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Controlling methylene blue aggregation: a more efficient alternative to treat *Candida albicans* infections using photodynamic therapy

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Methylene Blue (MB) has been widely used in antimicrobial Photodynamic Therapy (aPDT), however, the mechanisms of action (Type I or Type II) are defined by its state of aggregation. In this sense, the identification of the relationships between aggregation, the mechanisms of action and the effectiveness against microorganisms, as well as the establishment of the means and the formulations that may favor the most effective mechanisms, are essential. Thus, the objective of this study was to assess the *in vitro* aPDT efficacies against *Candida albicans*, by using MB in vehicles which may influence the aggregation and present an oral formulation (OF) containing MB, to be used in clinical aPDT procedures. The efficacy of MB at 20 mg L^{-1} was tested in a range of vehicles (water, physiological solution – NaCl 0.9%, phosphate saline buffer – PBS, sodium dodecyl sulfate 0.25% – SDS and urea 1 mol L^{-1}) in a *C. albicans* planktonic culture, when using 4.68 J cm^{-2} of 640 ± 12 nm LED for the irradiations, as well as 5 minutes of pre-irradiation time, together with measuring the UFC mL^{-1}. Based upon these analyses, an OF containing MB in the most effective vehicle was tested in the biofilms, as a proposal for clinical applications. When comparing some of the vehicles, sodium dodecyl sulfate was the only one that enhanced an MB aPDT efficacy in a planktonic *C. albicans* culture. This OF was tested in the biofilms and 50 mg L^{-1} MB was necessary, in order to achieve some reduction in the cell viabilities after the various treatments. The light dosimetries still need further adaptations, in order for this formulation to be used in clinical applications. The present research has indicated that the development of this formulation for the control of MB aggregations may result in more effective clinical protocols.

Introduction

Yeasts of the genus *Candida* are commensal microorganisms that are to be found in the vaginal mucosa, the gastrointestinal tract, in the skin, or in the oral mucosa of humans, without necessarily causing a pathogenic process.[1] Some etiological factors destabilize the natural balance of the oral microbiota and facilitate the developments of infection, such as nutritional deficiencies, the use of oral prostheses, metabolic diseases, immunosuppression, mucosal lesions, deficiencies in oral hygiene, broad-spectrum antibiotic therapy and long-term corticoid therapy.[2–5] Candidiasis is an opportunistic fungal infection that is considered to be the most common in humans, being generally caused by yeasts of the genus *Candida*, of which more than 80% of the g per cases of clinical infections are caused by *C. albicans*, *C. glabrata* and *C. tropicalis*.[6] These 3 are also the species that retain the greatest biofilm-forming capacities. Among the three species, *C. albicans* is the most pathogenic.[7] The treatment for oral candidiasis is carried out by using synthetic antifungal agents. However, they may exhibit limitations of use, such as adverse reactions (hepatotoxicity in a prolonged use), in addition to the development of a microorganism resistance, due to an indiscriminate use of antimicrobials, often rendering them as being ineffective in treatments.[8] Thus, antimicrobial photodynamic therapy (aPDT) has emerged as an alternative to conventional treatments.

This aPDT is capable of eliminating microorganisms, by using an appropriate combination of light and dye in the presence of oxygen.[9–12] The discovery that an aPDT could be used

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as a therapy for local infections is recent and its clinical applications are still under development.[10,13–16] Among the advantages of applying an aPDT as an antimicrobial therapy are the independent efficacies regarding an antimicrobial resistance, a minimal invasiveness, an absence of side effects when used within the appropriate parameters, a broad spectrum of action, since this photosensitizer can act on any microorganism.[9] There are expensive photosensitizers and formulations for an aPDT on the market, but in this study, when using methylene blue (MB) in the formulation as a photosensitizer, it became an economically viable technique. *Candida* spp. can be inactivated by an aPDT.[17–19] Several studies have reported the effectiveness of methylene blue as a photosensitizer *in vitro*.[20–22] However, the literature has only a few clinical trials that have shown the applicability of the parameters that are used in the *in vitro* and *in vivo* studies of an aPDT with MB in patients with pathologies that are usually caused by *Candida albicans*.[11,23] Although it is a widely available photosensitizer, due to its low cost and effectiveness,[24] the application parameters are somewhat controversial. Within the fungal infections that involve *C. albicans*, there are many divergences regarding the treatment parameters, such as the MB concentrations that range from 100.0–500.0 (mg L^{-1}), which can irradiate *C. albicans* by the most diverse of light sources, for instance by an LED device (LED with an emission maximum of about 660 nm), or by an InGaAlP laser at 660 nm. This is since MB absorbs light from 550 nm to 700 nm, so that the monomer has the maxima occurring at 664 nm, with the dimer occurring at 590 nm.[25,26]

Besides, the wavelengths range between 630 nm, 660 nm and 664 nm; the radiant exposure is from 7.5 J cm^{-2}–245 J cm^{-2}, while the irradiation periods vary from 1.15–11.45 minutes and the dark incubation periods can alter by 1 to 10 minutes, all according to Javed *et al.*[25]

In addition to these highly important issues, the delivery of MB should be considered to be a critical factor for therapy effectiveness, since depending upon the physicochemical environment in which it is found, MB may aggregate and its state of aggregation (monomers or dimers) modulates with the type of photochemical reaction that occurs, thus, affecting the therapy's effectiveness.[27,28] Carvalho and collaborators have shown that the medium involved has a significant influence on the phototoxic effects of an aPDT with MB, however, a satisfactory mechanism has not yet been elucidated.[29] On the other hand, Lyon *et al.* have demonstrated the synergistic effects for the use of an aPDT with MB and surfactant agents.[30] Furthermore, Prochnow and collaborators have described that the use of MB formulations in ethanol were more effective than when dispersed in water with biofilms containing *Pseudomonas aeruginosa*.[31] Meanwhile, Nuñez *et al.* reported that MB-urea mixtures have shown greater antimicrobial activities, when compared to the aqueous solutions of MB, when they were employed for *C. albicans*.[32] There are indications that the presence of other molecules, such as sodium dodecyl sulfate and urea, can control the amount of dimers in the solution.[27,32] Given these findings, the development of formulations for clinical applications that can control an MB aggregation and, consequently, more efficient clinical protocols, has been made possible. The objective of the present study was to evaluate the effects of different mediums in an MB aPDT efficacy, when implemented for *C. albicans* and based upon that, to propose and to evaluate the utilizations of oral formulations (OF) that can control an MB aggregation, so as to be used in future clinical protocols.

Results and discussion

Photodynamic therapy has been employed in antifungal therapies, with optimal results,[9,10,11,13–17,19,33,34,35] even for those species that are resistant to azole antifungals. However, although these studies are promising, Javed and collaborators[25] have shown that there is an inconsistency regarding the parameters used in photodynamic therapy as a treatment for *Candida albicans*. Within the variations of these parameters, such as duration of irradiation, the light source and the concentrations, it has become unfeasible to gather data and perform a coherent comparison, in order to identify the most efficient protocol. Due to this, the researchers of this study have decided to determine the most adequate experimental parameters within their laboratory conditions.

While maintaining the MB concentrations at 20 mg L^{-1} and preserving the duration of irradiation at 10 minutes (1.56 J cm^{-2}), the dark incubation periods (DIP) were tested between 1 and 20 minutes (1, 5, 10, 15, and 20). It could be noted that an increase in the incubation period, did not influence the inactivation effectiveness of *C. albicans* in a planktonic culture (Fig. 1A) and that no significant statistical differences were observed between the periods of incubation. These DIPs were required for the photosensitizer to contact the microorganisms and to bind to or penetrate the plasma membrane, thus, causing damages when photoactivated.[34] In the present study, the variation in DIP did not result in a greater inactivation of *C. albicans*. Other studies concluded that microorganism inactivations were indifferent to the variation in DIP, although the studies have used other photosensitizers and biofilms as their experimental models.[34,36] Since MB was not removed prior to irradiation, the microorganisms were irradiated in the presence of the photosensitizer solution, which may have resulted in the absence of the differences between the dark incubation periods. If the solution had been removed, only the MB that was bound to or uptaken by the microorganisms would have been photoactivated. Despite this, Chabrier-Roselló and collaborators, when using a methodology that removed the photosensitizer prior to irradiation, determined a DIP of 1 to 5 minutes.[36] Andrade *et al.* reported that the optimal DIP was of between 4 to 8 minutes, when the different species of *Candida* in suspension were evaluated.[37] Given that no statistically significant differences were observed between the assessed periods, a duration of incubation of 5 minutes was adopted for this current research, as has been used for MB[20,38,39] and other photosensitizers in some studies.[18,40]

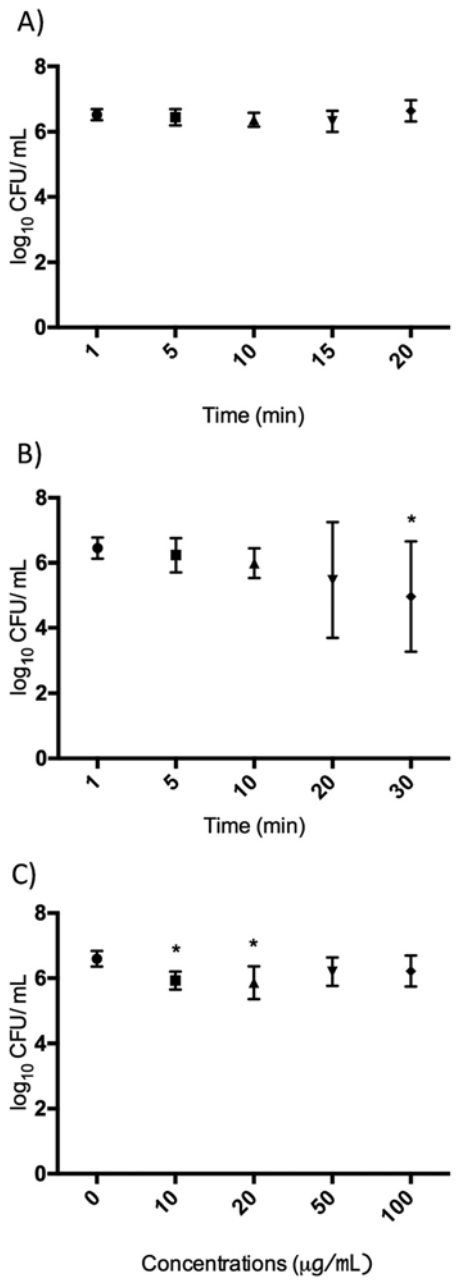

Fig. 1 Experimental parameters standardization in planktonic *C. albicans* culture. (A) Dark incubation period; (B) LED irradiation period (640 nm ± 12 nm, with 2.6 mW cm^{-2}); (C) methylene blue concentration. Experiments were performed in triplicate and 3 independent experiments (n = 9). Data presented as average ± SD. *p < 0,05 compared to control group.

In order to determine the irradiation period, 20 mg L^{-1} of MB and a DIP of 5 minutes were employed. It was possible to note that the longest duration of irradiation (30 minutes) was related to a higher effectiveness for the inactivation of *C. albicans* in the planktonic culture (Fig. 1B), displaying a statistically significant difference (p < 0.05) when compared to control. This result was consistent with what has been described in the literature. A study that was conducted by Hosseini and collaborators, when investigating *C. albicans*, showed that by doubling the duration of irradiation, this reduced the amount of viable cells by 20%.[41,42] Although Prates *et al.* investigated the susceptibility of *C. albicans* when using a laser (λ = 660 nm) device for their irradiation, they clearly noted that the duration of irradiation significantly influenced the results regarding the microbial death.[43] They used 100 mW cm^{-2} and 300 mW cm^{-2} when they compared times of 3, 6, and 9 minutes of irradiation, resulting in radiant exposures from 18 J cm^{-2} to 162 J cm^{-2}. The results that were obtained in the present study also corroborated with the investigations that were conducted by Williams *et al.*, who associated an increased bacterial cell death to the enhanced radiant exposures and not necessarily to the greater photosensitizer concentrations.[44]

When the optimal concentrations were determined, the irradiation period was maintained at 10 minutes (1.56 J cm^{-2}) and the DIP at 5 minutes (Fig. 1C). It was clear that the lowest concentrations (10 and 20 mg L^{-1}) were the most effective for the inactivation of *C. albicans* in the planktonic model, exhibiting significant differences (p < 0.05) when compared to the control group. McCullagh and co-workers, when evaluating the photodynamic inactivations of *Chlorella vulgaris*, a green eukaryotic microalgae, showed that 6 μmol L^{-1} was the optimal MB concentration, since at the lower concentrations, slower kinetics for the processes were observed and at the higher concentrations, aggregation was observed.[45] Their concentration values were 5 and 10 times smaller than the effective MB concentrations that were found in this current study. However, their evaluation methods were different, as well as their parameters of light. About 1 $\log_{10}$ CFU reductions were found, which can be considered low in comparison to other studies. Dai *et al.*[46] used the phenothiazinium salts toluidine blue O (TBO), MB, and new methylene blue (NMB) at 20 μM, using LumaCare lamp at an irradiance of 32.5 mW cm^{-2} (12.5 times higher than used in this study) and radiant exposures up to 9.75 J cm^{-2} (twice than here) and only NMB achieved a great inactivation with 4.43 $\log_{10}$ CFU reductions of *C. albicans.* When MB or TBO were used under the same conditions, only modest fungal inactivation was observed. Besides, Baptista *et al.*[47] used an intermediate MB concentration (50 μM) proving that this was effective. However, they used different light parameters. Their pre-irradiation time was 10 min and their exposure times were 12 min, 15 min and 18 min with the LED device, delivering radiant exposures of 129.6 J cm^{-2}, 162 J cm^{-2} and 194.4 J cm^{-2}, respectively (27 to 40 times higher than here in this study). They evaluated the potential molecular targets of an aPDT, depending on growth phase of *C. albicans*, in a lag (6 h) and at stationary (48 h) phases. A complete eradication in the number of viable young cells (6 h) and a 2 $\log_{10}$ reduction in the old cells (48 h) were noticed after 18 min of irradiation. After 12 min and 15 min of irradiation, the reductions in the cell viabilities in the lag and at the stationary growth phases were similar.

When regarding a single concentration of MB, the D/M values decreased in the following media sequence: saline solu-

Table 1 Dimer to monomer ratio (D/M)[a] of MB solutions

Concentration (mg L^{-1})	Water	Physiological solution 0.9%	OF
100	0.64 ± 0.003	0.86 ± 0.026	0.24 ± 0.000
50	0.50 ± 0.003	0.62 ± 0.010	0.22 ± 0.002
20	0.40 ± 0.006	0.49 ± 0.005	0.23 ± 0.014
10	0.37 ± 0.042	0.38 ± 0.009	0.22 ± 0.008

[a] D/M = A_{590}/A_{664}.

tion, water and the OF. When the MB concentrations were increased in the medium, the D/M ratio also increased, with the exception of the OF (Table 1). Regarding MB, the increases in concentration and the changes in the medium caused a greater electrostatic and hydrophobic interaction between its molecules, leading to aggregation, which resulted in the formation of dimers.[26] Previously, it has been described in the literature, MB was more active at low concentrations (around 3–6 μmol L^{-1}, *i.e.*, 1–2 mg L^{-1}), because of the self-quenching.[45] Besides, Toluidine Blue, another phenothiazinium dye, has also shown good results with 10 mg L^{-1}.[48] Although the literature has brought this information to the investigating MB world, existing clinical trials do not apply these concentrations of MB in a clinical practice. These clinical trials, when using an aPDT with MB to treat oral pathologies that are caused by *Candida albicans*, have used values of 1.4 mmol L^{-1} (450 mg L^{-1}) to 3 mmol L^{-1}.[11,23] MB aggregation seems to be well known, so it would be beneficial if researchers were more concerned about the medium in which it is generally delivered. Normally, this information is omitted, or it is unintentionally described, whenever the commercial brand is disclosed. There are clinical trials that use MB in a water and saline solution.[11]

Upon regarding this MB aggregation when related to the medium, the efficacies of an aPDT with MB have been evaluated in a range of mediums, as well as the dimer to monomer ratios (D/M) – Fig. 2. One may note that MB presented an efficacy against *C. albicans* when applied in an SDS solution, achieving a complete inactivation in a planktonic assay. On the other hand, MB when it was applied in all of the other media, such as water, PBS, NaCl and urea, showed no differences to control (without the MB). In the dark, MB caused no reductions of *C. albicans*, while SDS at 0.25% presented a 1 $\log_{10}$ reduction. Thus, the observed data was a result of the photodynamic effects. Since it is known that MB presents an aggregation and it may affect the efficacy of the photodynamic treatments, the dimer to monomer ratios were evaluated, in order to find a relationship between the aggregations and the efficacies. The data has suggested that a dimer to monomer ratio above 0.3 reduced the effects of the MB photodynamic treatments; while below 0.3, the values increased the efficacies of an aPDT. In a study that was conducted by Nuñez *et al.*,[32] their objective was to investigate the effects of urea on MB aggregations. In water, the D/M ratio was found to be 1, while at the same concentration, the value in this current study was 0.37 (Table 1). These differences may be attributed to the reduced cuvette path length that was used in this current research, which has allowed for the measurement of absorbances within the range of 0.2–1 u.a., where the relationships between the absorbance and the concentrations were linear, according to Beer's Law. Nuñez *et al.* based the D/M values upon the spectra that presented a maxima above 2 u.a. Besides, when compared with water, the D/M ratios were reduced from 1 to 0.8, when approximated in the urea (2 M), while in this study, no D/M changes were shown in the urea (1 M). The differences in the urea concentrations may have caused these differing results.

Given these findings, the researchers have proposed an oral formulation (OF) containing both MB and SDS, which was a patent requirement (BR1020170253902), with the purpose of controlling the MB aggregation. When using this OF, MB retained the same relative amount of dimers and monomers, regardless of the assessed MB concentrations (Table 1). In view of the shown D/M values, in order to investigate the relationships between the aggregations and the efficacies of an aPDT, the same concentrations of MB that were delivered in the OF

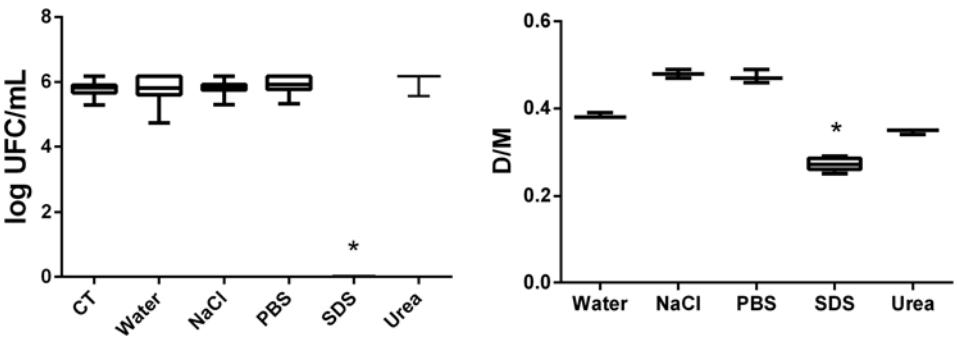

Fig. 2 The influence of the vehicle in the efficacy of MB mediated aPDT and dimer-to-monomer ratio (D/M). Left: aPDT in planktonic *C. albicans* culture, CT – no photosensitizer; MB at 20 mg L^{-1} in: water, NaCl 0.9% (PS – Physiological Solution), PBS – phosphate saline buffer, SDS – sodium dodecyl sulfate 0.25% and UREA 1 mol L^{-1}. Dark incubation period of 5 minutes, 30 minutes LED irradiation (640 ± 12 nm, 2.6 mW cm^{-2}, 4.7 J cm^{-2}). Experiments were performed in triplicate and 3 independent experiments (n = 9), *p < 0.05 in comparison to others. Right: Dimer-to-monomer ratio (D/M). Experiments were performed in triplicate and 3 independent experiments (n = 9), *p < 0.05 in comparison to others.

(lower aggregation) and in the saline solution (higher aggregation) were chosen for comparison. Since in planktonic culture studies, a suspension of *C. albicans* that was prepared in water and that an addition of this suspension to the OF would alter the D/M ratios, it seemed more appropriate to compare the effectiveness of the two systems of interest in an *in vitro* model of biofilms.

No decrease in the numbers of CFU mL^{-1} when compared to control (PS) were observed, when MB 20 mg L^{-1} was used in the physiological solution (MB 2 – PS) or in the OF (MB 2 – OF), Fig. 3. However, at 50 mg L^{-1}, it was possible to verify that there were significant differences ($p < 0.05$) between MB in the oral formulation (MB 5 – OF) and all of the other treatment groups. Although in the planktonic culture, 20 mg L^{-1} caused differences in the viability in relation to the control group (Fig. 1), it was necessary to use a higher concentration (50 mg L^{-1}), in order to verify significant differences in relation to control in the biofilms. This data was consistent with previous studies that have indicated microorganism resistances to the therapy, when they have been organized in the biofilms. Costa *et al.* inactivated planktonic *C. albicans*, when using 0.39 μmol L^{-1} of erythrosine. However, for a biofilm inactivation, 400 μmol L^{-1} was not enough to reach a similar reduction in the planktonic culture.[18] In general, a complete photo-inactivation after an aPDT has been reported when the microorganisms were presented in the planktonic form.[41,49] However, only a reduction in the number of microorganisms was observed when the microorganisms were organized in the biofilms.[18,41,49–51] This happened because when the microorganisms were organized in the biofilms, they were less susceptible to the actions of the antimicrobial therapies, when they were compared to the planktonic form, due to the presence of the extracellular polymer substances, the growth rate, the metabolic activities, the efflux pump cell wall compositions and the expressions of the specific genes.[18,51–55]

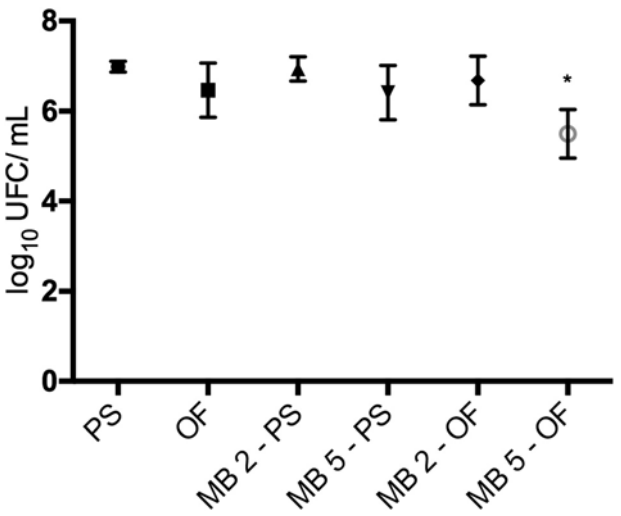

Fig. 3 *C. albicans* biofilm inactivation with MB at different levels of aggregation. PS = physiological solution – control group (NaCl 0.9%); OF = oral formulation without MB; MB 2 – PS = MB 20 mg L^{-1} in physiological solution; MB 5 – PS = MB 50 mg L^{-1} in physiological solution; MB 2 – OF = MB 20 mg L^{-1} in the oral formulation; MB 5 – OF = MB 50 mg L^{-1} in the oral formulation. Dark incubation period of 5 minutes, 30 minutes LED irradiation (640 ± 12 nm, 2,6 mW cm^{-2}, 4.7 J cm^{-2}). Experiments were performed in triplicate and 3 independent experiments (n = 9). Data presented as average ± SD. *statistically significant difference ($p < 0.05$) related to PS.

In spite of the fact that the OF contained 50 mg L^{-1} of MB, which caused statistically significant reductions in relation to control, total inactivations of *C. albicans* in the biofilms were not observed. It is known that microorganisms are considered to be inactivated by at least a reduction of 3 $\log_{10}$. However, this complete eradication of *C. albicans* on biofilms with an aPDT was not found in the literature. Freire *et al.*,[56] used MB (100 μM), or New Methylene Blue (NMB) (100 μM), combined or not with potassium iodide (KI) (100 mM) and this was exposed to 10, 20, 40 and 60 J on *in vitro C. albicans* biofilms. The best log reduction of CFU mL^{-1} on the biofilm grown cells was MB plus KI when using 40 J (2.31 $\log_{10}$); and NMB without KI, when using 60 J (1.77 log). In a study that was conducted by Fumes *et al.*,[57] when using chlorhexidine digluconate and an aPDT as treatments in biofilms that were formed by *Streptococcus mutans* and *C. albicans*, no statistically significant differences between the groups that were tested were found regarding *C. albicans*. In addition, chlorhexidine did not cause any reductions. So, the current researchers have assumed that the findings regarding an aPDT with this OF were effective.

It was possible to increase the microorganism inactivations by modifying the light exposure parameters. In this present study, a low irradiance and a low radiant exposure were used (2.6 mW cm^{-2} and 4.7 J cm^{-2}), when compared to other devices, such as lasers in which 100 mW was delivered at a 0.0028 cm^2 area, reaching an irradiance of 35.7 W cm^{-2}; this was, for instance, almost 14 times higher than the device that was used in this current research. Increasing irradiance by changing the LED device (keeping the same wavelength, but with a higher irradiance), or by using longer irradiation times (a higher radiant exposure), will increase the death of the microorganisms. Souza *et al.* (2010) showed that by increasing the radiant exposure from 15.8 to 39.5 J cm^{-2} with a 660 nm laser, it was able to inactivate *Candida albicans*.[38] Besides, Baltazar *et al.* conducted tests using Toluidine Blue (TBO) as a photosensitizer (70 mg L^{-1}, 40 mg L^{-1} and 10 mg L^{-1}) and a 630 nm light-emitting diode (LED) (18, 48 and 72 J cm^{-2}) as a source of light to target 12 *Trichophyton rubrum* isolates. The best results for their *in vitro* aPDT were 10 mg L^{-1} for the TBO (half of the concentration used here in this research) and 48 J cm^{-2} for the LED (10 times higher than used here in this research); these conditions were fungicidal and they inhibited >98% of the *T. rubrum* growth, depending upon the strain used.[48] Metcalf *et al.* studied another way to enhance the aPDT effects, through the fractionations of light, compared with continuous irradiation. The efficacy of an erythrosine-mediated aPDT of *Streptococcus mutans* biofilms, by fractionation of the light dose into 5 × 1 min doses, with a darkness period of 5 min between the light pulses, increased the amount of

bacterial killing by 1 $\log_{10}$, when compared with 5 min of continuous irradiation. Besides that, the fractionations of the light exposition, into 10 times of 30 s, with a darkness period of 2 min between the light pulses, resulted in a 3.7 $\log_{10}$ of cell kill, being an improvement of 1.7 $\log_{10}$, when compared with the continuous irradiation protocol.[58] The translations of this study to clinics will, therefore, need a standardization of the irradiation parameters, since smaller irradiation times are desirable. A laser device, or an LED cluster with a higher irradiance, should be evaluated.

The OF provided an enhanced effectiveness in the inactivation of the *C. albicans* biofilms, probably due to the lower amount of dimers. Usacheva *et al.* proposed that the dimers presented an essential role in the photodynamic inactivation of bacteria.[59] They showed that the ability to form dimers in the presence of bacteria increased the aPDT efficacy of the dye. However, the results of this study have contrasted to that, since the MB presence in the monomers was more effective to inactivate the *Candida* than the dimers. The MB aggregation, which was dependent upon its concentration and the employed dispersion medium, influenced the type of photochemical reaction that took place. In other words, Type I *via* the radicals or Type II *via* the singlet oxygen.[27,28] The samples underwent a singlet oxygen production estimation, in order to evaluate the mechanisms that took place (Table 2).

When the samples were excited at wavelengths of 610 nm and 625 nm, the production of singlet oxygen by the MB photoactivation was higher in the OF (31.8 and 50) than in the saline solution (19.3 and 37.2, for 610 nm and 625 nm, respectively). The excitation wavelength affected the absorption efficiency; *i.e.*, since the dimers presented an absorption maxima at 590, their formation reduced the light absorption of the LED device. In addition, these findings were related to the state of the MB aggregation. The dimer/monomer ratios of these solutions were 0.23 for the OF and 0.49 for the physiological solution, indicating that, in the latter, there were approximately 2.1× more dimers than in the OF. The obtained results have shown that there were 1.6× (31.8/19.3) and 1.3× (50/37.2) greater productions of singlet oxygen regarding the MB that was applied in the OF, than in the saline solution. Junqueira *et al.* reported that the monomers tended to produce more singlet oxygen, while the dimers tended to produce more radicals; put differently, there were different photochemical mechanisms for the monomers and for the dimers.[27] When the samples were excited at the 610 nm and 625 nm wavelengths, the production of singlet oxygen by the photoactivation of MB was greater, when it was employed in the OF than in saline solution. When using the 640 nm wavelength for the excitation, the production of singlet oxygen was similar, independent of the medium – and when 655 nm was used, the production was higher in the saline solution. At the 640 nm and 655 nm wavelengths, the absorption of 0.005% MB solutions in the OF was above 0.4. In other words, under these conditions, the values of the singlet oxygen production were lower than when using the physiological solution, probably due to the previously described factors. Singlet oxygen has been considered to be the main cytotoxic agent in the PDT in tumor cells.[60] In bacteria, it has recently been reported that photosensitizers, which present a high capacity of producing singlet oxygen, were more potent for an aPDT than the free radical production ones.[61] For the Candida species, there is no consensus regarding the main toxic agent. Marioni *et al.*[62] showed that an aPDT on Candida tropicalis, when using anthraquinones, acted mainly by the superoxide radical anion, rather than the singlet oxygen, while Di Palma *et al.*[63] found that singlet oxygen was the main cytotoxic agent in an aPDT that was mediated by zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine in *Candida albicans*. In this study, singlet oxygen seemed to be more important in the cell death than the other oxidizing species.

Table 2 Singlet oxygen production[a]

Wavelength (nm)	PS 0.9%	OF
610	19.3	31.8
625	37.2	50.0
640	48.9	47.1[b]
655	81.6	51.9[b]

[a] *I*/Abs ratio, where *I* represents the intensity of $^1O_2$ emission, and Abs characterizes the absorption at the excitation wavelength. [b] Absorption at this wavelength was above 0.3, resulting in a significant filter effect.

In the present research, the objective was to control the MB aggregation by using the OF and, thus, direct the Type II mechanisms *via* the singlet oxygen. As a comparison, MB was employed in the saline solution, since it increased the amount of dimers and it triggered the Type I mechanisms (radicals). For the same concentrations of MB, its delivery in the OF significantly reduced the amount of *C. albicans*, when compared to the physiological solution. The data that was obtained has suggested that the Type II mechanisms (*via* the singlet oxygen) were more effective in causing the death of *C. albicans*. Moreover, the MB that was employed in the OF, retained the same relative amounts of the dimers and the monomers, regardless of the assessed concentration, enabling the use of higher concentrations. These results have indicated that it is now important to develop formulations for clinical applications that promote the disaggregation of MB. However, in order for this protocol to be adapted for a clinical practice, it is necessary to re-evaluate the parameters, especially the irradiation, since their devices would be different and a 30-minute period of irradiation would render the procedure clinically infeasible.

The OF formulation has shown itself to be an efficient strategy to control the MB aggregation. In addition, the use of MB that was employed in the OF increased the effectiveness of the therapy. In view of these results, it is possible to state that, when in the form of monomers, MB was a more effective photosensitizer and that the Type II reactions (*via* the singlet oxygen) were the photochemical mechanisms that induced the *C. albicans* death more efficiently. This research has brought valuable information which might be used for the elimination

of microorganisms, by using photodynamic therapy in clinical applications. However, further studies are necessary, in order to adjust the light parameters for clinical applications.

Materials and methods

Biological studies

Suspension preparation. The *Candida albicans* yeast (ATCC 10231) was grown on a Sabouraud Dextrose Agar (Kasvi, Curitiba, Brazil) medium at 37 °C for 48 hours. After the preparation of a standard suspension on the 1.0 McFarland scale, the cell number certification was carried out by counting, when using a Neubauer Chamber.[64]

Suspension assays. Volumes of 0.20 mL of the *C. albicans* suspension (5×10^7 cells per mL) and of the MB solution were inoculated into 48-well microplates and they were maintained in the dark during the incubation period. The plates were then irradiated with an LED system (Condulai Indústria de Iluminação Ltda, São Paulo, Brazil) that emitted light at 640 ± 12 nm, with 2.6 mW cm^{-2}, being measured at the surface of the wells. Following this irradiation, an aliquot of each sample was taken and diluted (10^{-1} to 10^{-5}) and 20.0 μL of each dilution was seeded by streaking it onto Petri dishes containing the Sabouraud Agar Dextrose Culture Medium. The plates were incubated for 24 h at 37 °C and the number of colony forming units per mL were counted.[65] In order to obtain the most suitable experimental conditions, the following variables were evaluated in the suspension assays: MB concentrations (0 to 0.01% which is equivalent to 100 mg L^{-1} or 313 μmol L^{-1}), dark incubation periods (1 to 20 minutes), and duration of irradiation (0 to 30 minutes). After that, the best parameters found were used in order to verify the efficacy of MB in different mediums (water, Physiological Solution – NaCl 0.9%, phosphate saline buffer, sodium dodecyl sulfate 0.25% and urea 1 mol L^{-1}).

Biofilm assays. The suspension preparations were performed as previously described. A 48-well plate was treated with Bovine Fetal Serum (Vitrocell Comércio De Produtos Para Laboratórios Eireli, São Paulo, Brazil) for 24 hours. The Bovine Fetal Serum was removed and 0.10 mL of the suspensions were added to the wells, along with 0.30 mL of the Sabouraud Dextrose Broth Medium (Kasvi, Curitiba, Brazil). They were incubated for 48 hours at 37 °C for the biofilm formations. The supernatant was later discarded and the biofilms were washed with the Phosphate Buffer Saline (PBS) at pH 7.2, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 8.1 mmol L^{-1} Na_2HPO_4 and 1.47 mmol L^{-1} KH_2PO_4 (the salts were purchased from Synth, Diadema, Brazil). The biofilms were then exposed to the MB solutions (PS or OF) and the irradiation, according to the parameters that were established as stated below.

After the treatments, the solutions were removed, the biofilms were washed with PBS and they were disrupted by multiple pipetting in PBS, followed by shaking in a Vortex (Jand Química, São Paulo, Brazil) for 1 minute. The resulting suspensions were diluted, seeded, incubated and counted, according to the previously described procedures for planktonic assays.[66]

Solution studies

Determining the dimer-monomer ratio (MB aggregation assessment). The absorption spectra of MB were recorded in the different proposed media, by using a UV-Visible 1800 spectrophotometer (Shimadzu, Kyoto, Japan), in a region between 500 nm–800 nm, when using a 2 mm cuvette path length. The dimer-monomer ratios were determined by employing the absorbance values at 590 nm (dimer) and at 664 nm (monomer).[26]

Singlet oxygen production assessment. The MB solutions were excited by using a tunable Rainbow laser (Quantel, France), when employing the OPO system, based upon 5 ns that were pulsed with an Nd:YAG laser (Brilliant, Quantel, France). The decays that were related to the emission of $^1O_2$ (at 1270 nm) were detected by a Photomultiplier R5509-43 (Hamamatsu Photonics, Japan), then cooled in liquid nitrogen and stored in the computer. A band pass filter ($1225 < \lambda < 1325$ nm) was used, in order to eliminate the short and long wavelength photon emissions from the other sources. The singlet oxygen productions were calculated by the ratios between the emission intensities of $^1O_2$ and the absorbance of the samples at the excitation wavelength. The 20 mg L^{-1} MB solutions were employed in the saline solution and in the OF formulae. The productions of singlet oxygen were assessed at four different excitation wavelengths (610 nm, 625 nm, 640 nm, and 655 nm).

Statistical analysis. The statistical analysis was performed by using Prism Version 6.0 Software and the Shapiro–Wilk test was employed, in order to evaluate the data normality. The data that displayed the normality was expressed as a mean ± standard deviation and the ANOVA test, followed by Bonferroni post-test, were applied, in order to compare the values of the different antimicrobial treatments. The data not showing a normality was expressed as a median ±5–95% and the Kruskal–Wallis test, followed by Mann–Whitney test, were applied, in order to compare the values of the different antimicrobial treatments. A value of $p < 0.05$ was considered as being statistically significant.

Conclusions

The OF formulation has been shown to be an efficient strategy, in order to control an MB aggregation. In addition, the use of MB, when employed in the OF (MB 50 mg L^{-1}), increased the effectiveness of the therapy, when it was compared with the no formulating of MB. In view of the results, it is possible to state that, when in the form of monomers, MB was a more effective photosensitizer and that the Type II reactions (*via* the singlet oxygen) were the photochemical mechanisms that induced the *C. albicans* death more efficiently. This research has brought valuable information, which might be used in the eliminations of microorganisms, by using photodynamic therapy in clinical

applications. However, further studies are necessary, in order to adjust these light parameters for clinical applications.

Conflicts of interest

There are no conflicts to declare.

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