Nanostructured Carriers for Photodynamic Therapy Applications in microbiology

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The antimicrobial activity of Photodynamic Therapy (PDT) was first described in the beginning of the last century. In an unintentional experiment, an accidental contamination of a paramecium culture with acridine orange solution and subsequent light exposure, induced the death of this microbe culture. After that, subsequent experiments showed that some dyes, such as acridine orange, phenothiazines and phthalocyanines derivatives, when exposed to light, induce the production of a cascade of reactive oxygen species (ROS) that are responsible for the cytotoxicity of the therapy. Since then, several dyes, named photosensitized (PS), were described for PDT application in different clinical fields, such as treatments of oral and skin infections. One of the most important advantages in antimicrobial PDT described in the literature is the low risk of bacterial resistance after the therapy. This feature is due to the fact that ROS generated during PDT attack bacterial cells in nonspecific sites, avoiding the generation of PDT-resistant strains. Unlike that, the mechanism of action of the traditional antibiotics usually affects the activity/function of certain specific biomolecules, facilitating the development of resistant strains. Despite the low risk of selecting ROS-resistant bacteria strains, some authors reported that some PS are substrates for multidrug efflux pumps in bacteria. This information support the hypothesis that some strains could develop certain mechanisms for pumping out the PS molecules before the ROS generation. The most common PS described in the literature are the phenothiazine derivatives, such as toluidine and methylene blue and the new generation PS, the phthalocyanines and chlorin derivatives. For a specific substrate to be susceptible to the pump efflux it must be within a limited range of molecular sizes and some PS monomers or dimmers are especially susceptible to this resistance mechanism. One possible alternative to overcome this situation is to associate the PS molecules with larger molecular complexes, such as nanoparticles. Nanoparticles are not only able to improve the photophysical properties of the PSs, as discussed along the text, but they also may be designed to work as PS drug carriers that are kept attached to the bacterial surface. In addition, the nanoparticulated complex can be chemically adapted to increase the PS delivery specifically to bacterial cells instead of eukaryotic cells. In this chapter, we will present some examples of nanoparticulated PS carriers for antibacterial PDT application.

1. Introduction

Photodynamic Therapy (PDT) is suitable for the treatment of microbial infections and neoplastic tissues. The first reports of PDT were presented in the beginnings of 20th century, when, in an unintentional experiment, the accidental contamination of a paramecium culture with acridine orange solution and subsequent light exposure induced the cell death of this protozoan culture. After that, subsequent experiments showed that some dyes, such as acridine orange, phenothiazines and phthalocyanines derivatives, when combined with light exposure, induce the production of a cascade of reactive oxygen species (ROS) that promote the cytotoxicity effects of PDT [1].

The principle of PDT is the photodynamic process induced by the irradiation of certain photosensitive drugs, called photosensitizers (PS), with light at specific wavelengths, producing a series of reactive species, the actual effectors of the antimicrobial activity related to the therapy [2, 3]. During the PS photo-activation, the drug excitable electron is promoted to a higher energy level and the PS molecule reaches a higher energy level, known as the first excited singlet state. This singlet state can return to its ground state by emitting fluorescence or, by intersystem crossing, it can be converted into a long-lived triplet state. This long half-life state enables the triplet-PS to chemically interact with molecules in the surroundings [4].

To return to its ground state, the triplet-PS can transfer energy directly to the biomolecules present in the environment or, alternatively, it transfers energy to triplet oxygen, producing manly singlet oxygen, a reactive oxygen species (ROS) about 1000-fold more oxidant than triplet oxygen. Both triplet-PS and singlet oxygen are unstable molecules and for this reason they are responsible, in biological tissues, for the damage to biomolecules after PS irradiation. The generalized degradation of microbial biomolecules finally leads to the microbial cell death. Didactically, the direct reaction between triplet PS and biomolecules is called Type I reaction and the production of singlet oxygen by triplet PS is called Type II reaction [2, 5].

It is worth noting that the reactive species produced during PDT react non-specifically with several biomolecules (lipids, nucleic acids and proteins) only near to the site of photo-activation. In fact, as a result of their high reactivity, ROS generated during PDT have a very short half-life (in the microseconds range) and can then diffuse only through short distances [6, 7]. Therefore, the molecular damage is restricted to the sites where the PS is localized prior to photoactivation. Moreover, as the ROS attack may occur at several different sites within a molecule, the biomolecules are non-specifically damaged, a feature that difficult the generation of mutant PDT-resistant microbial strains. Unlike
PDT, the classical antibiotics generally targets specific molecular pathways, fact that could facilitate the development of resistant strains [8].

### 2. Photosensitizers available for Antimicrobial Photodynamic Therapy

Despite the early reports on the first successful experiments involving PDT described in the introduction section, surprisingly this therapy was not used for clinical applications till 20 years ago. Nowadays, PDT is a well established and approved therapy in several countries and there are many companies worldwide producing different photosensitizers formulations for antimicrobial purposes (Table 1).

As shown in Table 1, the most common available photosensitizers for clinical application nowadays are the phenothiazine derivatives, represented mainly by methylene blue and toluidine blue. A brief review in the literature will also confirm that these PS are the most studied in the context of antimicrobial PDT. Despite the antimicrobial effectiveness, the safety, and the good clinical results of these phenothiazine derivatives [9, 10], the lower capacity for ROS generation compared to others PS molecules, induced the development of others PS, such as phthalocyanines, chlorines and fullerenes derivatives, called new generation PS. In comparison to the phenothiazine derivatives, these new PS are activated by light at longer wavelengths and have an increased capacity for ROS generation, in special the singlet oxygen specie.

**Table 1**: Some antimicrobial photosensitizers formulations available on the market worldwide.

<table>
<thead>
<tr>
<th>Company</th>
<th>Country</th>
<th>Website</th>
<th>Principal Infections Treated</th>
<th>Principal Photosensitizer Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periowave</td>
<td>Canadá</td>
<td><a href="http://www.periowave.com">www.periowave.com</a></td>
<td>Gum Disease</td>
<td>Phenothiazine Derivatives</td>
</tr>
<tr>
<td>Helbo</td>
<td>German/UE</td>
<td><a href="http://www.helbo.de">www.helbo.de</a></td>
<td>Dental Infections</td>
<td>Phenothiazine Derivatives</td>
</tr>
<tr>
<td>Aptivalux</td>
<td>Brazil</td>
<td><a href="http://www.aptivalux.com.br">www.aptivalux.com.br</a></td>
<td>Dental Infections</td>
<td>Methylene Blue</td>
</tr>
<tr>
<td>DenFotex</td>
<td>United Kingdow</td>
<td><a href="http://www.denfotex.com">www.denfotex.com</a></td>
<td>Dental Infections</td>
<td>Phenothiazine Derivatives</td>
</tr>
<tr>
<td>Photopharmica</td>
<td>United Kingdow</td>
<td><a href="http://www.photopharmica.com">www.photopharmica.com</a></td>
<td>Infected Leg Ulcers</td>
<td>Phenothiazine Derivatives</td>
</tr>
</tbody>
</table>

As figure 1A shows, the light absorption peak of a phthalocyanine derivative (aluminium-phthalocyanine chloride, AlClPc) is located at longer wavelengths compared to the methylene blue absorption peak. This is an interesting feature of this new generation-PS since light at longer wavelengths has an increased potential to penetrate in biological tissues, allowing the treatment of deeper infections with PDT.

**Figure 1**: In section A, light absorption peak of two photosensitizers solutions: Methylene Blue (78mM) and Aluminum Phthalocyanine Chloride (5µM). In section B, we present the singlet oxygen generation of these two solutions after different laser fluencies irradiations.
Biological tissues strongly absorb electromagnetic waves in the range of red to near-infrared in the electromagnetic spectrum, i.e., between 600-1200 nm. Shorter wavelengths (<600nm) are absorbed by biological molecules, such as hemoglobin/myoglobin, and the wavelengths above this range (>1200nm) are intensely absorbed by water [11, 12]. Inside this wavelength window, the longer wavelengths can penetrate deeply inside the biological tissues. Therefore, PSs absorbing light at longer wavelengths may be photo-activated in deeper tissue layers and promote a more effective photo-activation in these regions. On the other hand, PSs that are activated by shorter wavelengths may produce the photodynamic effects only in very superficial tissue layers.

In addition to these features, new generation PS, such as phthalocyanines derivatives are more effective in the generation of singlet oxygen after light exposure, compared to the traditional phenothiazine derivatives. In Figure 1B we presented the result of an experiment were oxygen single generation (\(^{1}O_2\)) is monitored by the extinction of the benzofurane (BF) absorbance at 410 nm. This experiment is based on the \(^{1}O_2\)-mediated degradation of BF. In other words, a decrease in BF absorbance occurs when \(^{1}O_2\) is generated. As observed in Figure 1B, the phthalocyanine derivative generates more singlet oxygen when irradiated by laser at its specific wavelength compared to methylene blue, even in a situation that the molar concentration of the methylene blue (78 mM) molecule is higher compared to the phthalocyanine derivate (5 µM).

However, despite these positive photophysical properties related to the new PS generation, such as phthalocyanine derivatives, most of these drugs are hydrophobic compounds, and tend to form inactive aggregated complexes in aqueous media. This feature impairs the use of such drugs in the aqueous biological media [13, 14].

To overcome this drawback, some authors have proposed some chemical modifications in the PS molecules in order to increase the PS hydrophilicity and allow their use in aqueous media. However, despite the improvements in PS solubility in physiological solutions, the low hydrophobicity of these new molecules drastically impairs their interaction with biological membranes [15, 16]. The apolar portion of biological membranes, mainly formed by the fatty acid chains of phospholipids, forms a selective barrier that difficult the permeation of hydrophilic molecules across it and facilitates the passage for the hydrophobic molecules. These interactions between PS drugs and biological membranes were observed in both prokaryotic [15], and eukaryotes [17, 18] cells, and the PS internalization and cytotoxic effect of PDT is decreased with the increasing hydrophilicity of chemically modified PS.

### 3. Improving the photophysical and biological properties of photosensitizers for antimicrobial PDT in aqueous media with nanoparticles

Hydrophilic PSs have shown impaired properties in biological tissues when compared to the hydrophobic ones. One alternative to continue using the hydrophobic PS is to associate these molecules with specific drug delivery systems, such as the nanoparticulated carriers. For these purpose, some specific nanoparticles have hydrophobic compartments that maintain the PS molecules in a monomeric non-aggregated form inside or at the surface of the nanoparticle, keeping the PS active for properly absorbing light [19]. In addition, specific chemical modifications can be done in the nanoparticle surface in order to increase the PS delivery to the prokaryotic and prevent the absorption of these carriers by the eukaryotic cells [20, 21]. This strategy may prevent the possible side effects of the therapy.

In figure 2 is presented an example of fluorescence emission, which is a photophysical parameter of the PS, by aluminium-phthalocyanine chloride in different conditions. The graphic shows that the maximum PS fluorescence emission was achieve when the PS molecule was solubilized in ethanol, an organic solvent, and the minimum emission was detected in the aqueous solution. This feature is a consequence of the aggregation of this compound in aqueous media that decreases drastically the photodynamic effects of the PS. As it is not possible to use ethanolic PS solutions for biomedical application, an alternative to maintain the photophysical properties of the PS could be the formulation of nanoparticulated carriers for hydrophobic molecules. By the analysis of Figure 2, it is easy to note that the aqueous oil/water nanoemulsion formulation, with the same PS concentration used in the three formulations, maintains the fluorescence emission in a level near to the produced in the ethanolic formulation. This kind of formulation keeps most of the PS molecules in a monomeric non-aggregated and functional active form, and then allows the photodynamic action of these drugs to occur. Unlike that, the PS solubilized in the aqueous solution decreased drastically the fluorescence emission, and this kind of solution in unable to be used for photodynamic activation.
3.1. Liposomes

Liposomes are small micro- or nano-structured vesicles formed by a phospholipid bilayer around an aqueous core with a size ranging from 50 to 1000 nm. Liposomes vesicles are interesting and useful drug carriers because they can carry both hydrophilic molecules in their aqueous core and hydrophobic drugs among the fatty acid chains in the phospholipid bilayers [22, 23].

For photodynamic applications, the liposomes have been used in association with different PS types and have been successfully applied against several bacterial strains in both in vitro tests [20, 21] and clinical studies [24]. For this antibacterial propose, one suitable strategy used to increase the PS delivery to the bacterial cells is the use of liposomes formulations with cationic phospholipids, such as dimethyldioctadecyl ammonium bromide or sterilamine, that may confer a cationic superficial charge for these vesicles carriers. The positive charge allows the liposomes to strongly attach to the bacterial surface, since the bacterial cells have negative superficial charges [25].

This strategy could also induce a selective delivery of the PS drugs to the bacterial cells over eukaryotic human cells. This is due to the fact that cell membranes of both eukaryotic and prokaryotic cells have residual negative surface charges mainly due to the presence of phosphate groups on the membrane phospholipids [26]. However, the presence of negatively charged molecules such as peptidoglycans, lipoteichoic acid and lipopolysaccharide in the bacterial surfaces confers a more negative residual charge in bacterial cells compared to eukaryotic cells [21, 25].

As an example, the increased PS delivery to bacterial cells with cationic liposomes is presented in Figure 2. This figure shows the result for multi-species cariogenic bacterial suspensions that were treated with two different liposomal formulations containing a phthalocyanine derivative. The difference between the two formulations was the presence of positively charged phospholipids in the cationic liposomal solution. The second liposomal formulation had a slightly negative superficial charge. After thirty minutes of liposome exposure to bacterial cells, the culture suspensions were centrifuged, cells were lysed with an organic solvent and then the specific absorbance of the phthalocyanine derivative was quantified. As observed in the graph, the bacteria culture treated with the cationic liposome formulation, at a same initial PS concentration, had a three-fold increased PS up-take compared to the neutral liposome, after the experimental period. This graph exemplifies the functionality of these cationic liposomes as a suitable PS carrier to increase drug delivery to bacterial target cells.

Figure 2: Fluorescence spectra emission (Excitation 350 nm) of three different Aluminum Chloride Phthalocyanine (AlClPc) solutions, at the same molar concentration: The PS soublized in water or alcohol as solvent, or the PS formulated in a nanoemulsion solution. At the right part of the figure, a schematic representation where the shining blue species represents the monomeric functional PS species, and the gray species represents the aggregated non-functional PS species.
3.2 Polymeric Nanoparticles

Polymeric nanoparticles have been long known as drug delivery systems. Generally, polymeric nanoparticles for PDT have been produced with poly(lactic-co-glycolic acid) (PLGA), polyacrylamide, silica (and its derivatives), proteins (such as human serumalbumin and collagen), polysaccharides (such as alginate, chitosan and dextran) [27].

Particularly, PLGA has presented interesting results. For example, in a recent study on methylene blue-loaded PLGA nanoparticles for antimicrobial PDT, it was found that these polymeric nanoparticles were able to concentrate this PS on the cell wall of *Enterococcus faecalis* [28]. Once concentrated on the cell wall, the PS can be activated and promote lethal damages on the bacteria cell. This is an interesting effect and it is fully due to the nanoparticle structure, since the non-encapsulated PS cannot be properly concentrated on the bacteria cell wall when it has low affinity for this specific structure.

On the other hand, polymeric nanoparticles can be designed in such a way that their surface strongly binds the bacterial structures. This nanoparticle could then be used for entrapping several different kinds of PSs. It is worth noting that there are several kinds of polymers available on the market that can be used for producing PS-carrying nanoparticles. These polymers have different chemical groups that can be used for the chemical functionalization of the nanoparticle surface. The functionalization turns it possible to finely tuning the nanoparticles properties in order to adapt them to the specific kind of target bacteria or to control the PS release. This subject has been discussed in details elsewhere [27].

3.3 Nanoemulsion

Emulsions are systems basically composed of two immiscible liquids, one forming a continuous phase into which the other is dispersed as non-continuous droplets. In nanoemulsions these droplets are generally smaller than 300 nm in diameter. For biological applications, the continuous phase is generally an aqueous medium in which oily droplets are dispersed. This system is stabilized with the appropriate surfactants, i.e., with substances that reduce the interfacial tension (between the two immiscible liquids). When using biocompatible materials, this kind of formulation turns it possible to administrate oils even by the endovenous route, maintaining all the advantages due to the use of nanostructured particles. As in the case of liposomes and polymeric nanoparticles, the nanodroplets surface can also be adapted with several molecules, such as polymers or phospholipids, for example, in order to target them to a specific cell [29].
As well as liposomes, discussed above, nanoemulsions can also be used for improving the photophysical properties of hydrophobic PSs. However, it is worth to highlight here that nanoemulsions are particularly interesting topical drug delivery systems. Nanoemulsion can be used as skin permeation inducers. For applying PDT at superficial bacterial skin infections it is essential the PS to penetrate across the stratum corneum, reaching deeper layers of the epidermis. However, some PSs are not capable of crossing the stratum corneum, turning it difficult to reach the bacteria infecting the lower epidermis layers. This is particularly the case for hydrophilic PSs. In a recent study, the authors observed that the permeation of 5-aminolevulinic acid (5-ALA), and the subsequent detection of its metabolite protoporphyrin IX (PpIX) in the deeper layers of the epidermis, was 5-fold higher when this substance was incorporated to a nanoemulsion, in comparison to a conventional formulation [29].

4. May Nanoparticles carriers avoid the generation of PDT-resistant strains?

The development of antibiotic resistant bacteria is a worldwide concern and may be one of the most important clinical challenges in this century. Within this context, several epidemiological reports have shown that the number of bacterial resistance to the traditional antibiotics had significantly increased in the last decades [7, 30]. Moreover, these numbers are a major concern in specific locations, such as hospitals, where patients often have low immunological resistance against opportunistic infections. For example, the proportion of Staphylococcus aureus isolates from hospitals in the United States that were methicillin resistant (MRSA) increased from 35.9% in 1992 to 64.4% in 2003 [30].

To limit this emerging public health issue, an intense research for new alternative antibiotics has been presented in the literature. However, the recent advances in the search for new antibiotics in the last decades is not sufficient to face the increasing number of resistant bacteria strains developed during the same period [7].

Besides the new antibiotics developed, the antimicrobial PDT has been described as a possible innovative approach that could be employed to treat these resistant strains. This hypothesis was supported by several reports showing that photodynamic therapy is effective against traditional resistant strains in clinical trials [31], animal models [32], and in vitro [32, 33] evaluations. Moreover, intense ROS production during the therapy promotes an aggressive and random attack to the most different biomolecules in the microbe. This fact, in theory, should impair the ability of microorganisms to develop specific resistant strains against the ROS produced during PDT. Furthermore, the main bacterial antioxidant enzymes, such as the superoxide dismutase and catalase, are inactivated by the singlet oxygen produced during PDT [34], turning more difficult to the bacteria to create PDT resistance mechanisms.

Taken together, the information presented in the previous paragraph support the hypothesis that the development of resistant strains against ROS may not happen due to the typically multi-target random nature of ROS attack to the bacterial structures. However, this issue is still under debate, mainly because some reports have demonstrated that some PS drugs are selectively pumped out from the target bacteria by some specific bacterial proteins called multidrug pump efflux complex [35]. These multidrug efflux complexes have been recognized as a major mechanism of resistance in several bacterial strains and for several classes of antibiotics [36]. In PS efflux, the internalized molecules are bound by the efflux protein complex and then pumped out of the bacterial cells. In addition, a decrease in the PS uptake by bacteria in some conditions was also demonstrated [36]. This bacterial strategy may be considered as a resistance mechanism against specific PS during PDT. Therefore, the resistance mechanism can sometimes be related to the efflux of PS molecules, which have necessary a limited molecular weight (<600-700 Da) to serve as substrate to this transport mechanism [8]. However, when these compounds are heavier, these efflux strategies may not be useful to development of resistant bacteria.

This hypothesis was demonstrated by Giuliani et al. [8], that used a phthalocyanine derivative called RLP068/Cl, which have a molar weight >1,300 Da. The authors have shown that repetitive treatments of a Staphylococcus aureus strain did not induced the development of resistant strains. They supposed that larger PS molecules could not be internalized by the bacteria cells and that the PS molecules should accumulate in the superficial portions of the bacterial structures. In this way, the photoactivation would promote oxidative stress in these regions and consequently promote cell wall or membrane disruption and consequent bacteria lyses. This same mechanism may be true for the nanoparticles PS-carries, where the huge structures of the nanoparticles, compared to the single monomeric molecules, may not be completely internalized and the PS can then be kept in the bacteria surface. This option may impair the development of resistant strains, since the mechanism of nanoparticle-bacteria interaction occurs due to difference in superficial charges, molecular affinity, or adhesion properties of these structures to the bacterial surface. So, new resistant bacteria strains should have complexes structural modifications to limit the nanoparticle adhesion to their superficial structures. Another rising possibility is that the nanoparticulated PS carrier, once internalized by the bacteria, cannot be pumped out of the cell [28]. This is an interesting feature, but the nanoparticle must enter the bacterial cell in order to keep the PS inside the cell and avoid externalization.
5. Conclusions

The present chapter presents the Photodynamic Therapy (PDT) as a useful tool for antimicrobial applications. At the moment the literature presents several options of photosensitizers (PS) drugs available for PDT, and the associations of these PS with different nanoparticulated carriers have been shown as a suitable strategy to improve some photophysical and antimicrobial features related to the therapy. In addition, we presented a literature review about the possible low risk to development of microbial resistant strains against PDT. One of the goals of this chapter was to present a discussion about the improvement of the PS functionality, and the decrease in the risk of induction of resistant microbial species against PDT when these PS drugs are associated with nanoparticulated carriers.

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References


