

Chemical and Biological Systems of
the Activity Oxygen Singlete in the
Destruction of Microbial Cells in
Photodynamic Therapy

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Abstract

Singlet oxygen (1O_2) is an excited form of molecular oxygen that acts as an active agent in antimicrobial Photodynamic Therapy (PDT), a promising technique for the treatment of several types of infections. In this review, we present a summary of the physical-chemical and biological aspects of 1O_2 and the lethal photosensitization mechanisms mediated by it in PDT. The chemical structure of H_2O will be approached through molecular orbital theory. PDT techniques that enhance the formation of this radical using different photosensitizing dyes will also be introduced as well as biological targets of 1O_2 in microbial cells and the obstacles encountered in such structures. This knowledge is crucial to design new research methodologies that increase the chain of reaction of 1O_2 in PDT, consequently, increase the effectiveness of the therapy.

Introduction

Antimicrobial Photodynamic Therapy (PDT) on which the concept of non-toxic death is based is known as lethal photosensitization [1], where a previous impregnation of the microbial cells with the photosensitizing dye is necessary for subsequent exposure to a particular light source [2].

In PDT the elimination of microorganisms is related to the activation of the dye deposited in the target organism that after sensitization transforms molecular oxygen into 1O_2 that is cytotoxic [2,3]. During this process, photosensitive cellular components pass into an excited state when exposed to a complementary wavelength light that is characterized by the passage of the electrons to higher energy levels. In this excited state, the photosensitizer can interact with molecular oxygen by initiating the formation of highly reactive 1O_2 (Photoprocessing Type II) or interacting with other molecules, such as electron acceptors, resulting in the production of hydroxyls and other organic radicals (Type I photoprocess), which are toxic to the cell [4]. The products generated in these reactions can promote diverse damages in different components of the cell of the microorganism or change the metabolic activities of irreversible way resulting in the microbial death. In general, the process causes damage to the oxidative pathway, plasma membrane and genetic material of the microbial cell, but they are not toxic to host cells [5].

Oxygen accounts for almost half of the mass of the Earth's crust, two-thirds of the mass of the human body and nine-tenths of the mass of water. Large amounts of oxygen can be extracted by liquefaction through a process known as fractional distillation. Oxygen can also be produced by electrolysis of water or heating of potassium chloride ($KClO_3$). Almost all living organisms use oxygen for a generation of energy and for breathing [6].

Molecular oxygen has a fundamental triplet ($^3S_g^-$), that is, its occupied high-energy electronic level has two degenerate orbitals p^* (different orbitals with the same energy) occupied by two electrons, each of which occupies an orbital p^* [2]. They are with parallel spin, constituting, thus, a bi-radical, denoted by 3O_2 . This feature gives the oxygen a high reactivity, however its direct reduction by two electrons with antiparallel spins is prohibited by the rule of conservation of spin, making it relatively inert [5,6].

Hundreds of photochemical reactions involving oxygen have been studied in the laboratory, among them the photosensitization reaction that converts molecular oxygen into its singlet state (1O_2), excited and more reactive form of molecular oxygen. The excitation of O_2 to 1O_2 can occur when certain pigments absorb photons, entering a state of electronic excitation with energy transfer to O_2 , resulting in excited or reactive forms [7].

Studies involving 1O_2 have great biological relevance, since this species, due to the electrophilic character, reacts with molecules rich in electrons like proteins, lipids and DNA causing damages that result in loss of function and integrity of the cells [5].

Oxygen and molecular orbital theory

One of the explanations for $^1\text{O}_2$ reactivity is based on molecular orbital theory. The atom can be represented by allocating its respective electrons in the atomic orbitals using the Aufbau principle, which consists of first filling the lower energy orbitals with each orbital being able to contain up to two electrons, as long as they have opposing spins. In addition, Hund's rule states that if we have several orbits of the same energy (that is, degenerate), the electrons will be distributed so as to result in the largest possible number of unpaired spins [8].

The oxygen element has six electrons in the last layer and eight electrons in total. The O_2 molecule has 16 electrons. Its distribution in the molecular orbital is: $\sigma_{1s}^2, \sigma_{1s}^*, \sigma_{2s}^2, \sigma_{2s}^*, \sigma_{2p_x}^2, \pi_{2p_y}^2, \pi_{2p_z}^2, \pi_{2p_y}^1, \pi_{2p_z}^1$ [9]. As can be seen, molecular oxygen has two unpaired electrons in the degenerate molecular orbitals $\pi_{2p_y}^1, \pi_{2p_z}^1$, characterizing the triplet state ($^3\Sigma_g^-$) (Figure 1). These electrons tend to have the same spin and this fact restricts the reactivity of the molecule since the direct reduction of oxygen by two electrons is prohibited by the rule of conservation of spin [9].

The $^1\text{O}_2$ can be generated, in this context, by adding energy. In this state, the spin restriction is removed by moving one of the unpaired electrons, which undergoes a spin reversal. Thus, $^1\text{O}_2$ is much more oxidizing than molecular oxygen in its ground state [5].

There are two forms of $^1\text{O}_2$: a) one with two electrons occupying the same orbital, which is considered the first excited state ($^1\Delta_g^-$), has energy of 22.5 kcal above ground state and half-life in aqueous solvent of approximately 10^{-6} s. b) and a second excited state ($^1\Sigma_g^+$), with two electrons occupying different orbitals [4].

This form has energy of 37.5 kcal above the ground state being considered very unstable and with a very short life time (10^{-11} s) in aqueous medium and before reacting with other molecules, it becomes the first excited state, more stable (Table 1). Thus, the state $^1\Delta_g^-$ shows greater biological interest [5].

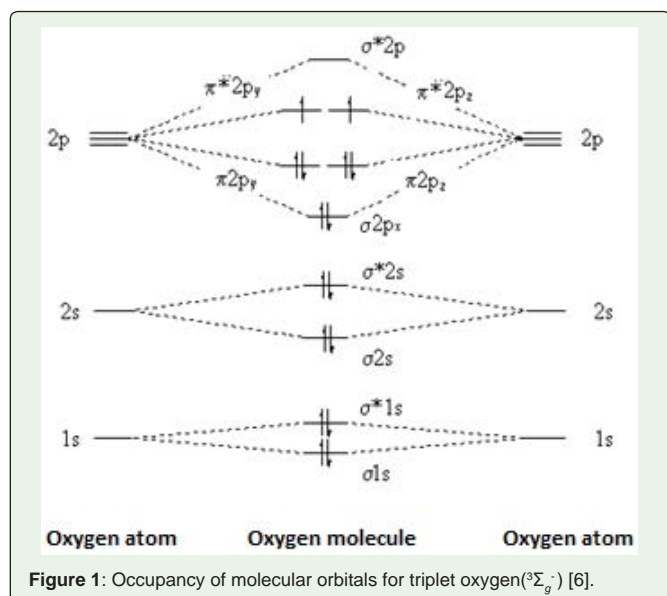


Figure 1: Occupancy of molecular orbitals for triplet oxygen($^3\Sigma_g^-$) [6].

Photo-sensitization reaction

Photodynamic therapy is a well-studied photochemical reaction for applicability in the health area. In these reactions molecules known as photosensitizers are used which are dyes previously used in the impregnation of cells. These are irradiated in the ultraviolet light spectrum or visible light at certain wavelengths and after absorbing photons, the photosensitizer molecule passes from the ground state S_0 to the singlet excited state S_1 . This state may decay to the ground state, emitting fluorescence or no light emission by interconversion and cross-over inter-systems [8]. Through the inter-system crossing process spontaneous inversion of the spin of the excited electron occurs. The photosensitizer molecule can pass from the S_1 state to the triplet excited state (T_1) characterized by the much longer life span than the S_1 state [10].

Once the T_1 is formed, this state can participate in various reactions can decay to the S_0 state with emission of phosphorescence, or react by photochemical mechanisms of type I or II. The excited molecule, therefore, can transfer its energy to other molecules or begin the direct reaction of its excited state. These processes are responsible for their photoactivity and phototherapeutic effects [10].

Photodynamic therapy is based on type I and II mechanisms. In the type I mechanism, the photosensitizing dye (S_0) in the S_1 state (singlet excited state) or T_1 (excited triplet state, generated by a spin inversion mechanism, called intersystem crossing) can, by oxidation and reduction reactions with different compounds organic (RH) is photo-reduced to radical anion, which by transferring an electron to the oxygen molecule, generates reactive species such as peroxides (ROO^\cdot), superoxide anion ($\text{O}_2^\cdot-$), hydroxyl radical anion (OH^\cdot), causing the destruction of the membrane or of macromolecules constituting the microbial cells. Finally the photosensitizer returns to its ground state [4].

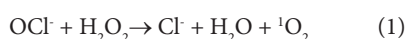
In type II mechanism the molecule in the T_1 state transfers its energy to the oxygen molecule whose fundamental state is triplet ($^3\text{O}_2$), finally forming its singlet excited state ($^1\text{O}_2$). Singlet oxygen is the intermediate factor in the photodynamic process, being the main responsible for the inactivation of the cell. $^1\text{O}_2$ can induce various chain reactions with molecular components of the cell, such as: DNA, proteins, cell membrane phospholipids, mitochondria and lysosomes, resulting in the death of the microbial cell [11]. These processes can occur simultaneously and the importance of each of them depends on the target molecule, the energy transfer efficiency from the sensitizer to the O_2 , the solvent and the concentration of O_2 . However, in photodynamic therapies depending on the type of photosensitizer, the occurrence of the type II mechanism may reach 90% [4] (Figure 2).

Table 1: Two electrons occupying the same orbital [5].

State	Orbital π^*	Energy (kcal. mol ⁻¹)	Lifetime (s)
$^3\Sigma_g^-$	$[\uparrow\downarrow]\pi_{2p_y}, [\uparrow\downarrow]\pi_{2p_z}$	0	
$^1\Delta_g^-$	$[\uparrow\downarrow]\pi_{2p_y}, [\uparrow\downarrow]\pi_{2p_z}$	22.5	10^{-6} s
$^1\Delta_g^-$	$[\uparrow\downarrow]\pi_{2p_y}, [\uparrow\downarrow]\pi_{2p_z}$	22.5	10^{-6} s
$^1\Sigma_g^+$	$[\uparrow\downarrow]\pi_{2p_y}, [\uparrow\downarrow]\pi_{2p_z}$	37.5	10^{-11} s

Measurement and detection techniques for singlet oxygen

The $^1\text{O}_2$ generated in the $^1\Delta_g$ state, because it is an electronically excited species, decays to the ground state by emitting light [10]. This emission can be measured by luminescence instruments, allowing the detection of different wavelengths emitted. The intensity of this emission is directly proportional to the concentration of $^1\text{O}_2$ and therefore provides a direct measure of the quantity produced [5,6]. This is exemplified in the hydrogen peroxide (H_2O_2) decomposition reaction in the presence of hypochlorite (OCl^-), emitting wavelengths in the range of 634 and 703 nm. This is due to the decay to the state $^3\Sigma_g^-$ of $^1\text{O}_2$ generated in the reaction (Equation 1) [5].



Some studies have shown to be efficient in the construction of new detection techniques. Hananya et al analyzed a chemiluminescence probe highly selective and sensitive to $^1\text{O}_2$ in living cells. The mechanism is based on the chemical reaction of the probe with $^1\text{O}_2$ to form dioxetane which spontaneously decomposes under physiological conditions, emitting green light of high intensity. The technique allowed to evaluate the production of intracellular $^1\text{O}_2$ produced by photosensitizers in PDT [12]. Yin et al developed an excitable luminescent probe of visible light using ruthenium dinuclear complex. The technique allowed the detection of $^1\text{O}_2$ with high sensitivity in alkaline and neutral media with potential of application in biological systems [13].

The use of $^1\text{O}_2$ detection techniques is extremely useful in research for the discovery of new FS more efficient in the process of PDT microbial cell death [13]. Mamone et al correlated the phototoxicity of plant extracts to prokaryotic or eukaryotic cells with the ability to produce $^1\text{O}_2$. The production of $^1\text{O}_2$ by the lighted extracts was measured indirectly by the fluorescence of the green singlet oxygen sensor, which proved to be highly sensitive to this reactive form, evidencing potential new photosensitizers from plant extract [14].

Biological targets of singlet oxygen

Oxygen in the singlet state is an electrophilic species capable of reacting with various biomolecules present in microorganisms. Preferred targets are those rich in electrons such as amines, sulfides, phenols, thiols, dienes, conjugates and amino acids. Some chemical reactions of $^1\text{O}_2$ can be highlighted: addition to conjugated dienes (Diels-Alder type cycloaddition, 2 + 4) usually resulting in the formation of endoperoxides; addition of double bond, forming "ene"

hydroperoxides and, with alkenes substituted by groups containing nitrogen or sulfur atoms, forming 1,2-dioxetanes. The $^1\text{O}_2$ can also react with phenolic compounds to form hydroperoxidenones and with sulphides, forming sulfoxides [5].

Biomolecules that tend to be damaged by $^1\text{O}_2$ are DNA, lipids and proteins, and DNA is the most studied due to genotoxic, mutagenic and carcinogenic effects [15]. More than 20 different types of DNA base damage were identified after exposure of this biomolecule to the various forms of oxidative stress. Among the nitrogen bases, guanine exhibits the lowest ionization potential and has been the preferred choice of studies of purine oxidation reactions, since there are efficient methodologies for their detection. $^1\text{O}_2$ is able to react significantly with guanine at neutral pH and is widely used in studies involving purine oxidation (Figure 3) [12]. The reactivity of nucleotides of DNA and RNA with $^1\text{O}_2$ in organic solvents decreases in the following order: guanine >> cytosine >> adenine >> uracil >> thymine [18].

It is estimated that more than 60% of $^1\text{O}_2$ generated in the biological system are involved in reactions with proteins, and the amino acids tryptophan, histidine, tyrosine, methionine and cysteine are the ones with the highest susceptibility (Table 2) [17]. Some products of the reaction of these amino acids present intermediates containing peroxide groups, which in the presence of metals can generate other more reactive species [15].

In addition to damage to DNA and proteins, there is also the process of lipid peroxidation. This can occur in biological membranes such as those of microorganisms when they are subjected to the action of free radical. These induce the formation of a radical in the lipid chain, which in turn initiates an auto catalytic process generating, as a primary product, lipid hydroperoxides (LOOH), among them fatty acid hydroperoxides, phospholipids (PLOOH) and cholesterol (CHOOH) [19].

LOOH are relatively stable but may participate in secondary reactions that inhibit or intensify their deleterious effects. When they are not reduced by antioxidant compounds, they can participate in reactions that promote the formation of reactive radicals such as peroxy radicals. These radicals can abstraction hydrogen from another lipid, undergo β -scission, generate cyclic peroxides and / or react with other peroxy radicals generating excited species, including $^1\text{O}_2$ [15,18,19]. Therefore, a single reaction of $^1\text{O}_2$ may have global effects, such as initiating lipid peroxidation and subsequent chain reaction of radicals, but in this case probably not due to lethal DNA damage, since $^1\text{O}_2$ does not react so easily with DNA in bacteria [20].

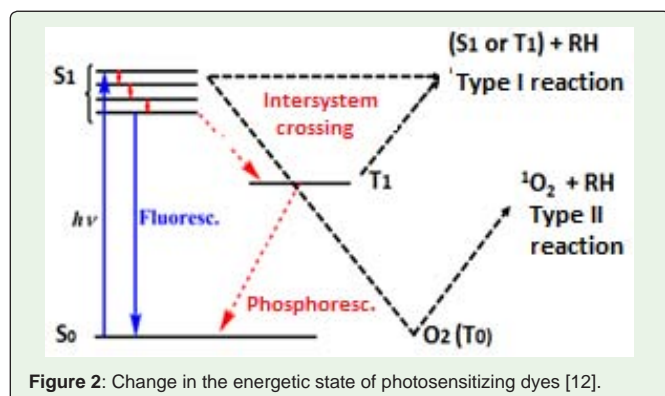
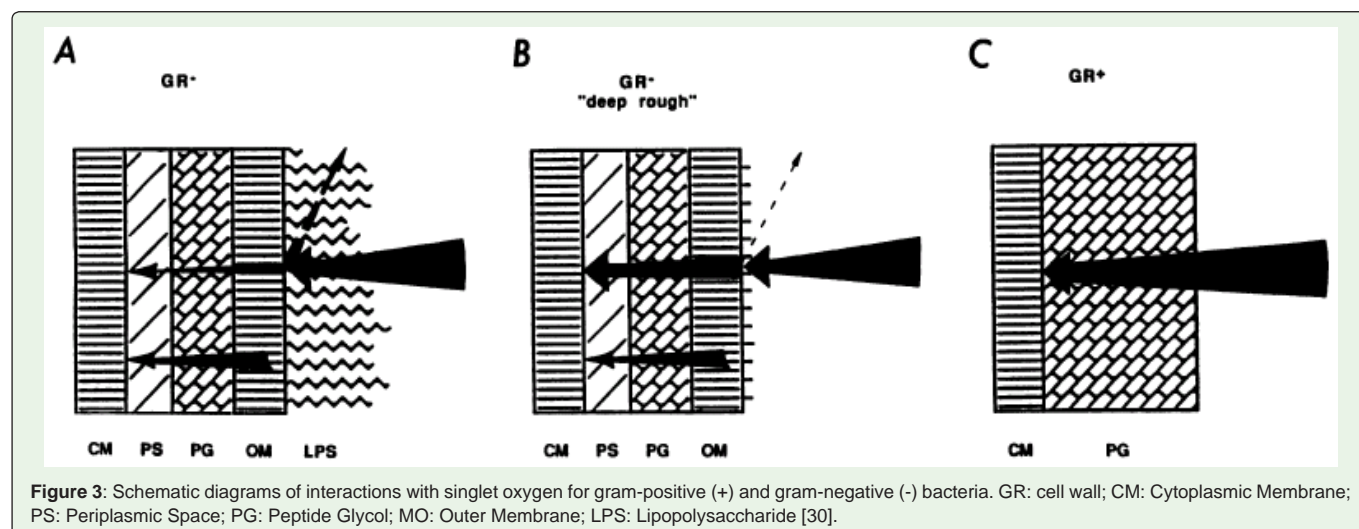


Figure 2: Change in the energetic state of photosensitizing dyes [12].

Table 2: Reaction constant data with $^1\text{O}_2$ (kr) of amino acid side chains at pH 7.

Amino Acid	$k_r (10^6 \text{ M}^{-1} \text{ s}^{-1})$
Triptofano	30
Histidina	20-70 ^a
Tirosina	8.0
Cisteína	8.9
Metionina	16

^aPhysical suppression (k_p). ^bDepends on pH, sendo K_a . $100 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in $\text{pH} > 8$ e $5 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at low pH [19].



Actions of singlet oxygen on microorganisms by means of photodynamic therapy

Photodynamic Therapy (PDT) has been widely used in the treatment of various types of cancers [21]. Compared with other cytotoxic therapies, PDT has the advantage of dual selectivity: not only the photosensitizer (FS) can be targeted to the cells or diseased tissue, but also the light can be accurately focused at the site of the lesion [21,22]. The procedure may be repeated several times if necessary, as there are no cumulative toxic effects and is usually non-invasive. In addition to the application in oncology, PDT also acts against several pathogenic microorganisms such as bacteria, fungi, yeasts and viruses that can be killed by visible light after treatment with an appropriate FS and light, in a process called Photodynamic Inactivation (PDI) or Chemotherapy Photodynamic Antimicrobial (PACT) [22].

The existence of antibiotic resistance among pathogenic bacteria is imminent. Studies to find an alternative antimicrobial therapy to which bacteria will not be able to develop resistance have been developed. Photodynamic therapy, mainly due to the production of $^1\text{O}_2$, has reduced or even eliminated this natural microbial resistance, as it happens with gram-negative bacteria resistant to penicillin. Therefore, a strain can be resistant to one or several classes of agents anti-bacterial, but if the photosensitizer is captured by it, there is the eradication of these microorganisms [23].

The bactericidal action of this new therapeutic option has been evidenced in different microorganisms such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Hemophilus influenzae*, *Escherichia coli*, *Candida albicans*, *Pseudomonas aeruginosa*, *Fusobacterium nucleatum* and *Streptococcus sanguis*, presenting superior results in black pigmented bacteria, because they present natural chromophores [24].

The most commonly used photosensitizers for PDT are halogenated xanthenes such as Bengal Rose, phenothiazines such as toluidine blue O and methylene blue (AM), acridines, chlorine conjugates and malachite green [22].

Chlorine is a derivative form of chlorophyll which has two important properties: a higher quantum yield of $^1\text{O}_2$ and an intense absorption band at wavelengths greater (650-660 nm) than the porphyrins (610-630 nm), where the biological tissues are more transparent to light. Other photosensitizing dyes with a high yield of $^1\text{O}_2$ are the phthalocyanines, being higher than that of a standard photosensitizer such as methylene blue. (Its central atom may be strongly related to the production of this type of oxygen). The Bengal Rose can act through irradiation at 532 nm with predominantly type II reaction, generating 80% of $^1\text{O}_2$ and the remaining 20% of superoxide anion [22-24].

Some FS are widely used in PDT due to their low toxicity characteristics in the absence of light in mammalian cells and selectivity to the target tissue such as porphyrins, chlorins, bacteriochlorins, and phthalocyanines [5]. Some studies have shown satisfactory results with the use of porphyrins in the microbial eradication in PDT with high production of $^1\text{O}_2$ [25]. Battistot al performed this analysis on *Helicobacter pylori* which proved to be a suitable target for PDT, since it spontaneously produces and accumulates porphyrins. The procedure was performed using the sialporphyrin as the FS in planktonic and biofilm cultures of *Staphylococcus aureus* [25]. The results showed significant bactericidal activity with more than 90% of effectively dead bacteria. Pucelik emphasizes the importance of the synthesis of new FS based on porphyrin and drugs as a vehicle for these dyes [26]. The results showed that the less polar halogenated and sulfonamide porphyrins were more easily absorbed by cells compared to hydrophilic and anionic porphyrins and, when incorporated into polymeric micelles, PDT effectiveness was significantly higher [26].

In general, the chain of chemical reactions catalyzed by $^1\text{O}_2$ causes damage to the oxidative pathway, plasma membrane and genetic material of the microbial cell, but are not toxic to host cells [5]. This is due to the more complex mechanisms of protection against oxidative stress in eukaryotic cells. These organisms have developed an endogenous, enzymatic, non-enzymatic system that would prevent or correct the oxidative damage that already occurs due to the natural process of aerobic respiration [27].

The antioxidant action can both prevent the formation of free radicals and, in their presence, the antioxidants are able to intercept them, preventing the attack on the lipids, amino acids of the proteins and DNA, avoiding lesions and loss of cellular integrity. Another way would be the production of enzymes responsible for removing damage to the genetic material of the cell or detoxifying the elements that may cause this lesion [24-29]. In this context, the diet also exerts an important non-enzymatic antioxidant system. The use of nutrients, especially those found in foods, can act in synergism in the protection of human cells and tissues [8,29].

The major targets of $^1\text{O}_2$ in mammalian cells are lysosomes, mitochondria and plasma membranes, while in microbial cells the damage to the outer membrane plays an important role, preventing DNA damage. The bacterial inactivation of Gram-positive (+) presents more expressive results than the gram-negative species (-), since these are significantly more resistant to many FS commonly used in PDT as well as the action of $^1\text{O}_2$. This can be explained by the cellular envelope of the gram-negatives that present as a complex outer membrane with lipid bilayers that function as a physical and functional barrier between the cell and its environment. This membrane, however, may form a chemical trap, since this layer is rich in established fatty acids and proteins that can react with $^1\text{O}_2$. The outer membrane and the lipopolysaccharide of gram-negative bacteria do not, however, allow a lethal action of $^1\text{O}_2$, since these components can be removed without killing the cells [28,29].

In addition to bacteria, fungi, such as *Candida albicans*, are even more resistant to PDT due to the presence of a nuclear membrane that may represent an additional barrier to photosensitizer penetration [24].

New methodologies in nanotechnology have been extensively studied. The development of different nanostructured carriers for FS allows a greater efficiency of PDT by itemizing chemical penetration of the dye and improving the permeability of the external bacterial membrane to it. This strategy may dribble the different barriers to the formation of $^1\text{O}_2$ in gram-negative and fungi [23,25].

A microemulsion study for toluidine Blue significantly reduced the growth of *Pseudomonas aeruginosa* with FS concentration and light intensity much lower than previous works that did not use nanostructured systems [20]. A study of lipid nanoparticle system development for PDT application against planktonic bacteria and biofilms showed a larger bacterial reduction than PDT with FS in aqueous solution, with a higher amount of $^1\text{O}_2$ [26] (Figure 3).

Figure 3A shows the action of singlet oxygen on gram-negative bacteria (GR-). The $^1\text{O}_2$ can reach the cell (larger arrow) and be deflected (dotted arrow) or penetrate the outside of the membrane barrier. A portion of the penetrating $^1\text{O}_2$ will reach the cytoplasmic membrane (thin arrow), while another part may react with unsaturated fatty acids and proteins in the outer membrane. $^1\text{O}_2$ reaction products with outer membrane components may be capable of causing cell death (lower arrow). Alterations in permeability resulting from the reaction of $^1\text{O}_2$ with the outer membrane may also increase its penetration into the cytoplasmic membrane [29].

Figure 3B exemplifies $^1\text{O}_2$ acting on gram negative, however, in this figure; the lipopolysaccharides are shown reduced to a few sugar residues, generating a greater permeability of the outer membrane.

Thus, there is a reduction in physical barrier capacity, as shown by small deviated arrows (dotted arrow) and greater penetration of singlet oxygen [21-29]. In turn, Figure 3C schematizes $^1\text{O}_2$ acting on gram-positive. The $^1\text{O}_2$ reaches the cytoplasmic membrane without restrictions, crossing the cell wall of peptidoglycan. With the lack of outer membrane in these bacteria, the byproducts would be formed at a lower rate in Gram-positive than in Gram-negative. The rate of cell death, therefore, depends only on the direction of $^1\text{O}_2$ to vital targets for the bacterium, without any need to invoke secondary reactions such as in the Gram-negative outer membrane [29].

In addition to the external barrier and the reactions shown above, microorganisms can also avoid the lethal action of $^1\text{O}_2$ by other mechanisms, such as the presence of defense components against oxidative stress. Some of them is the presence of carotenoids and glutathione. They are able to protect bacteria against the lethal effect of photosensitization, either by endogenous or exogenous photosensitizers. Glutathione is present in many organisms ranging from bacteria to humans and can be generally antimutagenic and protective agent. One of the functions of glutathione is to decontaminate intermediate electrophilic reagents such as peroxides and free radicals. It contains an amino acid, cysteine, which has been reported to react with $^1\text{O}_2$. Glutathione can be considered a potent protective agent against the death of bacteria by $^1\text{O}_2$ [29].

Conclusion

Singlet oxygen has shown an excited form of molecular oxygen with high reactivity, and can be used in several therapies both preventing the advancement of various types of tumors and also eradicating microorganisms that cause important pathologies, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Hemophilus influenzae*, *Escherichia coli*, *Candida albicans*, *Pseudomonas aeruginosa*, *Fusobacterium nucleatum* and *Streptococcus sanguis*.

$^1\text{O}_2$ is produced in amounts sufficient to control various types of infections without causing damage to host tissue through PDT. Some dyes have been shown to be very effective and with a high production of $^1\text{O}_2$ by type II mechanism, such as phthalocyanines and rose bengal [24].

Increasingly advanced studies and methods of detection of $^1\text{O}_2$ allow the investigation of new photosensitizing dyes for PDT with high production of $^1\text{O}_2$ safely for the patient being treated. In addition, the biological targets of this excited form of molecular oxygen are vital for the microorganism, ensuring less resistance to treatment as occurs with antibiotic therapy. The chemical reaction that occurs with different microorganisms shows that the action of $^1\text{O}_2$ is different for each species. Gram-negative bacteria are more resistant than Gram-positive bacteria and fungi also present an additional physical barrier to be broken [27,29].

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