is one of the most important issues, especially talking about the aPDT effectiveness. It is known that the biofilm porosities change from top layers to the bottom layers, while the biofilm mean pore radius decreases in the bottom layers. These aspects corroborate with the PS difficulty to access the deepest biofilm cells (dormant cells) decreasing the aPDT effect. Despite the ratios of viable cells to total biomass in the biofilm bottom layers (~39%) being around twice less than the cells in the top (~91%), deeper cells are one of the most important targets for the biofilm

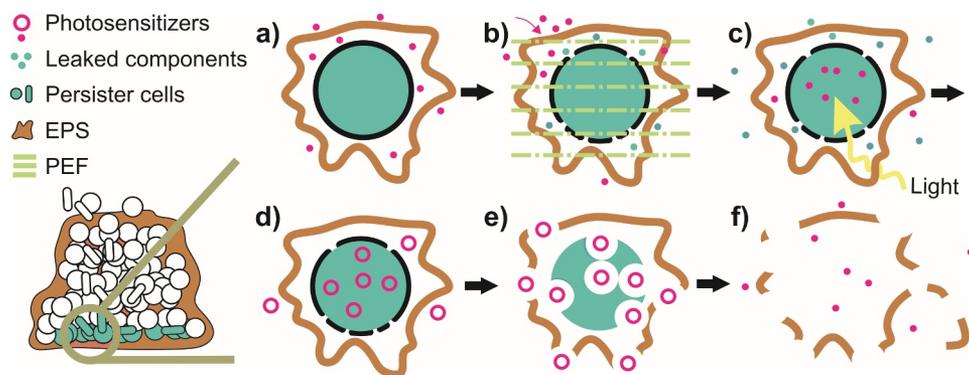


Figure 6. Hypothetical mechanism of action by the association of aPDT and PEF. **a)** Mature biofilm. **b)** *Persister* cells surrounded by EPS and the PS binding with the EPS and trying to reach the *persister* cells. **c)** Application of PEF, permeabilizing the PS diffusion through the EPS and cell membrane. **d)** Application of visible light, corresponding to the PS absorption. **e)** PS activation for ROS production. **f)** Production of ROS and its actions against both cells and EPS. **e)** The disruption of EPS, consequently also of the biofilm matrix and death of the microbial cell. PS returns to its ground state.

treatment. Since the bottom cells (dormant cells) have lower metabolism and are protected by EPS it became a challenge to find a way to reach and kill them. Therefore, electroporation could be a technique to overcome that, enhancing the biofilm permeability as well as increasing the amount of ROS produced. It is important to highlight the need to conduct more studies about the effect of aPDT and PEF against the QS molecules due to their importance for EPS production, cells virulence and pathogenicity, promoting biofilm development and resistance. It is also necessary to conduct thorough studies of the combination of two techniques in order to fully understand the benefits and to select the best parameters for biofilm treatment.

Executive summary

Conditions that should be *taken in* for aPDT and PEF application

- Understanding of photosensitizer chemical, physical and “biological” properties, once the clinician has to select a PS that seems most suitable for the patient’s needs (severity and location of infection) and with the best clinical features.
- PS should present 1) maximum absorption corresponding to the window between 650 nm and 850 nm, where tissue penetration is quite high; 2) energy for the triplet state that is sufficient for singlet oxygen production, 3) low photodegradation that is optimal for extended irradiation times, 4) amphiphilicity to ensure both transportations in the blood and penetration through the lipid layer of the cell membrane, hinder its precipitation or aggregation, 5) selectivity to accumulate in the target (microbial cells) tissue but not in normal (healthy) cells, restricting photo-induced damage to surrounding tissue and 6) should not cause mutagenic effects, irrespective of being irradiated or not.
- The main aspect to choose a light source is the spectral emission (corresponding to PS wavelength absorption), intensity/power and mode of light-delivery (via optical fiber or directly) both suitable for the localization and severity of the infection.
- Careful attention to physics and dosimetry of aPDT will help to minimize general toxicity and side effects.
- Develop an ideal photosensitizer is an important goal for clinical application of aPDT to be effective, but not prevent the use of supplementary forms of treatment that can be combined with aPDT, as PEF.

- Microbial inactivation by PEF, mainly nanosecond PEF, is governed by the treatment time (number of pulses \times duration of the pulse), knowing that microbial inactivation increases with an increase in the number of pulses. This approach could represent an alternative to aPDT, increasing and delivering the PS to the local of biofilm infection, special the ones that occur as wound. However, more fundamental evidence and technical developments are needed to understand the effects of PEF on biofilms and its synergistic effect in combination with aPDT.
- Define optimized and standard protocols of PEF/aPDT that can be suitable for a particular application on clinical, according to the tissue localization (wound or medical device) and species of microorganisms (multiple or single species) of the biofilm infection.

Acknowledgments

The author’s sincere thanks for the support from Center for physical sciences and technology (FTMC).

Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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