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Effects of ionic strength on the antimicrobial photodynamic efficiency of methylene blue

Silvia Cristina Núñez,*[a] Aguinaldo Silva Garcez,[a,b] Ilka Tiemy Kato,[a] Tania Mateus Yoshimura,[a] Laércio Gomes,[a] Maurício Silva Baptista[c] and Martha Simões Ribeiro[a]

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Antimicrobial photodynamic therapy (APDT) may become a useful clinical tool to treat microbial infections, and methylene blue (MB) is a well-known photosensitizer constantly employed in APDT studies, and although MB presents good efficiency in antimicrobial studies, some of the MB photochemical characteristics still have to be evaluated in terms of APDT. This work aimed to evaluate the role of MB solvent's ionic strength regarding dimerization, photochemistry, and photodynamic antimicrobial efficiency. Microbiological survival fraction assays on *Escherichia coli* were employed to verify the solution's influence on MB antimicrobial activity. MB was evaluated in deionized water and 0.9% saline solution through optical absorption spectroscopy; the solutions were also analysed *via* dissolved oxygen availability and reactive oxygen species (ROS) production. Our results show that bacterial reduction was increased in deionized water. Also we demonstrated that saline solution presents less oxygen availability than water, the dimer/monomer ratio for MB in saline is smaller than in water and MB presented a higher production of ROS in water than in 0.9% saline. Together, our results indicate the importance of the ionic strength in the photodynamic effectiveness and point out that this variable must be taken into account to design antimicrobial studies and to evaluate similar studies that might present conflicting results.

Introduction

Photodynamic therapy is a therapeutic approach that combines a chemical compound known as a photosensitizer and light to produce oxidative stress at the target area. Antimicrobial photodynamic therapy (APDT) has been intensively studied in the last few years, and substantial evidence has been collected to prove its efficiency against a broad spectrum of microbes including bacteria,[1,2] fungi[3,4] and viruses[5,6] *in vitro*. For topical APDT the photosensitizer (PS) can be directly delivered to the target and the light source can be focused on the mark without significant obstacles, with an added bonus in that the dye concentrations and light fluences that kill microorganisms are lower than those required to kill host cells or damage tissues.[7,8]

The phenothiazine dyes methylene blue (MB) and toluidine blue (TBO) used for APDT[1,2,9] are well known and have been employed for different uses in the medical field.[10–12] Some principles regarding the employment of these compounds are extensively studied by chemistry scientists and are somehow disregarded during their antimicrobial application, such as, for instance, the aggregation effect upon increasing dye concentration, the influence of the medium electrical charge and different dimer and monomer photochemical behaviours.[13–16] As an example we can point out the wide range of concentrations used in different antimicrobial studies.[17,18] On the topic of concentration, though toxicity to host cells is critical, of equal importance is the dye dimerization process, which is characteristic of this class of dyes.[9,19] MB and TBO absorption spectra are concentration-dependent; dye molecules often form dimers or higher aggregates in solution.[9]

The dimerization may increase with the amplification of the ionic strength and may increase or decrease due to the presence of charged interfaces, depending on the ratio between the dye and the interface.[14,15] Gram-negative bacteria promote increased dye dimerization,[9] and this class of microorganism is in general less susceptible to APDT; thus it can be hypothesized that the dimerization process may be a limitation on APDT efficiency with these dyes.

The different photochemical behaviour of MB dimers and monomers has also been reported[14] and it may be another important factor that would determine the clinical effectiveness of this therapy employing such photosensitizers. MB dimers tend to participate in type I reactions where the electron transfer between the triplet state sensitizer and

[a]*Center for Lasers and Applications, IPEN-CNEN/SP, São Paulo, SP, Brasil. E-mail: silvianunez@uol.com.br; Fax: +55-11-26722284; Tel: +55-11-996037089*
[b]*São Leopoldo Mandic, Campinas, SP, Brasil*
[c]*Department of Biochemistry, IQ-USP/SP, São Paulo, SP, Brasil*

biomolecules results in the generation of several reactive oxygen species (ROS) like superoxide, hydrogen peroxide and anion superoxide. On the other hand, MB monomers are usually involved in type II reaction where the energy transfer takes place between a triplet state sensitizer and molecular oxygen producing singlet oxygen ($^1O_2$), a highly reactive oxygen species.[14,15]

The solution used to dilute the dye and the medium where the photosensitizer will act may also affect the dimerization status of the dye[14,16] and, furthermore, depending on the dye solvent's ionic charge and its characteristics, the dissolved oxygen concentration on the solution may also play an important role, because more O_2 molecules would mean an increased probability of energy-transfer reactions (Type II reaction) due to the quencher ability of the O_2 molecules with dye-triplet state energy.[20] Several antimicrobial studies present no distinction regarding the solvent used to dilute the dye, some of them use water,[21–23] others saline solution[1,3,9] and even PBS (phosphate buffer saline)[24–26] and the solvent ionic charge may influence the photochemistry of MB rendering difficult the comparisons among these studies.

Therefore the purpose of this work is to investigate the effect of a small increase in the ionic strength of the medium represented by 0.9% saline solution on the MB dimerization process and its effect on the dye photodynamic antimicrobial activity. To test the microbiological effect of this variable, cultures of *Escherichia coli* were exposed to APDT and the solution effect was evaluated. Spectroscopic analyses of the dye were performed on the solutions to determine the dimer to monomer ratio during the course of the irradiation. Dissolved oxygen availability was measured in each condition. Singlet oxygen direct detection and indirect reactive oxygen species measurements were performed to qualify the best solution for ROS production. These analyses were performed in an attempt to define variables that should be noted when comparing results from different studies as well as to understand in which way we should work to improve APDT efficiency.

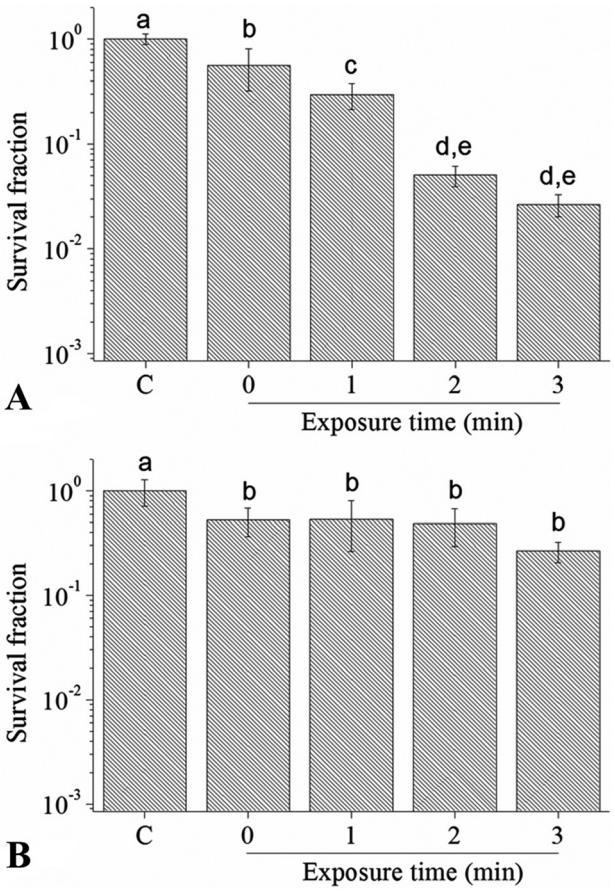

Fig. 1 Effect of MB (30 μM) on the photoinactivation of *E. coli* in (A) water and (B) saline solution. Irradiation was performed with a 660 nm laser, power of 40 mW and irradiance of about 100 mW cm^{-2}. Different letters mean statistically significant differences.

Table 1 Dissolved oxygen concentration measured in each solution

Solvent	Mean and standard deviation (mg L^{-1}) $n = 10$
Deionized water	7.48 ± 0.01
0.9% saline solution	7.37 ± 0.04

Results

The environmental control groups as well as the PS dark toxicity control group did not present any antimicrobial activity.

Comparing the bacterial killings between solutions using 30 μM of MB dissolved in water or 0.9% saline solution, a statistically significant difference for bacterial reduction in water was observed, as can be seen in Fig. 1A. Bacterial reduction using MB and saline solution under the investigated parameters was only significant when comparing the different irradiation times and the control group (Fig. 1B). There was no significant difference among other saline groups (Fig. 1B). On the other hand, the same conditions using water as the solvent presented a significant ($P < 0.05$) difference among all groups except between 2 and 3 minutes of irradiation as described in Fig. 1A. As we can perceive through the analysis of the results, less than 1% difference in the ionic strength of the solutions led to a more than 1 log difference on bacterial reduction.

The results on the oxygen availability for the solvents are presented in Table 1. Observe that the oxygen concentration in deionized water is significantly higher than in 0.9% saline solution ($P = 0.0049$). Water solubility of oxygen at 25 °C and a pressure of 1 bar is about 10 mg L^{-1}. Oxygen solubility is strongly temperature dependent and decreases at high temperatures; in our case all the data were collected at room temperature (22 °C). Also oxygen solubility is negatively correlated with the amount of dissolved solids; consequently the oxygen in fresh water exceeds that of salted water by 1–3 mg L^{-1} depending on the temperature.[27]

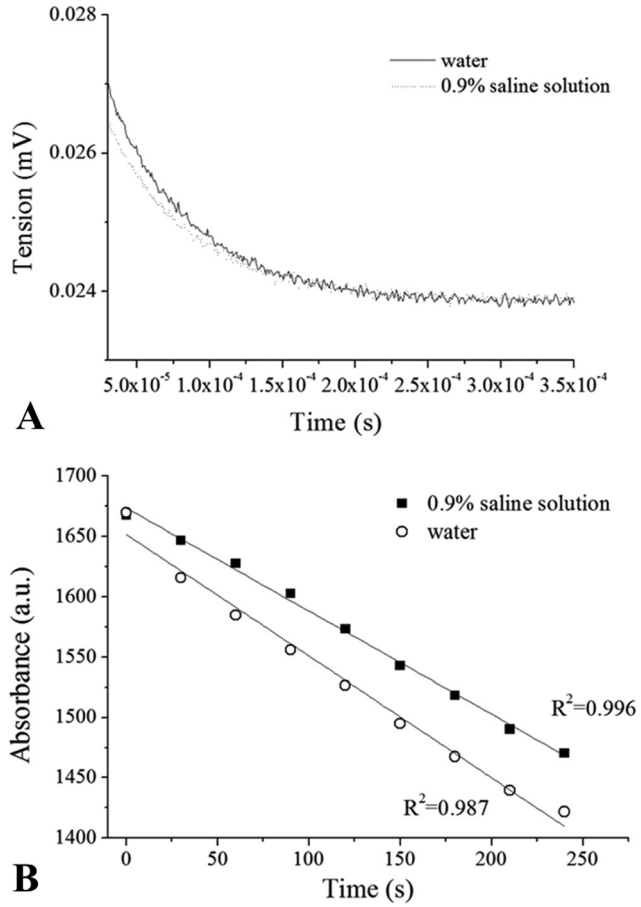

Fig. 2 Emission signal of singlet oxygen at 1270 nm in MB solutions (30 μM) prepared in D_2O and in D_2O with 0.9% NaCl (A) and oxidation of RNO by reactive oxygen species produced by illumination of MB in the presence of histidine to produce colorless products, measured by loss of absorbance at 440 nm (B).

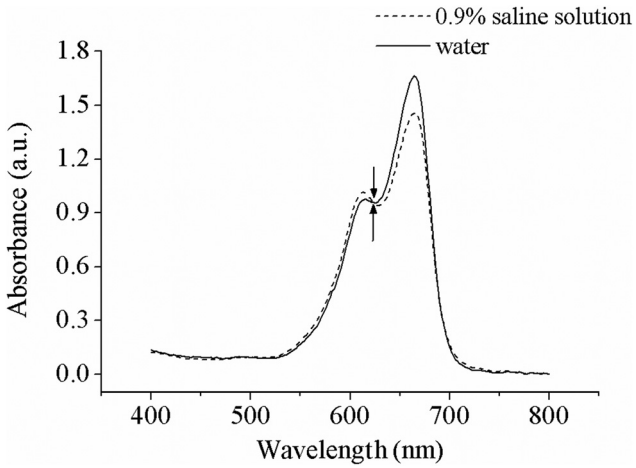

Fig. 3 Absorbance spectra of MB (30 μM) in water and in saline solution 0.9%. Note the presence of the isosbestic point around 624 nm for MB.

Measurement of the singlet oxygen emission at $\lambda = 1270$ nm revealed that MB in water generated about 2% more $^1O_2$ than 0.9% saline solution. Fig. 2A represents the graphical data for singlet oxygen emission. It can be observed that the RNO (4-nitroso-*N*,*N*-dimethyl-aniline) degradation also showed less formation of ROS when MB is dissolved in saline solution (Fig. 2B). From the two different measurement analyses, we observed a tendency of lower reactive oxygen species production when the photosensitizer is dissolved in saline solution, which represents a medium with a slightly higher ionic strength.

To compare the rates of RNO consumption, we assumed that the absorbance can be expressed by $A(t) = A_0 + \alpha t$, where $A(t)$ is the intensity of absorbance, A_0 is the maximum absorbance, and α is the decay constant. Then, the rate of RNO consumption can be calculated from the formulae: $V = dA(t)/dt$.[28] Thus, the relative rate (Vwater/Vsaline) of ROS formation in the presence of water is approximately 20% faster than in saline solution.

Fig. 3 represents the absorption spectra of MB in the two different solvents. Note that the dye presents an increased aggregation denoted by the smaller monomer band (absorption at 660 nm) when dissolved in saline solution. The isosbestic point, which is the wavelength where the change in concentration does not affect the absorbance, is around 624 nm for MB as we can see in Fig. 3. The point of intersection in the overlapping spectra as a function of concentration shows the presence of a mixture of two states (monomer and aggregate) and the presence of the isosbestic point is strong evidence of complexes formation.[29]

Fig. 4 displays the DA/MA (dimer absorbance/monomer absorbance) in MB/bacteria suspension. The comparison between water and saline at each time point revealed that the DA/MA ratio is significantly higher in saline than in water solution at all evaluated moments ($P < 0.05$). Sodium chloride in the solution, even in concentration as low as 0.9%, leads to the formation of a heterogeneous system, acting as a matrix of charges which induces the ordering of dye molecules, reproducing the same type of interactions as those observed in more concentrated homogeneous solutions. Thus, even in much diluted concentrations we can observe the formation of dye aggregates and the process becomes more evident with increased concentration.[9]

The statistical analysis revealed the same behaviour of the DA/MA on the two solutions upon irradiation. After 3 min of pre-irradiation time (PIT) the solutions did not present a significant difference in DA/MA compared to the initial moment. Interestingly, after irradiation we observed a disaggregation tendency in both solutions represented by a significant decrease in DA/MA (Fig. 4).

Discussion

Our study tried to elucidate the role of the dye solvent ionic strength in APDT with MB, and to accomplish this we used *E. coli*, a Gram negative microorganism as a proof of concept.

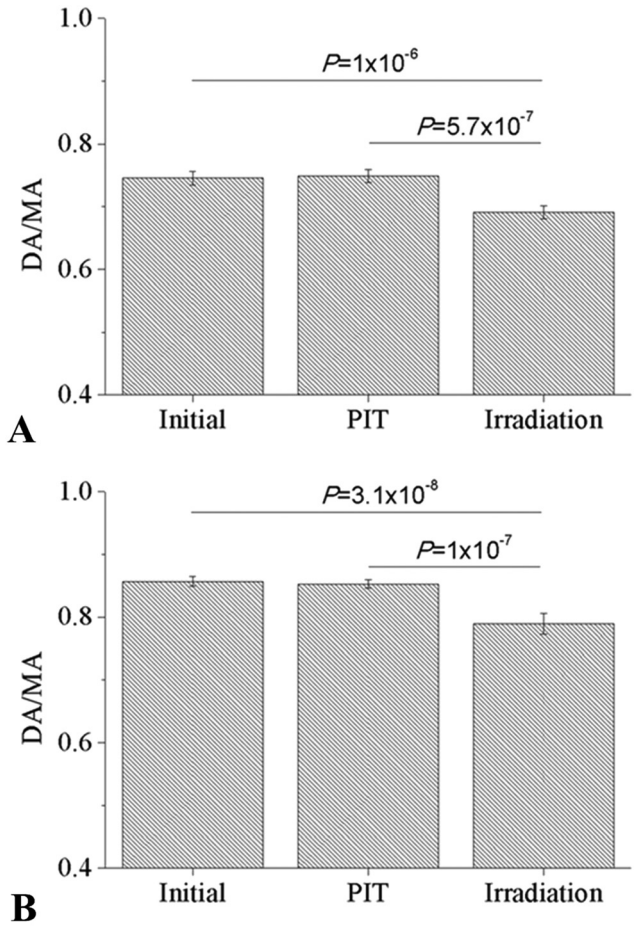

Fig. 4 DA/MA (dimer absorbance/monomer absorbance ratio) for MB (30 μM) in (A) water and (B) saline solution. Pre-irradiation time (PIT) represents measurements conducted 3 min after incubation of bacterial suspension with MB.

The results indicated that for MB the solution is involved in its antibacterial activity despite other parameters such as light and dye concentration. Our results also showed that the dye photochemistry is different regardless of the presence of the microorganisms.

The better photodynamic activity in a smaller ionic strength environment can be explained with different mechanisms and at least two of our results could be directly linked with the antibacterial effect. The first one is the smaller O_2 molecule availability and the second would be ROS formation, which was smaller on dye dissolved in 0.9% saline solution. These two results may be linked since less O_2 means a smaller probability of energy transfer and this parameter is considered very important in cancer PDT.[30] Although the difference in O_2 concentration between the two solvents seemed small (approximately 0.11 ppm) it would represent nearly 2.06×10^{18} less O_2 molecules. Besides that, according to our results it promotes a significant difference in the antimicrobial photodynamic activity of MB. Therefore, we can assume that any attempt made in order to increase oxygen tension in APDT target sites can be considered valid to improve antimicrobial results.

As mentioned above, the diminished molecular oxygen availability in the solution may contribute to less photodynamic effect. In fact, Demidova and Hamblin[24] showed lower photoactivity of TBO, Rose Bengal and chlorine upon increasing the bacterial concentration. The ratio between reactive oxygen species and targets was considered one important factor in such cases. Although the ratio is clearly an important issue, other phenomena take place at the same time, worsening the photodynamic effect. Indeed, the work of Maisch *et al.* showed that oxygen consumption by bacteria and also the molecular oxygen availability on the solution are directly linked with singlet oxygen production and with porphyrin antimicrobial activity against Gram-positive and also Gram-negative microorganisms.[31] According to the authors, molecular oxygen availability is extremely important for the photodynamic effect; therefore, *in vitro* as well as *in vivo* tests with low oxygen concentrations will render a lower antimicrobial effect. According to our results, less than 1% of NaCl in the solution not only diminishes the molecular oxygen in the solution but also diminishes the singlet oxygen signal and ROS formation. With 0.9% of NaCl in the solution we observed a 2% decrease in singlet oxygen emission. If even a small increase in ionic strength promoted by the salt leads to changes in the dye characteristics, in clinical situations where a much higher ionic strength is expected the alterations can be even more evident.

Also the different photochemical behaviour obtained from the dye dimers and monomers previously described can also be responsible for our results.[14,15] Solutions with higher ionic strength represented in our study by 0.9% of NaCl added into water led to an increased dimerization process (Fig. 3 and 4). MB dimers tend to participate in electron-transfer reactions; therefore the singlet oxygen production is smaller when increased concentrations of dye dimers are present.[13–15] The results obtained from direct and indirect singlet oxygen measurements in different solutions confirm these data, since the increased aggregation promoted by the NaCl in solution leads to a decreased intensity of the singlet oxygen emission signal and lower ROS generation.

The importance of the photochemical reaction type I or type II (electron transfer or energy transfer) is not clear. Microorganisms have evolved complex adaptive mechanisms for protection against ROS and oxidative stress, including enzymatic and non-enzymatic oxidant-scavenging systems,[32] and for the success of APDT, the amount of ROS formed has to surpass the bacterial defences. Radical formation is certainly very important for microbial reduction, although singlet oxygen seems to play a major role in the process according to our results and also the work of Tavares *et al.* The authors showed a major role of singlet oxygen employing $^1O_2$ quencher and free radicals scavenger to evaluate the importance of type I or II reaction against *E. coli* using a cationic porphyrin.[33] On the other hand, some papers have demonstrated contradicting results and pointed out that microorganisms may present different responses to the formation of singlet oxygen, and even depending on the type of photosensitizer,

the ROS formation may happen in different microbial targets.[34,35]

Our results are also important when comparing APDT studies that use different solutions to perform the photochemical tests.[1–3,21–26] Consistent with our results, the simple fact of using a different solution urges caution when comparing studies that used different dye solvents. Our microbiological study showed a significant difference in the same bacteria promoted only by the use of 0.9% saline solution or water, since illumination parameters and dye concentration were maintained equal in all assays.

According to our results, when MB is dissolved in an appropriate solution we will obtain a better photodynamic outcome. Irradiation parameters should be of utmost importance in modifying APDT parameters.[36] As we point out, increasing dye concentration may not be the best way of improvement since the aggregation tendency that comes with increasing dye concentration will also affect the DA/MA.[9] Fractioned light as used in cancer PDT to allow oxygen reposition on the target tissue should be tested. Previous work with *Streptococcus mutans* biofilm showed better results with fractioned light delivery.[37]

Materials and methods

Strain and inoculum preparation

Cultures of *E. coli* (ATCC 33694) in the stationary phase were obtained after 48 h of growth in brain and heart infusion (BHI) agar with aeration at 37 °C. Cells were harvested by swab and further suspended in either distilled water or 0.9% saline solution. Suspensions of 10^6 colony forming units per milliliter (CFU per mL) were standardized setting the solution transmittance by 95% at 600 nm (Spectrophotometer SP220, Biospectro, Brazil) with subsequent dilution at 1 : 10.

Effect of the solvent in APDT

The samples were separated according to the experimental purpose. In order to verify the viability of *E. coli* in water suspension and saline solution, two environmental control groups were set, one for water and another for saline solution, both containing 100 μL of bacterial suspension mixed with 100 μL of the appropriate diluents placed in a sterile 96-well microwell plate. Part of these bacterial suspensions was kept during the entire microbiological experiment resting in the dark, and thereafter was ten-fold serially diluted in PBS. Another part was diluted for plating immediately after preparation. Aliquots (10 μL) of each dilution were plated on BHI agar using the track-dilution method.[38] Plates were incubated for 12 h at 37 °C, and the colony forming units were counted and converted into CFU per mL values.

Another two control groups were prepared to evaluate the dark toxicity of 30 μM MB in water and in saline solutions. Suspensions were kept in the dark during the entire experiment and then were ten-fold serially diluted in PBS and incubated as mentioned above to obtain the CFU per mL values.

After the controls setting we prepared two treatment groups: Treatment Group Water and Treatment Group Saline. Bacterial suspensions were prepared in water or saline, with a final MB concentration of 30 μM. The bacterial–dye mixtures were kept in the dark for 3 min and then the irradiation was performed with a diode laser (InGaAlP) with λ = 660 nm ± 2 nm (Twinflex, MMOptics, São Carlos, Brazil), exposure time of 1, 2 or 3 min (energies of 2.4 J, 4.8 J and 7.2 J), and total output power of 40 mW as verified using an appropriate power meter (Scientech 373, Boulder Corp., USA). The laser probe was fixed on a holder that kept the beam area at 0.4 cm^2, which coincided with a single well size from the 96-well microwell plate. After each treatment, the groups were ten-fold serially diluted in PBS and aliquots (10 μL each) were plated on BHI agar using the track-dilution method mentioned above. Plates were incubated to obtain the CFU per mL values after 12 h. The irradiation parameters were chosen to allow an identification of possible differences between groups; therefore, a moderate reduction was expected.

Microbiological data analysis

Bacterial reduction is presented as survival fraction. The data were statistically analysed by a one-way ANOVA test followed by the Tukey test with the significance level accepted at 5% ($P < 0.05$). According to our microbiological results we decided to pursue an investigation on some variables that might have been responsible for the observed effects and we started with the evaluation of the total oxygen availability on each solution.

Dissolved oxygen in solutions

The dissolved oxygen availability on each solution was measured with a dissolved oxygen meter (Hanna Instruments, London, UK) with the appropriate calibration for altitude and salinity. Measurements were performed under magnetic stirring (150 rpm) in room temperature. The calibrated probe was introduced into the solution and after 30 s, the acquired values in part per million (ppm) were recorded. Since dissolved oxygen varies due to altitude (pressure), temperature and salinity, we investigated this variable to verify whether, even in small amounts, the presence of NaCl would diminish the O_2 molecular availability. Data were compared through statistical analysis (Student's t test) with $P < 0.05$ being considered a significant difference. After evaluating the molecular oxygen accessibility we decided to investigate ROS production in different solutions starting with singlet oxygen emission involved in type II PDT mechanisms.

Singlet oxygen emission

The singlet oxygen emission at λ = 1270 nm was measured with 30 μM MB (Sigma-Aldrich, USA) dissolved in D_2O, and in 0.9% saline solution prepared with D_2O. D_2O was used to enhance the singlet oxygen emission signal due to an increased lifetime of this molecule promoted by D_2O. Absorbance spectra of each solution were recorded on a computer-interfaced spectrophotometer (Shimadzu, Multi Spec-1501, Japan). Fluorescence spectra were recorded in a fluorimeter

(Spex 1681, Fluorolog, USA) in a right-angle mode, compatible-PC spectrometer. The spectral data were obtained in 1.0 cm path length quartz cuvettes, and further manipulation was performed with Origin® (Microcal, MA, USA) to endorse the correction of the concentration *via* absorption at λ = 532 nm in both solutions. For the near infrared (NIR) emission measurements, a Nd:YAG laser (λ = 532 nm) (Continuum, Surelite III, CA, USA) excited the sample into a fluorimeter (Edinburgh Analytical Instruments, WL, UK) connected to a NIR-photomultiplier tube (R5509, Hamamatsu Co., NJ, USA). The emission wavelength was selected using both a silicon cut-off filter and a monochromator. Singlet oxygen emission spectra with the maximum around λ = 1270 nm were obtained from each solution after the accretion of 20 laser pulses signal. Spectra were manipulated with Origin® (Microcal, MA, USA) software.

ROS detection

We also performed a chemical assay to detect ROS *via* oxidation of RNO. All chemicals were obtained from Sigma Aldrich (Milwaukee, WI) and used without further purification. A cuvette containing 15 mM L-histidine and 30 μM MB was blanked. RNO was added to achieve a measured absorbance of approximately 1 at 440 nm (13.3 μM concentration). Illumination was performed with broadband white light at an incident power of approximately 3 W, and loss of absorbance was measured every 10 s at 440 nm. RNO is used for the evaluation of the oxidation power of the system since it is easily bleached by ROS losing its main absorption band at 440 nm.[39]

Optical characteristics of the dyes

Optical characteristics of the dye regarding the dimer/monomer ratio (DA/MA) in different solvents were obtained through absorbance spectra in a spectrophotometer (Cary-Spectrophotometer Conversion, On-Line Instrument System Inc., USA) with 1.0 cm optical path length quartz cuvettes. Commercially available MB (Sigma-Aldrich, USA) was dissolved in either deionized water or saline solution at a concentration of 30 μM and their absorption spectra were recorded. After that, we performed a spectroscopic analysis of the dimer to monomer ratio using a 96-well plate in a plate reader spectrophotometer (SpectraMax M4; Molecuar Devices, USA). Two suspensions were prepared, one in water and another in saline solution, containing 10^6 CFU per mL of *E. coli* and 30 μM MB.

Spectra were analysed regarding DA/MA according to the following formula: MB: $\left[\frac{DA}{MA}\right] = A610\,nm/A660\,nm$ where DA means dimer absorption (610 nm), and MA monomer absorption (660 nm), for MB.[9] We performed 10 different measurements (n = 10) and calculated the mean and standard error for the experiment.

Conclusion

The results of this study may present a significant contribution to the evaluation of different trials performed in APDT. The variables involved in the process such as dye class, bacteria, and irradiation variables must be accredited, but the ionic strength of the medium and oxygen availability should also be analysed, so the puzzle of APDT may be put together and sooner we would be able to achieve more safe and effective results.

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