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Phenothiazinium Dyes as Photosensitizers (PS) in Photodynamic Therapy (PDT): Spectroscopic Properties and Photochemical Mechanisms

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Additional information is available at the end of the chapter

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1. Introduction

Oscar Raab demonstrated, in 1900, that the light incidence on dyes can induce cell death [1]. A photosensitizer is a chemical compound that is activated by light of a specific wavelength that leads to tumor destruction [2]. Indeed, Photodynamic Therapy (PDT) is considered to have its origin in 1900 with the classical experiments by the German scientist Oscar Raab. Raab noticed that the exposure of *Paramecium caudatum* to acridine orange and later subjection to light resulted in death of this organism. Raab and his supervisor Hermann von Tappeiner later coined the term "photodynamic therapy" and applied PDT successfully for the treatment of cutaneous tumors using eosin. From that concept, photodynamic therapy (PDT) [3,4,5,6], as we know today, was founded. Since then, the development of other studies, culminating with those performed by Dougherty and co-workers resulted in a non-invasive technique for cancer treatment and other diseases [7,8]. In fact, precancerous cells, certain types of cancer cells and microbial infections can be treated this way.

Interesting data regarding the application of PDT against several diseases have been reported, since the employment of this therapy in different diseases has increased significantly. In fact, PDT has been used with phenothiazinium [methylene blue (MB) and toluidine blue] as photosensitizers against AIDS-related Kaposi's sarcoma, promoting complete sarcoma remission with excellent cosmetic results [9]. PDT with MB (and LED as light source), which is a very inexpensive system, has been applied against *Leishmania*,

promoting significant reduction in the size of the lesions, diminishing the parasitic load in the draining lymph node and healing the lesions in hamsters experimentally infected with *L. amazonensis* [10]. This therapeutic alternative is very interesting due to the resistance of this organism to pentavalent antimonials (SbV), which constitutes the mainstay pharmacological alternative for leishmaniasis, due to emergence of drug resistance [11].

Tumor, which is also called neoplasm or blastoma, is the abnormal growth of tissues. Sick cells with genetic disturb develop more rapidly than the normal cells, which provokes the development of the tumor (that can be malign or non-malign cells). When the growth of the tumor is a very fast and chaotic process, with tendency to arrive in other organs, generally is a malign tumor [4]. Cancer is the general name of all malign tumors. This term cancer is originated from latin and means "crab". This name is due to the tendency of the tumor to be fixed in several biological tissues, which is correlated to the ability of the crab to be fixed in various surfaces [4].

Interestingly, the PDT procedure is easily performed in a physician's office or outpatient setting, which favors the application of this therapy in several environments, since PDT does not need great structural pre-requisites. In this context, it is important to notice that multicenter randomized controlled studies have demonstrated high efficacy and superior cosmetic outcome over standard therapies [12]. In fact, several cosmetic methodologies have been developed with PDT, such as resurfacing. For many non-oncologic dermatological diseases, such as *acne vulgaris*, viral warts and localized scleroderma, case reports and small series have confirmed the potential of PDT [12]. After the development of topical photosensitizers 5-aminolevulinic acid (ALA) or its methyl ester (MAL), PDT has gained worldwide popularity in dermatology, since these drugs do not induce prolonged phototoxicity as the systemic photosensitizing hematoporphyrin derivatives do [12]. PDT has essentially three steps. First, a light-sensitizing liquid, cream, or intravenous drug (photosensitizer) is applied or administered. Second, there is an incubation period of minutes to days. Finally, the target tissue is then exposed to a specific wavelength of light that activates the photosensitizing medication.

More than one million cases of skin cancer were diagnosed during 2008 in the U.S.A. and its worldwide incidence has risen throughout the last four decades. Squamous cell carcinoma (SCC) is the second most frequent skin cancer, only after basal cell carcinoma (BCC) [13]. In the 20th century, SCC was mainly linked to occupational sun exposure, whereas in the last decades the strongest link has been to ultraviolet (UV) radiation. On one hand, UVB exposure leads to direct DNA damage by pyrimidine dimer formation. On the other hand, UVA induces formation of reactive oxygen species which indirectly also cause DNA damage. Other factors such as the phototype, the genetic predisposition or the immune response are also involved in the carcinogenic process [13].

It is also important to notice that photoantimicrobial agents, that is, chemical compounds that exhibit increased inactivation of microorganisms when exposed to light, have been known also for over a century [14]. While there are several studies regarding the use of

photosensitizers against bacterial and viral targets, the clinical use of photosensitizers in antimicrobial therapy has been developed very slowly through small scale trials. This is particularly a surprise considering the efficacy exhibited, especially by cationic photosensitizers, against pathogenic drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [14]. Furthermore, the exponentially increasing threat of microbial multidrug resistance has highlighted antimicrobial photodynamic inactivation (APDI) as a promising alternative treatment for localized infections [15]. APDI involves the direct application of the PS to the infected tissue rather than being injected intravenously, as the usual procedure for cancer treatment with PDT [15].

The photodynamic process involves photophysical and photochemical steps, which can be applied with several aims, such as therapies against cancer or infections. PDT light sources include laser, intense pulsed light, light-emitting diodes (LEDs), blue light, red light, and many other visible lights (including natural sunlight). Photosensitizer drugs may become activated by one or several types of light. The optimal light depends on the ideal wavelength for the particular drug used and target tissue.

Electron and energy transfer in the excited state govern the efficiency of a variety of photoinduced processes, including photosynthesis, light to energy conversion in semiconductor devices, cell damage induced by solar exposition and photodynamic action [16, 17, 18]. It is well reported that photophysical behavior of a dissolved dye depends on the nature of its environment, i.e., the solvent influences the spectra characteristics of the solute molecules [19]. Several factors influence the visible spectral behavior of dissolved dye molecules, especially the solvent's polarity and its hydrogen-bond donor/acceptor capacities [19]. The properties can be determined by the solvent dielectric constant, ϵ , and solvatochromic parameters. The strong solvatochromic behavior can be observed for dye molecules with large dipole moment changes during transitions between two electronic states. The solvent can differentially stabilize the ground and/or the excited state in polar and non-polar solvents [19].

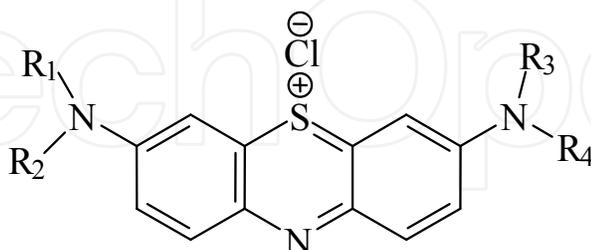
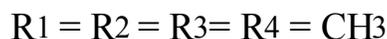
The series of phenothiazine [thionine, methylene blue (MB), azure A (AZA) and azure B (AZB)] derivatives (Fig. 1) are positive dyes used as a model for phototherapeutic agent as well as for dye sensitized solar energy converter [20, 21] due to their appropriate biological, chemical, photochemical and photophysical properties [22, 23, 24].

The intersystem cross quantum yield and the singlet oxygen formation for MB is 0.52 [25, 26, 27, 28, 29], the triplet lifetime is higher, approximately 3.0 μs , in air saturated aqueous solution, and up to 50 μs in nitrogen saturated aqueous solution. The singlet excited state has a lower lifetime, approximately 1,400 ps (Table 1), and it is due to the higher internal conversion and triplet formation, with a fluorescence quantum yield of 0.04 in methanol [30, 31, 32, 33]. In addition, MB and MB derivatives that have been used as photosensitizers in PDT showed a good biocompatibility (appropriate cytotoxicity and phototoxicity) when used *in vitro* to attack key organelles in cells [14, 21].

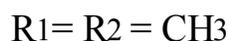
thionine



Methylene blue



azure A



azure B

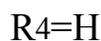
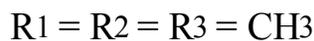


Figure 1. Thionine derivatives.

medium: water	
Dye	τ (ps)
Thionine	314,40
Nile blue	372,75
Azure A	421,28
Azure B	1268,56 (48,89%) e 306,00 (51,11%)
Toluidine blue	2179,65 (66,72%) e 358,03 (33,28%)
Methylene blue	328,84
medium: ethanol	
Dye	τ (ps)
Thionine	848,84
Nile blue	1170,03
Azure A	776,43
Azure B	724,78
Toluidine blue	643,89
Methylene blue	465,96

Table 1. Values of lifetime (τ) of some dyes at 25°C.

The Fluorescence decays of dyes were obtained by single-photo-counting technique. The excitation source was a Tsunami 3950 Spectra Physics titanium-sapphire laser, pumped by a Millennia X Spectra Physics solid state laser. The laser was tuned that a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 292 nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The spectrometer was set in L-format configuration, the emission wavelength was selected by a monochromator (680 nm), and

emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The software provided by Edinburgh Instruments was used to analyze the individual decays. The quality of the fit was judged by the analysis of the statistical parameters reduced- χ^2 and Durbin-Watson, and by the inspection of the residuals distribution.

The dyes stock solutions were prepared in ethanol (6.0×10^{-5} M) and aliquots of these stock solutions were added, via a calibrated Hamilton microsyringe, to volumetric flasks containing water or ethanol, and the solutions were stirred for 30 minutes. The final concentrations of dyes were 1.0×10^{-6} M. All measurements were performed at 25°C using a cuvette with 0.2 cm of optical path.

The excited state lifetime depends on the solvent [34,35]. The dependence of the lifetime on the viscosity and solvent dielectric constant indicates that the dye excited state deactivation process is slow as the medium viscosity increases. This effect is related to the partial inhibition or the higher friction on the dye substitute groups rotation, such as $-\text{CH}_3$, $-\text{NH}_2$, $-\text{N}(\text{CH}_3)_2$ and $-\text{N}(\text{CH}_2\text{CH}_3)_2$ [36]. The lifetime values are in agreement with the results reported in the literature. Lee and Mills [37] showed the lifetime values for methylene blue aqueous solutions (358 ± 20 ps). Grofcsik et al [34] measured the lifetime of Nile blue excited state and oxazine 720 in different solvents at 20 °C. The thionine dye photophysics is well known [38]. In an aqueous solution, thionine has a fluorescence lifetime of 320 ± 60 ps when excited at 610 nm [37,39]. In organic solvent, the increase of the thionine fluorescence lifetime (450 ps in ethanol and 760 ps in *tert*-butylic alcohol) results in a increasing of the fluorescence quantum yield [38]. The thionine lifetime differences observed in an aqueous medium and ethanol is quite high, which shows the effect of microenvironment polarity on the excited state decaying [38]. In our experiments, in an aqueous medium thionine has a useful lifetime of 314.4012 ps, which is in agreement with the results presented in the literature.

The Nile blue lifetime in ethanol and water are 1420 and 418 ps, respectively. These results are higher than those that we found in our work. However, it should be taken into account that the temperatures used in our experiments are different from those whose results are different. It was shown that the lifetime of Nile blue depends on the temperature due to the intermolecular charge transfer [34,35,36]. This charge transfer process is facilitated by the presence of NH_2 groups in molecule structure, such as on the Nile blue structure, which may change the lifetime values. Grofcsik et al [34] studied these probes in different solvents where it was observed that there is a relationship between the solvent permittivity and the excited state lifetime. It was shown that the lifetime is higher in nonpolar solvents, where protic solvents decreases the excited state lifetime. This behavior was observed in both dye molecules that were studied, which have a similar chemical structure.

Grofcsik et al [34,35] have shown that there is a relationship between the excited state lifetime of Nile blue and Oxazine 720 with the acidity of the medium. As the hydrogen ion concentration increases it is observed a decrease of the excited state lifetime [40]. It was also observed for methylene blue, azure A, azure B and azure C [41]. The reason for the rapid decay in acid medium is due to the formation of dications from the monocations reaction in the excited state with hydrogen ions. These results indicate that the reaction in the excited state the additional protons are located on the nitrogen atom of the ring and not on the

terminal amine groups [40]. It is believed that for other compounds the results may be the same due to the similarity in the chemical structure of such molecules.

Dutt et al [42] studied the fluorescence lifetime of cresila violet, Nile blue, oxazine 720 and Nile red, using different solvents, such as alcohols, polyalcohols, amides and some aprotic solvents. The authors showed that the lifetime values for these dyes are approximately 3.5 ns for n-alcohols, which are higher than that for the Nile blue (1.62 ns in ethanol). This result is in agreement with our studies. When it is considered the behavior of bipolar solute in polar solvents, the hydrodynamic and dielectric contribution must be taken into account [42]. However, it is not well known how to measure these hydrodynamic and dielectric contributions individually. In the case of the four dyes, when in the presence of amides and aprotic solvents, as described above, the contributions are reasonably described by the hydrodynamic friction, where to describe the rotating relaxation in the presence of n-alcohols; the dielectric friction must be included.

Chen et al [43] studied the quantum yield of the methylene blue singlet oxygen as a function of the medium pH values. The authors showed that the protonated acid (3MBH₂⁺) triplet state is similar to the base (3MB⁺) triplet state, and the quantum yield of the singlet oxygen formed is much higher in basic medium than that in acidic medium. The singlet oxygen formation increases as the pH of the medium is increased, while the singlet state lifetime decay the triplet state formation do not depend on the pH changes. It can be explained by the population decay rate of the singlet state due to the internal conversion to the fundamental state, and the intersystem crossing to the triplet state, which are much higher than the protonation rate [43,44]. Also [43] studied the behavior of methylene blue, 1,9-dimethyl-methylene blue and toluidine blue in aqueous medium and methanol. The triplet state formation and the singlet oxygen quantum yield in water were very similar to that for methylene blue and for 1,9-dimethyl-methylene blue. The kinetic studies results for the singlet state decay of methylene blue in water and in methanol were 0.37 and 0.62 ns, respectively, where for toluidine blue the results were 0.28 and 0.40 ns, respectively. In the case of methylene blue the decay useful life of the singlet excited state in methanol is approximately two times higher than in water. The authors showed that there is no influence of the solution concentration on the singlet state lifetime, where the differences on the lifetime decays that were observed in water and methanol are not related to the methylene blue dimerization in water. The methylene blue lifetime decay decreases with the increase of the dielectric constant of protic solvents due to the interaction of the methylene blue with the polar solvent [45]. In protic alcohols and in aqueous solutions the methylene blue excited state lifetime is higher than of the fundamental state. Therefore, the differences between the singlet and triplet states decrease as the relaxation rate is increased. In the presence of aprotic solvents, such as acetone, acetonitrile, and dimethyl sulfoxide, the dipole excited state is lower in the fundamental state, where the energy differences observed is higher and the relaxation lifetime is longer [46].

The use of these dyes as singlet oxygen photosensitizer in PDT, as well as tumor cells removal are being investigated [47,48,49,50]. It is known that under laser irradiation in the presence of photosensitizer dyes, the tumor cells undergo necrosis or apoptosis and the rate

of tumor cell removal through apoptosis increases [51,52,53]. This behavior has been related to the presence of singlet oxygen in the tumor cells [54,55]. The increase of cell removal through apoptosis is of great importance in the PDT treatment [50,56]. There are no side effects in the cell removal through apoptosis because it is a controlled cell removal process, where there is no inflammation of the laser irradiated tissue. In some cases changes in the PDT mechanism has been observed, type I via free radical and type II via oxygen singlet, which could be related to the interaction among the dyes and the cellular system [57,58,59,60]. These changes involve the aggregation of two or more dye molecules in the same site [61].

2. Photodynamic Therapy (PDT): Mechanism of action

Selective tumor destruction without damaging surrounding healthy tissues can be reached by using PDT, which is treatment, activated by light, which requires the combination of three elements: a photosensitizer, visible or near-infrared light, and oxygen [62,63,64,65,66,67,68,69]. However, the precise mechanisms of PDT are not yet fully understood but two general mechanisms of photoinduced damage in biomolecules have been proposed: Type I and Type II [62,70,71]. Type I is the photodynamic mechanism in which the excited molecule induces radical formation that causes damage to biological targets (membranes, proteins and DNA), and an electron transfer event is the initial step [16]. In Type I mechanism, the photosensitizer in the excited state interacts directly with a neighbor molecules, preferentially O₂, producing radicals or radical ions through reactions of hydrogen or electron transfer [72]. Frequently, these radicals react immediately with the O₂ generating a complex mixture of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical and hydroxyl radical, which are capable to promote oxidation a great number of biomolecules [62].

It is believed that ¹O₂ produced through type II reaction is primarily responsible for cell death. It is known that several factors including the PS, the subcellular localization, the substrate and the presence of O₂ contribute to this process [71]. The lifetime of ¹O₂ is very short (approximately 10-320 nanoseconds), limiting its diffusion to only approximately 10 nm to 55 nm in cells [73]. Type I photoreaction of some PSs are primarily responsible for sensitization through radical formation under hypoxic conditions. In the presence of oxygen, ¹O₂ mediates photosensitization process, but the supplemental role of H₂O₂, OH• and O₂• must also to be considered. Only substrates situated very close to the places of ROS generation will be firstly affected by the photodynamic treatment because the half-life of ¹O₂ in biological systems is under 0.04 μs and its action radius being lower than 0.02 μm [71]. This assumption is due to the fact that ROS are highly reactive and present a very short half-life. Type II is the photodynamic mechanism in which the photooxidation is mediated by singlet oxygen (¹O₂), where an energy transfer reaction from the photoexcited molecule to molecular oxygen is the initial step [16,62]. The process involves the excitation of the photosensitizer from a ground singlet state to an excited singlet state, where intersystem crossing to a longer-lived excited triplet state will occur. It is also important to point out that molecular oxygen is present in tissue with a ground triplet state. When the photosensitizer

and an oxygen molecule are in proximity, an energy transfer can take place that allows the photosensitizer to relax to its ground singlet state, and create an excited singlet state oxygen molecule. Additionally, energy is transferred from triplet protoporphyrin IX to triplet oxygen, resulting in singlet ground state protoporphyrin IX and excited singlet oxygen, which reacts with biomolecules, which can damage some cells in the treatment area. Singlet oxygen is the usual name associated to the three possible excited electronic states immediately superior to the ground state of molecular oxygen (triplet oxygen) [3].

Due to the short half-life and diffusion distance of singlet oxygen in aqueous media, PDT can be considered a highly selective form of cancer treatment, as only the irradiated areas are affected, provided that the photosensitizer is nontoxic in the absence of light [74]. This combination of light/photosensitizer/oxygen as a mode of disease treatment has expanded from an initial focus on cancer tumors to include application in certain non-neoplastic diseases including age-related macular degeneration (AMD), coronary heart disease, periodontal diseases, and microbial infections [75].

Singlet oxygen is a very aggressive chemical species and will very rapidly react with any nearby biomolecules, being that the specific targets depend directly on the physical-chemistry properties of the photosensitizer used in the photodynamic process, which will result in no desired side effects, such as destructive reactions that will kill cells through apoptosis or necrosis. Therefore, depending on whether Type-I or Type-II mechanisms take place, the therapeutic efficiency of PDT may be completely altered. Therefore, the ratio of apoptotic versus necrotic cell death in tumors treated with PDT may depend on the competition between electron and energy transfer in the reaction site [16].

Oxidative stress generated by the photodynamic action occurs because in biological systems the singlet oxygen presents significantly low lifetimes, where the lifetimes of the singlet oxygen is lower than 0.04 μs , implying that its radius of action is also reduced, being usually lower than 0.02 μm [3]. Reactive oxygen species (e.g. hydroxyl radicals or superoxide) are their high reactivity and low specificity with a broad spectrum of organic substrates [76]. Various methods have been employed for the generations of hydroxyl radicals such as O_3/UV , $\text{H}_2\text{O}_2/\text{UV}$, TiO_2 photo-catalysis and photo assisted $\text{Fe(III)}/\text{H}_2\text{O}_2$ reaction.

3. Photosensitizers

3.1. Phenothiazinium dyes

The phenothiazinium dyes were first synthesized in the late 19th century—e.g. both Methylene Blue (Caro) and Thionin (Lauth) in 1876—during what might be considered to be a “gold rush” period of chemical experimentation after the discovery of the first aniline dyes [77]. Among photobactericidal compounds, the phenothiazinium photosensitizers methylene blue (MB) and toluidine blue O (TBO) have often been used as lead structures, being effective photosensitizers with singlet oxygen quantum yields of approximately 0.40

and exhibiting low toxicity levels in mammalian cells [14]. Members of the phenothiazine class are known to cross the blood-brain barrier and to be relatively nontoxic [78,79].

The biomedical use of phenothiazinium dyes has begun with specimen staining for microscopy by various medical scientists, among whom were famous scientists such as Romanovsky, Koch and Ehrlich. The idea of structure–activity relationships in stains developed in this era, particularly by Paul Ehrlich, laid the foundations for modern medicinal chemistry, and these principles should be followed by those attempting the properly organized photosensitizer synthesis [77]. Cellular uptake is determined by a combination of charge type/distribution and lipophilicity, both of which characteristics may be controlled by informed synthesis. Due to the expansion of PDT into the antimicrobial milieu, a far greater scope for photosensitizer design exists now. For example, in the field of blood product disinfection, an ideal candidate photosensitizer would be effective in the inactivation of bacteria, viruses, yeasts and protozoan, but would remain non-toxic and non-mutagenic in a human recipient. It is hardly surprising that none of the currently available agents fits all of these criteria [77].

Phenothiazinium dyes are cationic compounds with high redox potential that interacts with visible light inactivating several kinds of pathogenic agents in fresh plasma. Phenothiazinium dyes present great reactivity with the proteins and lipoproteins (cell membranes) and nucleic acids. These cationic compounds have limited capability to permeate the cell membrane as function of their elevated hydrophilic character [80]. Phenothiazinium dyes present significant action against encapsulated virus and some virus without capsule, such as parvovirus B19. As function of its genotoxic action, the employment of phenothiazinium dyes is prohibited in several countries, such as Germany⁸⁰. On the other hand, the Methylene Blue is a highly hydrophobic compound with higher chemical affinity to the nucleic acids, which denotes its potential to application against virus.

Phenothiazinium dyes are photocytotoxic, and can cause photoinduced mutagenic effects [81]. In living systems, DNA acts as an important target for phenothiazinium dyes. It has been proved that these dyes can photosensitize biological damage. Azure B (AZB) is an easy available phenothiazinium dye, and has been widely employed both in metal determination and DNA staining detection. Owing positive charges on its molecular structure, AZB can bind to the DNA polyanion in living systems through electronic interactions. So, the study of the interaction of AZB with DNA in vitro is of importance.

Methylene Blue, MB (Figure 2) is a phenothiazinic dye current applied in PDT as therapeutic agent or photosensitizing compound. MB has a recognized antimicrobial effect in the dark (citotoxicity property) which can be increased, at oxygenated environment, by the incidence of light with a wavelength corresponding to its electronic absorption band [82,83].

Methylene Blue is a well-known photochemical oxidant. The photoreduction reaction of this dye by various types of electron donors has been studied quite often, and in most cases an electron transfer mechanism was proposed for explaining the observed results [84].

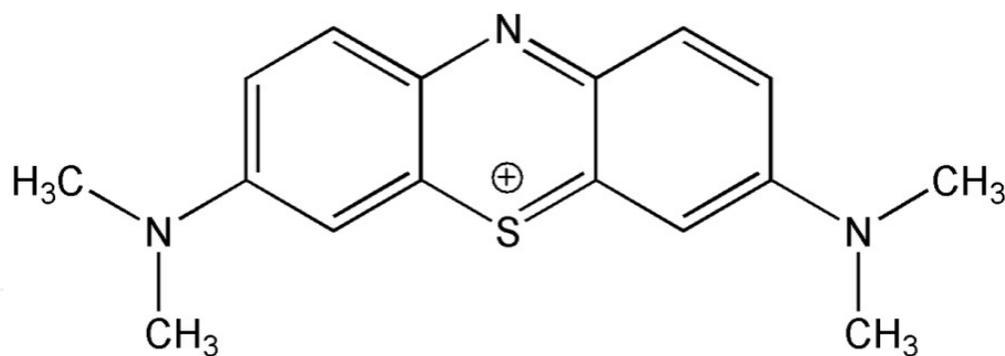


Figure 2. Structure of Methylene Blue

This molecule is particularly interesting for application in PDT due to its known physical chemical properties. For example, MB is a positive charged dye with three aromatic rings (6-members) very soluble in ethanol. It is already used clinically in humans for the treatment of methemoglobinemia, without significant side effects. Besides these characteristics, MB presents a quantum yield of singlet oxygen formation around 0.5, with a low reduction potential, intense light absorption in the region of 664 nm in water (within the phototherapeutic window). Also, it displays a high photodynamic efficiency causing apoptosis of cancer cells, by mono or polychromatic light excitation). Currently, MB is used by several european agencies for disinfection of blood plasma, due to its efficiency in photodynamic inactivation of microorganisms such as viruses [85], including HIV, hepatitis B and C [86,87].

MB has been clinically used as a photosensitizer drug for PDT in the treatment of different types of tumors [88]. Phototherapeutic application examples include treatment of bladder cancer, inoperable esophagus tumors, skin virulence, psoriasis and adenocarcinomas [89]. Additionally, an important point to be considered is the extremely low cost of a treatment based on this dye compared with other available photo-drugs.

Although MB possesses a positive charge and the planar structure with delocalized charge, it has a tendency to form dimers, trimers or type H aggregated systems in the presence of certain additives, cell organelles or solvents, for example, water [90,91,92,93]. The development of self-aggregates compromises its photodynamic activity, impairing the production of singlet oxygen, principal phototoxic species in PDT. In self-aggregated states auto-quenching processes occur where the excited monomers have the energy suppressed by collisions with other monomers that constitute the aggregate [94,95,96,97,98].

Often, treatment protocols require unusual preparation methods, or conditions that may have many distinct characteristics of the most ideal conditions. One example is that the MB in diluted aqueous solution, with concentration around 2×10^{-5} mol L⁻¹, is found in monomeric form. However, its uses in topical treatments require concentrations higher than 6×10^{-2} mol L⁻¹, where self-aggregation and its consequences are significant [82].

Therefore, it is important to investigate the phenomena of MB self-aggregation present in solvent mixtures and / or interaction with biomolecules [90]. This study aims to investigate

changes in MB spectroscopic properties caused by self-aggregate formation induced by solvent mixtures.

The MB is an oxazinic dye soluble in water or alcohol. It presents a quantum yield of oxygen singlet formation of about 0.5 and low reduction potential [25]. It is a dye with low toxicity, which absorb in the UV-visible light ($\lambda_{\text{m\acute{a}x}} = 664 \text{ nm}$; solvent: water) and shows good photodynamic efficiency to kill cancer cells, which can be excited by monochromatic and polychromatic light within the therapeutic window [82]. It is a hydrophobic dye, which forms aggregates when in the presence of aggregation agents such as polyelectrolyte, or when in the presence of solvents that induces the aggregation process. The aggregate formation changes photosensitization efficiency, decreasing the amount of singlet oxygen produced by light stimulation. The most important application of methylene blue (MB) is its use in PDT as a photosensitizer agent, in oncology and potentially in the treatment of other diseases, such as Leishmaniosis.

Teichert et al.[99] used *Candida albicans* strains that are resistant to the conventional treatment of *Candida* infections, which were collected from HIV-positive patients. These strains were inoculated in the oral cavity of rats that, subsequently, were submitted to the topic application of 1 mL of Methylene Blue at concentrations of 250, 275, 300, 350, 400, 450 and 500 $\mu\text{g mL}^{-1}$. After 10 minutes of dye application, the authors employed the diode laser with wavelength of 664 nm with potency of 400 mW (687.5 seconds), resulting in an energetic density of 275 J/cm^2 [100]. After one unique application, it was realized microbial culture exam of the respective samples and the individuals were sacrificed to the histological analysis of the tongue. The results obtained in this procedure demonstrated a complete elimination of the microorganisms, when the dye concentrations of 450 e 500 $\mu\text{g mL}^{-1}$ were employed. In the histological analysis, the rats that were treated with PDT had no inflammatory signals. The tongues of the control group rats presented high level of infection by *Candida* which was located in the keratin layers [100]. The respective authors concluded that the PDT is a potential alternative to the treatment of the fungi infection, emphasizing, as advantages of this technique, its topic character, simple methodology and, mainly, the unspecific characteristic of PDT, i.e., the possibility of to be applied to a great number of microorganisms. Moreover, PDT can be applied several times without risk of selection of resistant yeasts [100].

Azures A, B and C, are examples of photosensitizer agents, which have the cationic derivatives, such as the Azure Bf4. The organic ions can interfere through fluorescent radiation absorption that is emitted by excited molecules, resulting in a photobactericidal effect on the *Staphylococcus aureus* and *Enterococcus faecium* colonies. This behavior is related to the light stimulation wavelength because the organic compounds present in the system absorb electromagnetic radiation. However, only organic compounds that present double bond conjugated system, such as azure A, B or C, are capable to absorb the visible light radiation.

It was observed that red visible light (600-700 nm) and nearinfrared are the wavelengths that can penetrate the human skin. The phenothiazinic dyes, such as Azures, absorb light in such

wavelengths with high intensity. They show the formation of aggregates due to the presence of aggregation agents such as polyelectrolytes, or due to the presence of solvents that favors the aggregate formation, such as water. The aggregate formation changes the photosensitization process efficiency, decreasing the amount of singlet oxygen produced by the light stimulus. The self-aggregation phenomenon can be minimized by adding charged groups in the dye structure, which results in an electrostatic repulsion interaction, increasing the hydrophilic behavior of the dye, such as Azure B and Azure BF₄.

Azures are phenothiazine compounds. This class of dye has low toxicity in the dark, constant composition, being synthesized with high yield. Azures present great selectivity to the tumor cells and significant photo stability, being not maintained in the body for long interval of time. These dyes can be applied through endovenous and topic ways. Azures present high bactericide ability, being very auspicious compounds to be applied as photosensitizers in PDT, especially due to their favorable photodynamic properties and low cost [101,102]. Azure dyes (including Azure B) are recalcitrant compounds used in the textile industry. For instance, Azure B has been used in a selective assay for detecting lignin peroxidase, the oxidative enzyme with the highest redox potential produced by white-rot fungi [103,104].

Azure B is a very sensitive dye and extremely susceptible to detect slight alterations in its chemical environment, presenting significant solvatochromic processes. Physico-chemical properties of Azure B have motivated the employment of Azure B as a chromogenic reagent for the spectrophotometric determination of several compounds, which are relevant to biological and environmental chemistry such as periodate [105]. This cited method is simple and rapid, offering advantages of sensitivity and wide range of determinations, without involvement of any stringent reaction conditions, being successfully applied to the determination of periodate in solution and in several river water samples. In its time, Azure-C (AZC), and related phenothiazine compounds has been widely used for accelerating the oxidation of NADH, but not in connection to the NAD⁺ reduction process.

Thionine has been a subject of many studies, as for example in a photochemical and electrochemical biosensor [106,107,108,109,110] and in photovoltaic cells [111]. Thionine is a positively charged tricyclic heteroaromatic molecule, which has been investigated for its photoinduced mutagenic actions [112,113], toxic effects, damage on binding to DNA [114] and photoinduced inactivation of viruses [115]. Thionins consist of 45–47 residues bound by three to four disulfide bonds, which includes α 1-purothionin, β PTH, and β -hordothionin (β HTH) [116,117,118].

It has the ability to immobilize proteins and DNA and act as molecular adhesive [119]. Biophysical and calorimetric studies with three natural DNAs of varying base compositions, have shown the intercalative binding and high affinity of thionine to GC rich DNAs [120]. Thionine presented a high preference to the alternating GC sequences followed by the homo GC sequences contained in different synthetic polynucleotides [121]. AT polynucleotides presented a lower binding affinities but the alternating AT sequences had higher affinity compared to the homo stretches. The intercalation and the sequence of specific intercalative

binding of thionine were shown by fluorescence, viscosity experiments and circular dichroic studies, respectively [121].

Studies based on absorbance, fluorescence, circular dichroic spectroscopy, viscosity, thermal melting and calorimetric techniques were used to understand the binding of thionine, with deoxyribonucleic acids of varying base composition, where strong binding of thionine to the DNAs were shown. Strong hypochromic and bathochromic effects and quenching of fluorescence were observed that showed strong binding of thionine to the DNAs [97]. The binding process is exothermic, which is associated to a large positive entropy changes and a negative enthalpy, and it showed that nonelectrostatic contributions are very important for the association of thionine to DNA. Studies on the interaction of thionine with sodium dodecylsulfate (SDS) micelles have shown that thionine binding affinity to SDS micelles was decreased with increasing temperature due to the thermal agitation [122].

The spectroscopic characteristics of thionine aggregates have shown that it depends on the concentration of thionine and on the chemical nature of the solvent [123]. Two peaks can be observed, at 597 nm and at the lower wavelength side of the 597 nm peak, and they related to the monomeric species and to the aggregate formation, respectively [124]. The understanding of the thionine aggregation process is very important for some application, such as in photovoltaic cells, where the reverse homogeneous redox reaction can be inhibited due to the presence of a surfactant in the system. The presence of a surfactant interferes in the thionine aggregation and polymerization process [125].

Several works about sensors have shown that the changing of spectroscopic and electrochemical properties of organic molecules, such as Toluidine Blue O (TBO), a phenothiazine dye, may indicate that there are some interaction with mediators and biological molecules [126,127,128]. Photochemical and electrochemical properties of TBO have been used to develop new photovoltaic devices for energy conversion and storage [129]. The aggregation behavior of such dyes in phase solution can be studied by using several optical rotation and circular dichroism techniques, as it can be seen in some studies of the interaction of TBO molecules on the DNA surface [130]. It was suggested that both intercalative and electrostatic interactions of TBO with DNA, where it was pointed out that the electrostatic interaction play an important role on the formation of the bridged structure of TBO with DNA [131].

TB can also be used as an oxygen radical inactivation, biological sensitizer and complexing agent in biological systems avoiding pathological changes [132]. Due to its low toxicity and high water solubility in salt form, which has an intense absorption peak in the visible region [133], it has been used in pharmaceutical formulations for cancer treatment [134]. Studies on the micellar solutions have shown that the aggregation properties and distribution behavior of toluidine blue in the presence of surfactant depend on the electrostatic interaction. In the case of surfactin, a natural surfactant, TB molecules can be located in the palisade of surfactin micelle [135].

Nile blue (NB) belongs to a class of molecules whose basic framework is that of a benzophenoxazine, a class which also includes Nile red, a phenoxazinone, here termed red

Nile blue (RNB) and Meldola's Blue. It has been found to be localized selectively in animal tumors [136] and can retard tumor growth [137,138]. NB has been used as a photosensitizer for oxygen in PDT applications [139,140], in processes that depend on solvent polarity [141,142], as a stain for *Escherichia coli* in flow cytometry [143], as a DNA probe [144] and many other applications [145,146,147,148]. Due to their high fluorescence quantum yield together with their solvatochromism, they have been used as stains and imaging agents. These dyes present relatively low solubility in aqueous medium as well as their fluorescence is reduced significantly in the presence of polar medium, which opens up new possibilities to develop aqueous analogues of these benzophenoxazines [149]. Together with the increase of the solubility in water, it is believed that the self-assembly process to form aggregates can be disrupted resulting in an enhancement of the fluorescence intensity [150].

NB shows thermochromic and solvatochromic behavior in its ultraviolet/visible spectra [151]. The variation in the absorption spectrum is due to the equilibrium between the monocation and the neutral molecule, where the monocationic form is the more stable in most solvents. In strong basic conditions the neutral form is observed, where in strong acidic conditions the dicationic and tricationic forms can be observed [152]. The fast decay processes study can be used to get information on the effect of medium condition, basic and acidic, on determining the excited state lifetime on the picosecond scale. It was shown that the reason for the faster decay in acidic conditions results from the formation of dications by reaction of excited state monocations with hydrogen ions [153].

Despite the photophysics of NB in pure solvents is well characterized in literature, the NB interaction with microheterogeneous systems, such as micelles, reverse micelles (RMs) and DNA is still not well understood. Electrochemical studies have shown that NB-DNA duplexes modified microelectrode can be used as a rapid and sensitive method to detect TATA binding to DNA in the presence of other proteins [154]. However, there are no many works done on its interaction with DNA [155,156,157,158]. In a work done on the interaction of NB with biomimicking self-organized assemblies (SDS micelles and AOT reverse micelles) and a genomic DNA (extracted from salmon sperm) (SS DNA), it has been shown that there are two different binding modes of NB with genomic DNA, electrostatic and intercalative modes [144]. There was no explanation for the mechanism related to these interaction modes. The electrostatic mode is believed to be responsible for electron transfer between the probe and DNA, which may result in a quenching process of the NB fluorescence emission intensity when in the presence of low concentration of DNA. The intercalative mode is believed to be the subsequent release of quenching due to the intercalation of the dye in DNA base pairs. In another study, it was shown that binding affinity of the probe is higher with SDS micelles than with the DNAs within its structural integrity in presence of the micelles. The complex rigidity of NB with various DNAs and its fluorescence quenching with DNAs has shown a strong recognition mechanism between NB and DNA [159].

NB was immobilized in two different surfaces, a nonreactive surface (SiO_2), with its conduction band at much higher energies, and a reactive surface (SiO_2), with a conduction band situated at lower energies. The former is used to directly probe the excited-state dynamics of the dye undisturbed by other competing processes. The latter is used to study

the charge injection process from the excited dye into the semiconductor nanocrystallites, acting as an electron acceptor. The transient absorption measurements of NB adsorbed on SiO₂ colloids (inert support) show that the NB aggregates have a relatively short-lived excitonic singlet state ($\tau = 40$ ps) (Table 1). The lifetime of the excited singlet of the monomer in aqueous solution is ~ 390 ps. NB aggregates that were immobilized on reactive surface also inject electrons into SnO₂, resulting in the formation of the cation radical, (NB)₂⁺, of the NB aggregates and by the trapping of electrons in SnO₂ nanocrystallites. The monophotonic dependence of the formation of (NB)₂⁺ on SnO₂ surface supports the charge transfer from NB aggregates to SnO₂. The rate constant for this heterogeneous electron transfer process is $\sim 3.3 \cdot 10^8 \text{ s}^{-1}$ [160].

4. Aggregation of photosensitizers and its influence in PDT

Most of these dyes form aggregates in the ground state [161,162,163], even when the dye concentration is low (approximately 10^{-6} M) and in the presence of salts and aggregation inducing agents, such as anionic micelles, heparin, polyelectrolyte, liposome and vesicles. The planar structure of such dyes is a key factor that contributes to the approaching and dimerization of the dyes [164,165].

The presence of hydrophobic ligands in the dye structure facilitates the aggregate formation in polar medium. The effects of the planar structure of the dye, hydrophobicity and the interaction with cell membranes were observed in photosynthetic systems II of plants [166] and other systems [167]. Some studies have shown that the interaction among phenothiazines and cyclodextrins results in the aggregate formation with different sizes depending on the cyclodextrins cavity size [162,168].

Studies that were conducted previously have shown that methylene blue molecules form aggregates and the photophysical behavior changes depending on the ground state aggregation. It results in a decreasing of the fluorescence intensity and on the singlet oxygen formation [49]. These studies have shown that the interaction with micelles is responsible for the dimerization process and not the interaction with monomer of the surfactant, as it has been postulated in some works [169]. In this stage of the work it is important to study the nature of the aggregates formed in different negative interfaces and in biological systems, more specifically in micelles, vesicles and mitochondria.

It is well known that dimerization and medium composition effects changes the energy transfer process among triplets species and molecular oxygen and other triplet suppressors [170,171,172,173,174,175,176,177,178]. Some studies carried out using thionine and MB have shown some of these effects [179]. Azure A, azure B, thionine e MB are dimerized with different dimerization constants.

The aggregation of ionic dyes cannot be assigned to a specific type of chemical interaction. There is a significant contribution of several influences, such as van der Waals interactions, intermolecular hydrogen bonds and pi-electrons interactions, being that, frequently, it is not trivial to evaluate the specific contribution of each one of these interactions [180].

The quantum behavior of extended aggregates of atomic and molecular monomers, containing from a just a few up to thousands of subunits, is attracting increasing attention in chemistry and physics, being that prominent examples are aggregates of large dye molecules, chromophore assemblies describing the photosynthetic unit of assemblies of ultra-cold atoms [181].

According to their structure, dyes, such as phenothiazinium, exhibit J- or H-aggregates, which present very typical J- or H-absorption bands [182]. The aggregate absorption band is red-shifted in relation to the monomer absorption. These are the J-aggregates showing a very narrow band whose position is well-predicted by a theory ignoring intramolecular vibrations. By contrast, other dyes showed a shift towards the blue (i.e. higher absorption energies) and were termed H-aggregates (hypsochromic shift). Unlike the J-band, the line shape of the H-band generally shows a rich vibrational structure and has a width of the order of the monomeric band [183]. The J-band is polarized parallel to the rods, while the H-band is polarized perpendicularly to the rod long-axis [184].

Self-organized J-aggregates of dye molecules, known for over 60 years, are emerging as remarkably versatile quantum systems with applications in photography, opto-electronics, solar cells, photobiology and as supramolecular fibres [185].

5. Future perspectives

Photodynamic Therapy has been used in clinical applications with significant success. Several studies have focused on the suitable conditions to improve the clinical results, such as the optimization of the incident light intensity. Indeed, the importance of irradiance is a determinant of PDT-induced pain. The increased use of low irradiance PDT may have a considerable impact on pain, which currently is the main limiting factor to successful delivery of PDT in some patients [186].

Another area of improvement of the PDT application is focused on the increase of the aqueous solubility of the photosensitizers. In fact, the photosensitizers require being suitable to several types of administration in biological medium. In this context, interesting photosensitizers that present low water solubility, such as C₆₀, constitute an area of scientific efforts. C₆₀ can be accumulated selectively in the target point. However, the biological application of C₆₀ is limited due to its poor solubility in water [187]. To improve the solubility of C₆₀ in water, several water-soluble derivatives have been synthesized. Furthermore, other solubilization methods for C₆₀ have been explored using cyclodextrins, calixerenes, micelles, liposomes, and poly(N-vinyl-2-pyrrolidone) (PNVP). In general, core-shell polymer micelles can be formed spontaneously by amphiphilic diblock copolymers due to association between hydrophobic blocks in water. The hydrophobic drugs can be incorporated into the hydrophobic core of the polymer micelle, and thus, the drugs can be solubilized in water. Nanosized water-soluble core-shell type polymer micelles can allow long circulation in the blood stream avoiding reticuloendothelial systems (RESs) and can be utilized for their enhanced permeability and retention (EPR) effect at solid tumor sites.

The production of ROS can be affected by factor, such as the aggregation and photobleaching of the photosensitizer. In fact, photosensitizers such as, for example, magnesium protoporphyrin (MgPpIX), have demonstrated that the aggregation and photobleaching reduce the photodynamic efficiency [188].

Low-level laser therapy has been used to speed up healing process of pressure ulcers due to its antiinflammatory, analgesic, anti-edematous, and scarring effects, since there is no consensus on its effect on infected ulcers [189]. It is an interesting topic to be evaluated in novel studies.

It is known that Gram positive bacteria are more sensitive to PDT as compared to Gram negative species. However, the use of cationic photosensitizers or agents that increase the permeability of the outer membrane allows the effective killing of Gram negative organisms [190]. Some photosensitizers have an innate positive charge, but some approaches are focused on to link photosensitizers to a cationic molecular vehicle, such as poly-L-lysine [190].

Photodynamic therapy has been also applied in dentistry, in endodontic treatments, with auspicious results regarding the control of microbial infections associated to this type of odontologic therapy [191].

The increasing application of PDT has motivated the development of other therapeutic techniques, with similar principles. We can mention the case of Sonodynamic Therapy (SDT). In 1989, Umemura and co-workers first pioneered the development of non-thermal ultrasound activating a group of photosensitizers for treating tumor, which is called Sonodynamic Therapy (SDT) [192]. They reported that the photosensitive compounds activated by ultrasound can kill cancerous cells and suppress the growth of tumor. Otherwise, they also thought highly of that the ultrasound could reach deep-seated tumor and maintain the focus energy in a small volume because of exceedingly strong penetration ability and mature focusing technology [193]. Particularly, SDT was developed from the well-known PDT but only put up low phototoxicity. Therefore, in recent years, along with the lucubration the SDT has attracted considerable attention and has been considered as a promising tumor treatment method [192].

Regarding the development of photosensitizers, it is important to register the relevant role of phthalocyanines. Phthalocyanines (Pcs) are highly delocalized p-conjugated organic systems and exhibit wide variety of roles in a various high technological areas such as semiconductor devices, liquid crystals, sensors, catalysts, non-linear optics, photovoltaic solar cells and PDT [194,195]. They are among the most important promising chemical compounds by advantage of their stability, photophysical, photochemical, redox and coordination properties. The properties of Pcs depend on their molecular composition with the number; position and nature of substituents and type of central metal play an important role in controlling their properties [194].

Indeed, Pcs have many considerable physical and chemical features, which have motivated the interest of several investigators because of their physico-chemical properties [195]. The presence of different substituents on the Pc ring also leads to increased solubility and supramolecular organizations with improved physicochemical characteristics, depending of

the interest in terms of application [194]. In fact, phthalocyanines are very versatile chemical systems, which allow great variability of adjustment of properties in the process of chemical synthesis. This great number of structural possibilities has been utilized in many fields, since the different phthalocyanines can be applied in quite different areas, such as gas sensors, semiconductor materials, photovoltaic cells, liquid crystals, optical limiting devices, molecular electronics, non-linear optical applications, Langmuir-Blodgett films, fibrous assemblies and PDT [195].

6. Conclusions

The selection of photosensitizers that are more able to generate an efficient photodynamic action is one of the main questions involving PDT in the present days. The novel generations of photosensitizers is aiming to obtain the maximum quantum yield through the therapeutic window, avoiding spectral ranges that are absorbed by endogenous dyes, such as hemoglobin and melanin. In this way, the previous knowledge regarding the spectroscopic behavior of the new prototypes of photosensitizers is a relevant pre-requisite to the advancement of the types of applications and its respective repercussions, since the efficacy of the methodology depends on the capability of generations of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are intrinsically related to the optical profile of the photosensitizers.

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