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Sonophotodynamic Inactivation: The power of light and ultrasound in the battle against microorganisms

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ABSTRACT

The inactivation of pathogenic microorganisms that are a threat to the human being is an old challenge that needs to be overcome, mainly because microorganisms are able to develop resistance that leads to the failure of the treatment. Moreover, the biofilm living form is a complex microorganism community that is less susceptible to antimicrobials agents. For this reason, the search for alternative therapies that are not able to induce resistance and have the ability to inactivate the biofilms is unquestionably needed. In this review, Photodynamic and Sonodynamic Inactivation (PDI and SDI) are suggested as promising antimicrobial approaches. However, the use of the combined therapy, called Sonophotodynamic Inactivation (SPDI), is reinforced and encouraged for microbial diseases. As long as SPDI has been demonstrated to be more effective in inactivating microorganisms than PDI and SDI, it is allowed to reduce the time and parameters of the treatment, turning this therapy safer for mammalian cells.

Introduction

Antimicrobial challenges

The inactivation of pathogenic microorganisms that are a threat to the human being is an old challenge for health professionals and needs to be overcome. Antimicrobial drugs have been effectively used in the treatment of infectious diseases for decades. However, it is well known that microorganisms can undergo mutations and develop resistance, decreasing the effectiveness of these agents [1–3] dramatically. The widespread use and the regimen of such drugs lead to the emergence of resistant strains [4]. The emergence of resistant microorganisms and their impact on healthcare have been raising concerns since the late 1940s and 1950, and since then, antibiotic resistance has been explicitly framed as a global public problem [4]. The Interagency Coordination Group on Antimicrobial Resistance of the United Nations, in 2019, presented an alarming report entitled “No time to wait: securing the future from drug-resistant infections”. According to this document, at least 700,000 deaths occur worldwide a year caused by drug-resistant infections, including 230,000 deaths from multidrug-resistant tuberculosis [5]. This scenario may even progress to a global 10 million deaths per year by 2050, even more than deaths caused by cancer, if no concrete actions are taken to contain the antimicrobial resistance [6].

Besides the widespread use of antimicrobial agents, another negative characteristic of these drugs is related to the mechanism of action. Antimicrobials act mainly on the inhibition or regulation of enzymes involved in a relevant cell function, as in cell wall or protein synthesis or nucleic acid metabolism and repair; and others on the direct disruption of the membrane structure. Due to these mechanisms of action, each antimicrobial targets only a specific microorganism biomolecule [5]. Such high specificity results in a high inactivation rate for the microorganisms presenting the target biomolecule, but also in the selection of the microorganisms showing resistance to this mechanism of action. According to the World Health Organization (WHO) [7], in 2019 there was a list of 32 antibiotics being developed to fight against the top microorganisms that were considered priority. However, only six of them are innovative and the quality of them is not fully well known. Moreover, the WHO claims that the absence of effective treatments for resistant infections will lead to an increase in the number of clinical procedures, such as surgery and organ transplantation and, also, in the number of deaths. In this sense, the development of alternative strategies for microorganism inactivation, including the multi-drug resistant microorganisms, is presently one of the top priorities in healthcare research [5].

Photodynamic inactivation (PDI)

In this context, Photodynamic inactivation (PDI) or antimicrobial photodynamic therapy (aPDT) proves to be an attractive alternative therapy for the local treatment of infectious diseases and microbial decontamination [8-10]. PDI employs the photodynamic activity for the inactivation of microorganisms (e.g., bacteria, fungi, or virus) presented

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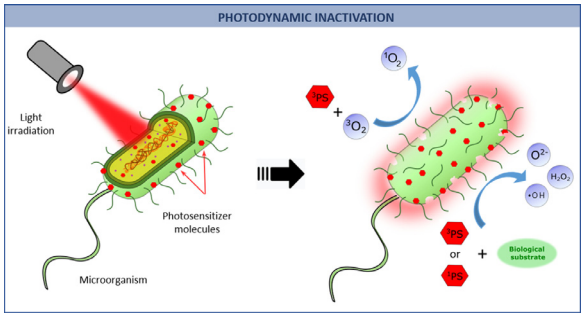

Fig. 1. Photodynamic inactivation. PDI is based on the interaction between a non-toxic photoactive drug (photosensitizer, PS) and low intensity visible light in the presence of molecular oxygen ($^3O_2$) dissolved in the cells; such interaction triggers a series of physical and chemical reactions that result in the mammalian or microbial cells death.

in the body or in the environment [11-13]. In literature, it is possible to find other terms attributed to this technique, such as: photodynamic inactivation (PDI), photodynamic antimicrobial chemotherapy (PACT), photoactivated disinfection (PAD), photodynamic disinfection (PDD) or lethal photosensitization [9,13]. The PDI is based on the interaction between a non-toxic photoactive drug (photosensitizer, PS) and low intensity visible light in the presence of molecular oxygen ($^3O_2$) dissolved in the cells; such interaction triggers a series of physical and chemical reactions that result in the mammalian or microbial cells death [8,9] (Fig. 1).

The antimicrobial therapy initiates with the PS administration, for which, the PS is incubated in contact with the target cells resulting in the sensitization of them. After incubation, the PS-loaded target cells are irradiated with a specific light wavelength, giving rise to the photodynamic activity. The ground state of the most molecules is the ground singlet state (S_{00}) characterized by two electrons with opposite spins orientation (↑↓), however, there are other molecules such as $^3O_2$, whose ground state is the ground triplet state ($3\sum_g^-$) characterized by two electrons with same spins orientation (↑↑). After illumination, the PS molecule in rest absorbs part of the delivered light dose remaining excited (activated PS). This means that an electron of the PS goes from S_{00} to an excited singlet state (S_{nv}) of higher energy. Due to the instability of the molecule in an excited state, the electron could undergo a series of non-radiative decays (e.g., vibrational relaxation and internal conversion) until the excited state of lower electronic and vibrational level (S_{10}), followed by the release of the energy excess through non-radiative decays or fluorescence (radiative decay), returning to S_{00}. Another way for the electron in S_{10} releases the excess of energy is through intersystem crossing to the excited triplet state T_{1v}, which involves the spin inversion, getting two electrons with equal spins. In T_{1v}, the electron undergoes a vibrational relaxation until T_{10}, where the PS molecule can interact with the surrounding biological substrate via two mechanisms. The "Type I" mechanism includes the electron transfer between the PS molecule and the substrate, producing radical ions which interact with the surrounding $^3O_2$, resulting in the formation of reactive oxygen species (ROS) such as superoxide anions (O^{2-}), hydrogen peroxides (H_2O_2) and hydroxyl radicals (OH). The "type II" mechanism involves the direct energy transfer between the PS molecule and the $^3O_2$, since both are in a triplet state, resulting in the formation of singlet oxygen ($^1O_2$), a powerful oxidizing ROS [8,10]. After charge/energy transferring, the electron in T_{10} returns to S_{00}. Another way for the electron in T_{10} returns to S_{00} is emitting the excess energy as phosphorescence (radiative decay) [8]. Finally, the ROS induced by each mechanism leads to the oxidative stress of proteins, fats and other molecules within the photosensitized cells, followed by the microorganisms' death in the treatment area [9,13] Fig. 2 shows the Jablonski diagram, which summarizes these events involved during PDI.

The contribution of the "type I and II" mechanisms will depend on the PS structure, $^3O_2$ concentration and PH of the biological substrate [13]. For most PS molecules, the "type II" mechanism is considered the one that determines the efficiency of PDT, however, as oxygen is depleted, the "type I" mechanism begins to prevail.

The cell damage and microbial kill triggered by ROS depends on the location of the PS accumulation, which occurs preferentially in the therapeutic target (e.g., bacteria), binding to microbial cells instead of the host mammalian cells [8,14]. The most common cell damages proposed in the literature has been via three mechanisms: (i) damage to the cell membrane (or virus envelope), which leads to leakage of cellular contents following inactivation of the membrane transport system; (ii) DNA damage due to the inhibition of cellular metabolic processes; and (iii) inactivation of essential protein/enzyme resulting in loss of enzyme activities, protein oxidation and formation of protein-protein cross-links. In addition to the functional changes, morphological alterations can also result from photodynamic damage such as: alteration of the mesosome structure [10,13].

A fact that could compromise the effectiveness of PDI against different pathogens is the interaction of the PS with the cellular components. The PS needs to penetrate the cell wall of the bacteria and end up in the cytoplasm; however, the permeability barrier related to the structure and organization of different classes of microbial cells limits the simple diffusion of PS into the bacterial cytosol [10,12,15]. Usually, antimicrobial PDT is more effective against Gram-positive bacteria than Gram-negative bacteria, because of the differences in the cell wall structures of these two groups. In Gram-positive bacteria, the cytoplasmic membrane is surrounded by a porous peptidoglycan layer and lipoteichoic acid that allows the PS to cross. The Gram negative has a thin peptidoglycan layer and an outer membrane that contains lipopolysaccharide, phospholipids, and proteins. The periplasmic space between the cytoplasmic and outer membranes contains transport, degradative, and cell wall synthetic proteins. The outer membrane of these bacteria contains numerous strongly negatively charged molecules, which reduces the permeability and attachment of neutral PSs or repels anionic PSs, making this group of bacteria less susceptible to PDI than the first one. On the other hand, the fungal cell wall is made up of a thick layer of beta-glucan and chitin with a permeability barrier intermediate between Gram-positive and Gram-negative bacteria [10,15].

Different organic and inorganic PSs have been used in applications of PDT and PDI, these are summarized in Table 1 [16,17]. The PSs that are commonly used in PDI includes a wide range of compounds, most of which are cationic (positively-charged) [12]. In vivo and in vitro reports established that the best way for optimizing selectively for microbial cells over mammalian host cells, was to apply a PS with a pronounced cationic charge on their functional groups. In this way, bacteria that possess a negative charge on the surface can be easily bounded to such cationic PS [10,14,15].

The potential of PDI comes with the findings that this photonic therapy works even on antibiotic-resistant microorganisms, and up to now, there is no evidence that microbes are becoming resistant to PDI even after numerous repetitions [15,18-20]. However, the management of infection diseases by PDI also depends on how microorganisms are organized. Most pathogens are found in nature living as a biofilm. Biofilm is a community of microorganisms highly structured and complex, where cells are attached to a biotic or abiotic surface and involved in an extracellular matrix produced by them [21]. This microbial community is found in a variety of biological environments, such as, on the surface of catheters, prosthesis, mucosa, teeth and are responsible for the development and persistence of the infection. Moreover, microorganisms in this living form are able to interact with each other by quorum sensing molecules, which enable them to coordinate several behaviors, such as the secretion of virulence factors [22]. Taking these features into account, biofilms are a challenge for any type of treatment, since they are more virulent and, mainly, because of the presence of the extracellular matrix that difficult the drug penetration, including the pho-

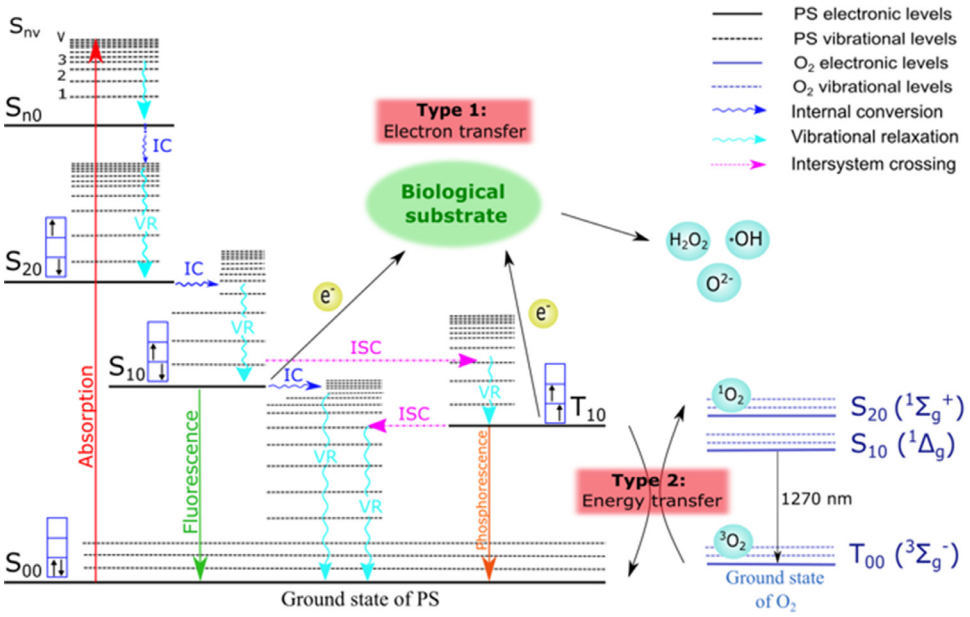

Fig. 2. Jablonski diagram. The solid horizontal lines represent the electronic singlet and triplet levels of the photosensitizer (black) and the oxygen (blue). The dotted horizontal lines represent the vibrational levels of the photosensitizer (black) and the oxygen (blue). IC: Internal conversion, VR: Vibrational relaxation, ISC: Intersystem crossing. The subscript "n" indicates the electronic level, while "v" indicates the vibrational level.

Table 1
Organic and inorganic PSs applied for PDT* and PDI** [16,17]. NPs: nanoparticles. NDs: nanodots.

Organic PSs	Metal-Free Inorganic Nanomaterial-Based PSs	Metallic Inorganic Nanomaterial-Based PSs
Small Organic PSs	Carbon Nanomaterial–Based PSs	Pure Metal-Based PS
Porphyrins *, **	Carbon dots*	Au NPs, Ag NPs, Pt NPs*
Phthalocyanines*, **	Graphene quantum dots*	
Indocyanine Dyes*	Fullerenes*, **	
Phenothiazinium chromophore*, **	Graphitic carbon nitride (g-C_3N_4) *	
BODIPYs*, **	Silicon Nanomaterial-Based PSs	Metal Carbide-Based PSs
Curcumin*, **	Black Phosphorus	Ti_3O_2 nanosheets, W_2C NPs*
Furocoumarin*		Metal Oxide-Based PSs
Perylenequinone*		TiO_2-Based PSs*
Perinaphthenone**		Monometal Oxide-Based PSs* (ZnO NPs, Cu_2O
Vitamin B2 derivatives**		nanocrystals, Fe_2O_3 NPs, BiOCl nanosheets, Tungsten
Nobel metal complexes		oxide nanowires.)
Ru(II) Complexes*		Bimetal Oxide-Based PSs*
Ir(III) Complexes*		(β-$SnWO_4$ NPs, Bi_2WO_6 nanosheets, Bi_2WO_6 NPs)
Au(III) Complexes*		
Organic Frameworks Compounds		
Metal-Organic Frameworks*		
Covalent Organic Frameworks*		
Hydron-Bonded Organic* Frameworks*		
Polymer-based PS		Metal Sulfide–Based PSs
Polyfluorene*		Monometal Sulfide-Based PSs* (Cu_2S nanocrystals,
Polythiophene*		CuS NPs, Co_9S_8 NDs)
poly(N-phenylglycine) (PNPG) *		Bimetal Sulfide-Based PSs* ($CuInS_2$/ZnS core-shell
		nanocrystals, PVP-conjugated $CuMo_2S_3$ nanocrystals,
		PVP-functionalized CuSbS NPs)

*Photosensitizers used in PDT
**Photosensitizers used in PDI

tosensitizer uptake. These characteristics make biofilms less susceptible to PDI than planktonic cells. Pérez-Laguna et al. (2018) [23], verified that PDI mediated by rose bengal was capable to reduce 6 $\log_{10}$ of *Staphylococcus aureus* suspension using low concentrations of the PS (0.31 μg/mL); however, a maximum reduction of 3.3 $\log_{10}$ was reached using 8 μg/mL of rose bengal against staphylococcal biofilms. Wu et al. (2017) [24] evaluated in vivo Chlorin e6 at 0.01%, 0.05% or 0.1% to mediate PDI against *Pseudomonas aeruginosa*. Authors observed that PDI has a transient efficacy in inactivating *P. aeruginosa* in vivo, and repetitive PDI treatments are required to fully resolve the infection. Wu et al. (2020) [25] observed that PDI mediated by toluidine blue O at the concentration of 0.1 mg/mL was more effective to inactivate *Streptococcus mutans* and *Lactobacillus acidophilus* in suspension than the biofilm living form. Ribeiro et al. (2013) [26], used a nanoemulsion (NE) in the attempt to increase penetration of Aluminum-Chloride-Phthalocyanine (ClAlPc) into *Candida albicans* biofilm. Authors demonstrated that the combination of cationic NE-ClAlPc with 100 J/cm^2 of light decreased cell metabolism by 70%, while the same PDI parameters reduced the metabolism by 92.3% of planktonic cells. For this reason, the use of additional strategies to improve PDI efficacy against microbial biofilms have been employed, such as the use of ultrasound to disrupt the biofilm.

The ultrasound: a powerful tool for the improvement of PDI

Ultrasound (US) is sound waves with frequencies above the only audible limit of human hearing. The human ear detects sound waves from 16,000 up to 20,000 hertz. However, the ultrasound used in medicine

has frequencies from 750,000 up to 3,300,000 Hz [27]. The ultrasound effects and interactions in the target tissue depend on the features of the ultrasound waves. For this reason, knowledge about the ultrasound waves is essential for the correct and successful application of this therapeutic tool. The frequency, wavelength and amplitude of the waves will determine the ultrasound effect on the tissue. The ultrasound that is able to capture images of the internal tissues of the body has a frequency ranging from 2 to 15 MHz, with low amplitude. On the other hand, the frequency of the ultrasound used in therapies varies from 0.75 to 3.3 MHz with high amplitude, delivering more energy to the tissue per pulse [27].

The antimicrobial effect of ultrasound was first demonstrated against the algal microorganism *Spirogyra* in 1927 [28]. Then, other studies evaluated and proved the ultrasound antimicrobial efficacy, alone or combined with other chemicals and strategies, against a diversity of microorganisms, being applied for food decontamination and for the inactivation of bacteria present in medical devices [28]. Sarkinas et al. [29] assessed the efficiency of the ultrasound treatment (300 W and 600 W, 28 kHz, 10-30 min) against suspensions of *Listeria monocytogenes, Bacillus cereus, Escherichia coli* and *Salmonella typhimurium* bacteria and also on phytoviruses. Authors observed that the effectiveness of the US was influenced by the power of ultrasonic waves, exposure time and bacteria type. The US was able to eliminate vegetative cells of gram-positive and gram-negative bacteria from 1.59 to 3.4 log in bacterial suspensions and some *phytoviruses* in fruits. Seth et al. (2013) [30] evaluated the combined effect of US with Ciprofloxacin to treat rabbits with *Pseudomonas aeruginosa* biofilm-infected wounds. They found that US had a significant impact on biofilm-infected wounds, being able to reduce the bacteria viability and to improve the wound healing and host inflammatory dynamics. Another investigation [31] assessed in vitro the effect of US at 40 kHz with 1:9 of duty cycle, 600 mW/cm^2 of intensity and 30 min of application in combination with Colistin and Vancomycin on resistant *Acinetobacter baumannii* biofilm. Authors obtained reductions higher than 2 log CFU/mL for colistin plus vancomycin with US compared to the group without US application, after 12 h of incubation. Bacterial viability decreased continuously for 24 h, being the reduction equivalent to 3.77 log CFU/mL higher for the group that received the US in comparison with the group without US [31]. Moreover, US was also evaluated for the inactivation of *Candida albicans* biofilms in combination with Amphotericin B-Loaded Poly(Lactic-Co-Glycolic Acid) Nanoparticles [32]. The use of the loaded Amphotericin B combined with US at 42 kHz, 0.30 W/cm^2 of intensity during 15 min resulted in significant reductions in the metabolic activity, biomass and in the production of phospholipase and proteinase enzymes, in comparison with the isolated application of Amphotericin and US [30]. Wierzbicki et al. [33], during the pandemic of SARS-CoV-2 virus in 2021, performed a modeling study suggesting that ultrasound vibrations can cause coronaviruses damages, when it was applied at the same parameter of medical diagnostic imaging devices. Authors observed the mechanical response of the virus to ultrasound waves by computer simulations, and they found that US frequencies between 25 and 100 MHz provoked collapse and rupture to the virus' shell and spikes after a fraction of a millisecond, simulating the virus in the air or in the water [33].

Based on this ability, a good use of ultrasound in the battle against microbial is in combination with a sensitizer (sonosensitizer, SS). This modality of treatment is known as Sonodynamic Inactivation (SDI) [34], also known as Sonodynamic Therapy (SDT), Sonodynamic chemotherapy (SDCT), Sonodynamic antimicrobial chemotherapy (SDACT) or Sonodynamic Excitation (SDE). Its principle is very similar to the PDI, which involves the combination of three distinct components: the sensitizing drug, ultrasound, and molecular oxygen. The sensitizer increases the efficacy of US's antimicrobial effects and the specificity of the treatment, since the sensitizer accumulates into the target cells, then, the sono-reaction occurs in this area where the SS is linked. The attractive feature of SDI over PDI emerges from the deeper penetration of the ultrasound waves into the target tissue in comparison with light. Then, SDI is more effective in treating deeper and less reachable infections than PDI [34].

The sonodynamic action is divided in three main steps [35]. First, US has to be generated to concentrate energy in the focal area, where sensitizers are. Then, sonochemical reactions are induced by the Cavitation phenomenon and mechanical forces. Ultrasonic cavitation is a single and dynamic phenomenon of ultrasound, where it acts on the media, producing microbubbles that are excited, then vibrated, contracted and, in some cases, there is the collapse of the microbubbles. Depending on the US intensity, cavitation will happen in a different way, being divided in non-inertial cavitation (also known as stable cavitation), and inertial cavitation. The non-inertial cavitation occurs when a low-intensity ultrasound is applied in a liquid media, characterized by the production of bubbles that do not firmly collapse, having a higher lifetime. Non-inertial cavitation produces bubbles with high energy, which oscillate and are able to generate radiation force and microjet. For this reason, they are capable of interacting with cells, biomolecules and structures that are closed to them, such as the cell membrane. When the microbubbles reach the cell membrane, there is the production of transient pores, then, the surrounding drug, such as a sonosensitizer, can penetrate into the cells. On the other hand, inertial cavitation occurs when a high-intensity ultrasound is applied in the liquid and a strong bubble dynamic process happens. Besides that, the inertial cavitation bubbles absorb high quantities of energy and release this energy in a minimal area, which increases the local temperature and pressure, there is the formation of free radicals, the appearance of strong shock waves and high-speed micro-jets in the media. This physical scenario in combination with the production of chemical radicals is highly harmful to the target cells, having their organelles and biomolecules deeply affected. [35]. In summary, the cavitation regimens induce the elevation of the temperatures and pressures, production of hydroxyl radicals and hydrogen atoms. Also, the high pressure and temperature are able to decompose the solutes in the media. Finally, the ROS produced through the bubble collapse leads to chemical reactions in the liquid. Another phenomenon that is also linked to the production of ROS is called sonoluminescence. Sonoluminescence is the light emission generated by the bubble collapse and it has been hypothesized to excite the sonosensitizer [35]. However, the observation of this phenomenon is very controversial, and it has not been effectively proved. Fig. 3 summarizes these US effects and the SDI mechanisms.

In the available literature, studies have evaluated SDI against a variety of microorganisms, most of them in planktonic conditions. Nakonechny et al. (2013) [36], observed that when rose bengal was activated by the ultrasound against *S. aureus* and *Escherichia coli*, this treatment reduced 3-4 log the bacteria concentration. Zhuang et al. (2014) [37], investigated the effect of hematoporphyrin monomethyl ether (HMME)-mediated SDI over *S. aureus*. Authors verified reductions in the viability higher than 95% of the bacteria. Wang et al. (2014) [38] investigated the SDI for the eradication of methicillin-resistant *S. aureus* (MRSA) in suspension using curcumin and ultrasound. The viability assay showed that the reduction in the viability of MRSA was proportional to the concentration of curcumin. A reduction equivalent to 5-log was achieved using 40 μM of curcumin for 50 min followed by sonication at 1.56 W/cm^2 during 5min. Wang et al., in 2016 [39], investigated the sonodynamic effectiveness mediated by hypocrellin B for the inactivation of MRSA. Authors observed reductions equivalent to 5-log using 40 μM of hypocrellin B and the ultrasound was applied at 1.38 W/cm^2 during 5 min. Author also showed that SDI mediated by hypocrellin B caused alteration in the membrane integrity of MRSA and reduced the growth of this bacterium. Costley et al., in 2017 [40], evaluated a Rose Bengal-antimicrobial peptide conjugate as a sonosensitizer against *S. aureus* and *Pseudomonas aeruginosa* suspensions, and authors achieved 5 and 7 log of reductions, respectively. In the study conducted by Wang et al. [41], in 2020, authors evaluated a novel Titanium dioxide (TiO_2) nano-composite as a sonosensitizer, and they found that this sensitizer has good hydroxyl radicals and singlet oxygen yields

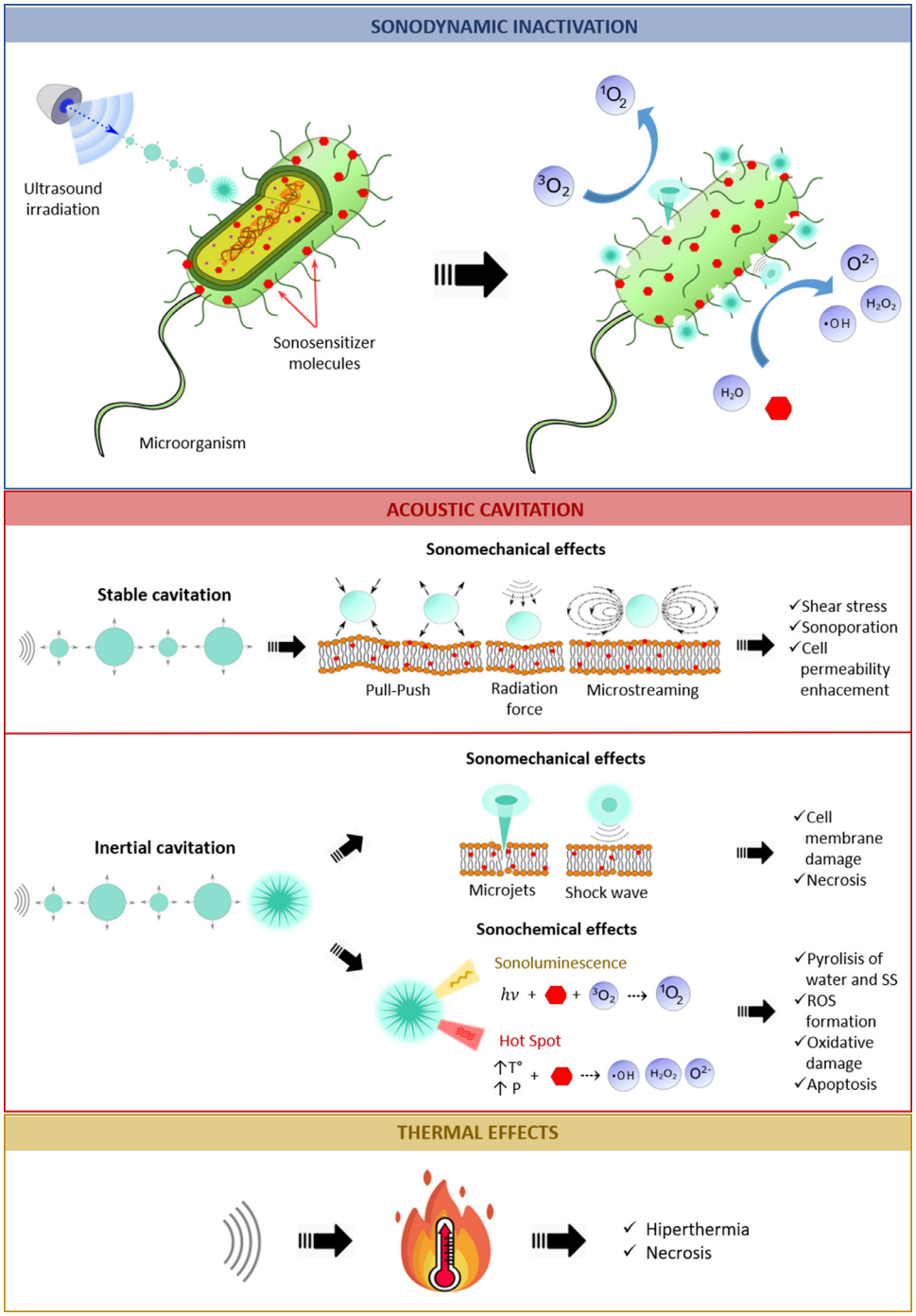

Fig. 3. Sonodynamic inactivation: sonochemical reactions induced by the Cavitation phenomenon and mechanical forces. Non-inertial cavitation (when a low-intensity ultrasound is applied) produces bubbles with high energy, which oscillate and are able to generate radiation force and microjet, and they are capable of interacting with cells, organelles and biomolecules. Inertial cavitation (when a high-intensity ultrasound is applied) increases the local temperature and pressure, there is the formation of free radicals, the appearance of strong shock waves and high-speed micro-jets in the media. This scenario is highly harmful to the target cells. Another phenomenon that is also linked to the production of ROS is Sonoluminescence, that is the light emission generated by the bubble collapse and it has been hypothesized to excite the sonosensitizer. Besides that, the increase in the temperature is also responsible for cell death.

when exposed to the ultrasound, and it showed great antibacterial effect, achieving 92.41% of *S. aureus* reduction. Zhang et al. [42] also evaluated SDI for antimicrobial proposes, in this investigation authors used hematoporphyrin monomethyl ether as a sonosensitizer in a range of concentrations (10, 20, 30 and 40 μg/mL) and evaluated different periods of US exposure (2, 4, 6, 8 and 10 min) for the inactivation of *Porphyromonas gingivalis* suspension. The combination of 3 W/cm^2 of US for 10 min and the sonosensitizer at 40 μg/mL was able to reduce 4.7 og of the bacteria and authors observed ROS production by SDI. In 2020, Maryam Pourhajibagher et al. [43] assessed the sonodynamic activation of a nanomicelle curcumin (Cur) in comparison with Cur for the inactivation of *S. mutans*. Authors showed that the nanomicelle Cur-mediated SDI exhibited lower cytotoxicity and apoptotic properties and higher uptake by the bacteria, increased ROS generation. Besides that, when SDI was mediated by the nanomicelle, greater reductions of the *S. mutans* in comparison with Cur was observed. A range of studies have been considered some strategies to improve SDI effectiveness, such as increasing cavitation, association with immunotherapy, hypoxia and light association. Wang et al. published a review listing some studies regarding this aspect [44]. Another strategy that has been evaluated is the use of nanoparticles in combination with SDI, and the reviews of Sun et al. [45] and Wang et al. [46] address this promising approach.

The use of ultrasound and a sensitizer for cancer therapy (SDT) has also been investigated and a comprehensive review in this field is the one published by Gong and Dai in 2021 [47], where authors approach the challenges and limitations of PDT, describe the development of US

equipment for SDT proposes and list the design of sonosensitizers. In this review authors are very optimistic about the clinical feasibility and efficacy of SDT.

Light and ultrasound: a good combination!

Based on these aspects and records, a more recent antimicrobial strategy that combines the advantages of PDI and SDI is called Sonophotodynamic Inactivation (SDPI), also known as antimicrobial Sonophotodynamic Therapy (aSPDT) or Sonophotodynamic Chemotherapy (SPDCT). SPDI is a very promising and interesting antimicrobial that synergies the SDI and PDI effects. It is hypothesized that a PS or SS class has the dual capability to be excited by both sources, light and ultrasound [48]. In the literature, it has been described organic and inorganic sensitizers that could mediate SPDI, and the revisions written by Sadanala et al. (2014) [49] and Zheng et al. (2020) [50]. Interestingly, a quick search on Scopus website (www.scopus.com), the world's largest abstract and citation database of peer-reviewed research literature, performed in April of 2021, the combination of words "photodynamic and microorganism or bacteria'' resulted in 3,039 documents. Additionally, the search for the words "sonodynamic and microorganism or bacteria" resulted in 44 documents, while the combination of words "photodynamic and sonodynamic and microorganisms or bacteria" came out with only 24 archives, showing that this approach is new and has not been fully investigated, as the same way as PDI and SDI. Table 2 lists some SPDI studies.

In the treatment of cancer, this modality is currently being evaluated and studies have proved that the sensitizers can be effectively excited by both sources (light and US), being the combined treatment (SPDT) more effective than the isolated therapies (PDT or SDT) [51]. Some reviews about this treatment for the oncology management can be cited and they address different aspects of the therapy. The paper published by Tzerkovsky et al. [52], in 2019, authors summarize in vitro and in vivo studies involving SDT and SPDT with a range of sensitizers (hematoporphyrin, 5-aminolevulinic acid, chlorin derivatives). From the selected papers revised, authors found that US has been used in the frequency of 1–3 MHz and intensities ranging from 0.7 to 5 W/cm^2, with or without the application of light, and it has been proven that these associations increase the cytotoxic effect of the sensitizer against malignant pathology of the mammary gland, stomach, esophagus, prostate, lung and brain. In the book chapter published in 2020 by Lopes de Mello et al. [53], authors approach the effects of the US in biological systems; the concepts, theories and mechanisms involved in each treatment (PDT, SDT and SPDT), and also describe studies performed in the oncology area. This chapter highlights the need for standardized studies evaluating SPDT to be able to compare the results obtained by SDT and PDT therapies and to conclude the real synergistic effect of light in combination with US. The reviews published by Yang et al. (2019) [54], McHale et al. (2016) [55] and Sadanala et al. (2014) [49] are very interesting papers that explain the concepts, advances and challenges of SPDT and describes the sensitizers that have been evaluated. Some studies evaluating SPDT can be cited, such as, McCaughan et al. (2011) [48], demonstrated that the excitation of the chlorins by light and ultrasound was more toxic to the cancer cells than SDT or PDT. Authors attribute this result to the improved ROS generation and higher sensitizer uptake caused by the transient pores in the cell membrane. In another study, Li et al. [56], observed that compared with SDT and PDT, SPDT increased the reduction of the viability, DNA damage, and inhibition of the clonogenicity of mammary cancer cells, using Chlorin e6 as PS. In the study conducted by Ponce et al. [57], authors evaluated the ability of light, US and light+US to excite and degrade protoporphyrin IX (PpIX). Authors concluded that the application of light+US improved the PpIX decay rate and had greater scope than light or US sources, suggesting that the use of US+light in the presence of the PS is a promising approach for cancer treatment. All these works demonstrated that the use of both sources to activate the sensitizer is more exciting and effective than the application of light or US only.

However, few investigations were performed for antimicrobial proposes. Table 2 contains some antimicrobial studies evaluating SPDI. Alves et al. [58], observed that SPDI mediated by Photodithazine or Rose Bengal was more effective for the inactivation of *Candida albicans* biofilm than the isolated application of PDI or SDI. In another study, Alves et al. [59] compared the effectiveness of PDI, SDI and SPDI mediated by Curcumin against *S. aureus* biofilm. Moreover, authors also evaluated strategies to improve these treatments, such as, the use of potassium iodide (KI) with Curcumin, the use of sodium dodecyl sulfate (SDS) in combination with the PS and a pre-treatment with ultrasound. It was demonstrated that SPDI was more effective than PDI and SDI. SDS achieved the most significant viability reductions, followed by KI and ultrasound pre-treatment. The work published by Niavarki et al. [60] aimed to compare PDI and the PDI associated with the US, both mediated by methylene blue (MB), to inactivate *Enterococcus faecalis* biofilms formed in root canals. Authors observed that the penetration depth of MB was higher when the US was applied in association with PDI. Also, the viability reduction of *E. faecalis* was more significant in the group US+PDI. Pourhajibagher and Bahador [61] proposed to study the effects of SPDI, SDI and PDI mediated by a Curcumin-decorated nanophytosomes on the viability, biofilm degradation, metabolism and pathogenicity of *Aggregatibacter actinomycetemcomitans*. The sensitizer was synthesized and characterized, and researchers used a blue laser and an ultrasound apparatus to perform the treatments. Pourhajibagher and Bahador showed that SPDI exhibited greater capacity to inactivate, to degrade the biofilm, to reduce the metabolic activity and the expression of genes related to virulence of the bacteria (qseB and qseC) in comparison with SDI and PDI. In another investigation, Maryam Pourhajibagher et al. [62] assessed the efficacy of PDI, SDI and SPDI, mediated by chitosan nanoparticles-indocyanine green (CNPs-ICG), in comparison with chlorhexidine (CHX) against bacteria biofilms responsible for periodontitis on the surfaces of titanium dental implants. Authors demonstrated that SPDI was more effective to reduce bacterial biofilm than SDI and PDI, being as effective as CHX.

. For this reason, these studies demonstrated that SPDI is more effective than the therapies alone. Besides that, as long as SPDI is more effective, it allows to reduce the time of treatment, to decrease the parameters of US, light and sensitizer concentration, turning this therapy safer for mammalian cells.

Conclusion and future perspectives

In conclusion, the need to search for alternative antimicrobial therapies that are not able to induce resistance in the microorganisms and, also, that are able to inactivate even the biofilm living form, is unquestionable. PDI is a promising approach and had revealed good results for antimicrobial proposes, however, this treatment has some limitations, such as light penetration, mainly when microorganisms are organized as biofilms, and also, decreased effectiveness against gram-negative bacteria, since the PS has limited access to enter in the cell. The use of ultrasound instead of light to excite the PS, called SDI, appeared to overcome the limitations of PDI, since the ultrasound waves exhibit greater penetration and reach deeper layers of the biofilms. Also, ultrasound has the ability to disrupt the biofilm through mechanical forces. However, for SDI to achieve great results, the use of the US in high intensity parameters could lead to tissue damage due to non-inertial cavitation. For this reason, the use of light in combination with the US is a promising alternative to overcome limitations of PDI and SDI, since the US exhibits excellent penetration ability and light is well known to activate the sensitizer. These aspects reinforce and encourage the use and evaluation of SPDI for microbial disease. Moreover, based on all studies that evaluated SPDI in comparison with SDI and PDI, the combined treatment was more effective, and it allows to reduce the time of treatment, to de-

Table 2
Original contribution in SPDI.

Authors	Microorganism	PS parameters	Light parameters	US parameters	Efficacy (Viability reduction, CFU/mL)
Alves et al. (2018) [58]	*Candida albicans*	Photodithazine 25,50, 100 mg/L (Planktonic) 175, 200 mg/L (Biofilm) 20 min Rose Bengal 1.5, 10 μM (Planktonic) 100, 200 μM (Biofilm) 30 min	LED 660 nm 25 J/cm^2 (Planktonic) 50, 113 J/cm^2 (Biofilm) White LED 25 J/cm^2 (Planktonic) 50, 113 J/cm^2 (Biofilm)	1MHz 2.5 W/cm^2 50 % 100 Hz 5 min	**Planktonic:** (SDI) 4.35 $\log_{10}$ using 25 mg/kg; eradication using 50 and 100 mg/kg. (PDI) 5.23 and 5.87 $\log_{10}$ using 25 and 50 mg/kg, respectively; eradication using 100 mg/kg. **Biofilm:** (PDI+SDI) 2.08 and 3.39 $\log_{10}$ using 175 and 200 mg/kg, respectively. **Planktonic:** (SDI) 5.01 $\log_{10}$ using 1uM; eradication using 5 and 10 μM, respectively. (PDI) 5.24 and 5.39 $\log_{10}$ using 1 and 5 μM, respectively; eradication using 10 μM. **Biofilm:** (PDI+SDI) 1.45 and 1.91 $\log_{10}$ using 100 and 200 μM, respectively.
Alves et al. (2021) [59]	*S. aureus*	Curcumin (Cur) 80μM 20 min	LED 460 nm 70 J/cm^2	1MHz 3 W/cm^2 20 % 100 Hz 32 min	**(Curcumin)** 1.67, 2.39 and 3.48 $\log_{10}$ for SDI, PDI and SPDI, respectively. **(Curcumin+SDS)** 2.6, 2.57 and 7.43 $\log_{10}$ for SDI, PDI and SPDI, respectively. **(Curcumin+KI)** 3.73, 4.04 and 5.48 $\log_{10}$ for SDI, PDI and SPDI, respectively.
Niavarzi et al. (2019) [60]	*E. faecalis*	Methylene blue 100 μg/mL 5 min	LED 660 nm 0.27 mW/ cm^2 16.2 J/cm^2	Various 970 Level 5 power E5 tip 20 s	**(Apical part)** 90.7 and 98.6% for PDI and PDI+US, respectively. MB penetration depth of 32.78 and 325.87 μm for PDI and PDI+US, respectively. **(Coronal part)** 56% and 98.3% for PDI and PDI+US. MB penetration depth of 54.99 and 456.84 μm for PDI and PDI+US, respectively.
Pourhajibagher and Bahador (2021) [61]	*A. actinomy cetemcomitans*	Curcumin-decorated nanophytosomes (Cur-NPhs)	Diode laser 450 nm 150 mW/ cm^2	1MHz 2 W/cm^2 100 Hz 5 min	**(Cur-NPhs)** 13.6 $\log_{10}$ for SPDI, degradation of 65% and reduction in metabolic activity of 89.6% using a Cur-NPhs concentration of 50×10^{-4} g/L.
Maryam Pourhajibagher et al. (2020) [62]	A.actinomycetemcomitans P.gingivalis P.intermedia	Chitosan Nanoparticles-Indocyanine green (CNPs-ICG)	Diode laser 810 nm 250 mW 31.2 J/cm^2	1MHz 1.56 W/cm^2 100 Hz 1 min	**(CNPs-ICG)** 8.8, 6.65 and 6.54 $\log_{10}$ for SPDI, SDI and PDI, respectively. **(ICG)** 7.12, 5.63 and 5.29 $\log_{10}$ for SPDI, SDI and PDI, respectively.

crease the parameters of US, light and sensitizer concentration, turning this therapy safer for mammalian cells.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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