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ORIGINAL ARTICLE

Antimicrobial photodynamic therapy against pathogenic bacterial suspensions and biofilms using chloro-aluminum phthalocyanine encapsulated in nanoemulsions

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Abstract Antimicrobial photodynamic therapy represents an alternative method of killing resistant pathogens. Efforts have been made to develop delivery systems for hydrophobic drugs to improve the photokilling. This study evaluated the photodynamic effect of chloro-aluminum phthalocyanine (ClAlPc) encapsulated in nanoemulsions (NE) on methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* suspensions and biofilms. Suspensions and biofilms were treated with different delivery systems containing ClAlPc. After the pre-incubation period, the drug was washed-out and irradiation was performed with LED source (660±3 nm). Negative control samples were not exposed to ClAlPc or light. For the suspensions, colonies were counted (colony-forming units per milliliter (CFU/mL)). The metabolic activity of *S. aureus* suspensions and biofilms were evaluated by the XTT assay. The efficiency was dependent on the delivery system, superficial load and light dose. Cationic NE-ClAlPc and free-ClAlPc caused photokilling of the both strains of *S. aureus*. For biofilms, cationic NE-ClAlPc reduced cell metabolism by 80 and 73 % of susceptible and resistant strains, respectively. Although anionic NE-ClAlPc caused a significant CFU/ml reduction for MSSA and MRSA, it was not capable of reducing MRSA biofilm metabolism. This therapy may represent an alternative treatment for eradicating resistant strains.

Keywords Antimicrobial photodynamic therapy · Drug delivery systems · Phthalocyanine · MRSA

Introduction

The antimicrobial photodynamic therapy (aPDT) has emerged as an alternative treatment of skin and mucosa lesions and infections [1]. However, unlike antitumor PDT, which has

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been used in some countries as an established therapeutic option, aPDT is still in the experimental phase [2]. In this therapy, a non-toxic light-sensitive compound (known as photosensitizer—PS) is applied on the lesion and, after an incubation period to permit accumulation of the drug in the bacterial cells, the area is irradiated with a visible light source of appropriate wavelength with maximum absorption by the PS [1]. With light excitation, the PS undergoes a transition to a more energetic state in which it is capable to react with molecules present in the medium and transfer electrons or hydrogen, leading to the production of reactive oxygen species (ROS; type I reaction), or transfer energy to molecular oxygen, generating the highly reactive singlet oxygen (type II reaction) [3]. The products of these reactions cause lethal oxidative damage to the bacterial cells.

The growing interest in aPDT is due to its ease of application and the fact that the ROS generated during the photodynamic reaction have a multifunctional nature and can damage multiple cellular structures, from membrane to organelles, reducing the chances of development of PDT-resistant strains [4]. Unlike chemical agents, which have a continuous action and may induce the habituation of microorganisms to these drugs, the toxic products of aPDT are only generated when the PS is excited by a light source of specific wavelength, permitting a better control of its toxicity. In addition, the ROS have a short life in the biological systems and minimal diffusion, which restricts their oxidative damage to the site of PS application [4]. Therefore, the use of photodynamic therapy with capacity to kill pathogenic bacteria without inducing resistance has been suggested as an alternative especially for microorganisms resistant to conventional treatments with antimicrobial agents. The development of antibiotic-resistant bacteria has become a major health problem worldwide, as it is responsible for significantly higher morbidity and mortality due to certain infections and increased healthcare costs [5]. Among the several types of antibiotic-resistant pathogenic microorganisms, methicillin-resistant *Staphylococcus aureus* (MRSA) is noteworthy. This microorganism has developed resistance to antimicrobial agents by several mechanisms that range from mutation to transduction [6]. It has been associated with life-threatening infections in immunocompromised patients, which may lead to fatal bacteremia and septicemia [7], and is frequently found in infections related to prostheses, catheters, and other biomaterials [8]. MRSA are often found in biofilm formations, which act as protective coatings that attract various staph strains to form a microbial structure involved by a extracellular polymer matrix responsible for reducing the capacity of antibiotics to reach the microorganisms, increasing their the resistance to the treatment [9].

This way, strategies have been developed to increase the permeation of the light-sensitive agents into the bacterial biofilm structure and ensure the action of aPDT. The first step is the choice of the PS, which should preferably be a drug with an effective production of singlet oxygen and high oxidative power, such as phthalocyanines [10]. However, phthalocyanines are not water-soluble and need delivery systems, such as polymeric micelles, liposomes, nanoparticles, and nanoemulsions (NE). In addition, the inclusion of charges on the surface of these delivery systems has also been suggested to increase the physical attraction to the bacterial cells [11]. It is also known that in addition to acting directly on the bacterial cells, the products generated by aPDT also act on the extracellular matrix of biofilms [12], increasing the photodynamic efficiency. Therefore, the aim of this study was to evaluate the photodynamic effect of chloro-aluminum-phthalocyanine (ClAlPc) encapsulated in cationic and anionic NE compared with ClAlPc diluted in organic solvent (free ClAlPc) on the inactivation of planktonic and biofilm cultures of methicillin-susceptible *S. aureus* (MSSA) and MRSA.

Methods

Photosensitizer and light source

ClAlPc encapsulated in NE was kindly donated by the Center of Nanotechnology and Tissue Engineers, Photobiology and Photomedicine research Group of the University of São Paulo at Ribeirão Preto, Brazil. The NE preparation was obtained by spontaneous emulsification as previously described [13]. Initially, the surfactants were dissolved in 10 mL of spectroscopic acetone at 55 °C under magnetic stirrer. At the same time, the phthalocyanine was dissolved directly in Miglyol 812 N oil at 55 °C and was added to the phospholipid organic solution at a concentration of 1.0 mg/mL. The aqueous phase was obtained by dissolution of the biopolymer Poloxamer 188 (Sigma-Aldrich Co., St. Louis, MO, USA) in ultra-pure water. The NE was formed by slow injection of the organic phase into the aqueous phase under magnetic stirring (300 rpm for 30 min) at 55 °C. The solvent was then evaporated under reduced pressure at approximately 75 °C, and the volume of the NE was concentrated to the initial volume of the aqueous phase. The amount of hydrophilic and lipophilic surfactants was fixed at 1 % with a 1:1 weight ratio. The photophysical, chemical, and biological properties were evaluated and presented similar response, as observed in another study of the research group [13]. The final concentration of ClAlPc in the NE was 31.8 μM and two NE with different superficial charges was developed, cationic and anionic. In order to compare this new delivery system with the free phthalocyanine, ClAlPc was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in DMSO to give a 600-mM stock solution. Before the experiments, this solution was further diluted in PBS to obtain the same final concentration as the NE (31.8 μM).

Irradiation of PS was carried out using a light system composed of red-emitting diodes (LEDs) (LXHL-PR09; Luxeon III Emitter; Lumileds Lighting, San Jose, CA, USA) with the lamps uniformly distributed into the device in order to provide a uniform irradiation of the plate. The LED device provided a maximum emission at 660 nm and the irradiance delivered was of 38.1 mW/cm^2. The light doses used were 15, 25, or 50 J/cm^2, resulting in approximately 8, 13, and 26 min of irradiation time, depending on the bacterial strain.

Bacterial culture

The bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA): MSSA (ATCC 25923) and MRSA (ATCC 33591). Both isolates were maintained in Tryptic Soy Broth—TSB (Acumedia Manufactures Inc., Baltimore, MD, USA) medium and frozen at −70 °C until use. For the experiments, these bacteria were individually inoculated in 5 mL of TSB and grown aerobically overnight at 37 °C. Each culture was harvested after centrifugation at 2,000 rpm for 10 min, washed twice with sterile distilled water, and resuspended in PBS. Cell suspensions were standardized by spectrophotometer (Biospectro, EquiparLtda, Curitiba, PR, Brazil; model: SP-220) calibrated at 600 nm wavelength to give final concentration of 1×10^7 cells mL^{-1}.

Photodynamic inactivation studies in planktonic suspensions

Aliquots of 150 μL of the standardized MSSA and MRSA suspensions were individually transferred to separate Eppendorfs and centrifuged to remove the supernatant. The cells were incubated with either the cationic and anionic NE containing the ClAlPC or the free ClAlPc for 30 min in the dark. After this pre-incubation time, the ClAlPC was washed out and 300 μL of PBS were added to the cells. The samples were transferred to a 24-well plate that was placed on the LED device for illumination at light fluences of 15 and 25 J/cm^2 for MSSA and 25 and 50 J/cm^2 for MRSA. Controls were established in order to determine whether ClAlPc alone and its delivery systems (dark toxicity controls) or light alone (light control—LC) induced any effect on bacteria viability. Therefore, additional wells containing MSSA and MRSA suspensions were exposed to the drug under identical conditions to those described above, but not to LED, and others were exposed only to irradiation. A negative control (NC) was also established consisting of bacterial suspensions not exposed to ClAlPc or LED. Table 1 presents the groups distribution.

The viability of the planktonic suspensions was evaluated by two methods: the number of colony-forming units per milliliter (CFU/mL) and XTT assay. For the first method, aliquots of the contents of each well were serially diluted tenfold in sterile saline to give dilutions of 10^{-1} to 10^{-3} times the original concentration. Triplicate 25 μL aliquots were plated onto Mannitol Salt agar plates. All plates were aerobically incubated at 37 °C for 24 h and thereafter colony counts of each plate were quantified (CFU/mL) using a digital colony counter (CP 600 Plus; Phoenix Dentsply Ind. e Com. Equipamentos Científicos Ltda., Araraquara, SP, Brazil). For analytical purposes, CFU/mL values were transformed into logarithm ($\log_{10}$). For the transformation into $\log_{10}$, the number counted and the dilution used were placed in a formula (ufc/mL=number of colonies$\times10^n/q$), in which the "n" represents the absolute number of the dilution (0, 1, 2, 3, or 4), and "q" represents the amount (mL) used for each dilution when plated (0.025 mL).

Table 1 Distribution of the control and experimental groups

Group	Treatment
NC	Negative control
LC	Light control
C−NE	Dark control—anionic NE-ClAlPc
C+NE	Dark control—cationic NE-ClAlPc
C ClAlPc	Dark control—free-ClAlPc
PDT 15-NE	PDT anionic NE-ClAlPc with 15 J/cm^2
PDT 15+NE	PDT cationic NE-ClAlPc with 15 J/cm^2
PDT 15 ClAlPc	PDT free-ClAlPc with 15 J/cm^2
PDT 25-NE	PDT anionic NE-ClAlPc with 25 J/cm^2
PDT 25+NE	PDT cationic NE-ClAlPc with 25 J/cm^2
PDT 25 ClAlPc	PDT free-ClAlPc with 25 J/cm^2
PDT 50-NE	PDT anionic NE-ClAlPc with 50 J/cm^2
PDT 50+NE	PDT cationic NE-ClAlPc with 50 J/cm^2
PDT 50 ClAlPc	PDT free-ClAlPc with 50 J/cm^2

The XTT assay is a metabolic assay based on the reduction of a tetrazolium salt (XTT, Sigma-Aldrich). For this assay, 158 μL of PBS were prepared with glucose at 200 mM, 40 μL of XTT plus 2 μL of menadione at 0.4 mM (Sigma-Aldrich) were mixed and transferred to each well. The plates were incubated in the dark at 37 °C for 3 h. After this 100 μL of the reacted XTT salt solution was transferred to a 96-well plate and cell viability was analyzed by proportional colorimetric changes and light absorbance measured by a microtiter plate reader (Thermo Plate—TP Reader) at 492 nm.

Biofilm growth and photodynamic inactivation studies in biofilms

Colonies of recently grown bacteria isolates on Mannitol Agar were transferred to a Falcon tube containing RPMI 1640 and

incubated overnight in an orbital shaker (AP 56, Phoenix Ind. e Com. Equipamentos Científicos Ltda., Araraquara, SP, Brazil) at 120 rpm and 37 °C. Cultures were centrifuged, and the supernatants were discarded. The cells were washed twice and finally resuspended in PBS. Standardized cell suspensions were obtained as described for the planktonic cultures. The resulting standardized suspensions were standpoint for every further procedure. Aliquots of 100 μL of MSSA and MRSA standardized suspensions were transferred to a 96-wells microtiter plate and incubated for 90 min at 37 °C in the orbital shaker at 75 rpm for the adhesion phase. After this period, each well was washed twice with PBS and 150 μL of freshly prepared RPMI 1640 was added to each well. The plates were incubated for 48 h at 37 °C in order to generate single-species biofilms.

After this period, the wells were carefully washed twice with 200 μL of PBS to remove non-adherent cells. Aliquots of 150 μL of ClAlPc encapsulated in NE or free were added to each appropriate well directly over the biofilm. The photosensitizers were left in contact with the biofilms for 30 min and then were washed out. Aliquots of 150 μL of PBS were added to the wells before irradiation. The biofilms were illuminated using light fluences of 15 or 25 J/cm^2 for MSSA and 50 or 100 J/cm^2 for MRSA. The same experimental conditions described previously to planktonic cultures were performed in biofilms. The photodynamic effects against the biofilms were also evaluated using the XTT assay, as described previously.

Statistics

The results collected after the evaluation of aPDT against planktonic suspensions (log CFU/mL and XTT absorbance values) and biofilm cultures (XTT absorbance) presented heterocedasticity. The Kruskal–Wallis and Multiple Comparison based on pairwise ranking test were used to detect differences in CFU/mL and XTT absorbance values among the groups. Differences were considered statistically significant at $p<0.05$.

Results

Effect of aPDT on colony counts of planktonic suspensions

The photodynamic antimicrobial effect of ClAlPc encapsulated in cationic and anionic NE and free ClAlPc against MSSA (CFU/mL) is presented in Fig. 1. The bacterial growth was directly dependent on the delivery system used (NE or free), superficial charge of the NE (cationic or anionic) and light fluence (15 or 25 J/cm^2). Free ClAlPc and cationic NE-ClAlPc promoted photokilling of MSSA when irradiated with the light fluence of 25 J/cm^2. When combined with the light fluence of 15 J/cm^2, cationic NE-ClAlPc and free ClAlPc caused 5 and 6 $\log_{10}$ reduction of bacterial cell survival, respectively ($p<0.05$). Although anionic NE-ClAlPc did not cause photokilling of MSSA, 2 and 4 $\log_{10}$ reduction of bacterial cell survival was observed when irradiated with light fluences of 15 and 25 J/cm^2, respectively ($p<0.05$). The dark-NE (C−NE, C+NE) and LC did not cause a significant decrease of bacterial growth and only the combined use of PS and light was capable of reducing the number of colonies.

For MRSA, the delivery system, superficial charge, and light fluence also had a direct influence on colony counts after aPDT (Fig. 2). Cationic NE-ClAlPc and free ClAlPc promoted photokilling of MRSA when illuminated with 50 J/cm^2, which is twice the light fluence required for eradication of MSSA. Illumination of both delivery systems with 25 J/cm^2 promoted ~5 $\log_{10}$ reduction on CFU/mL. Anionic NE-ClAlPc in combination with the lower light fluence did not differ significantly from the negative control ($p>0.05$) and caused a significant reduction of bacterial cell survival (~1 $\log_{10}$) only when illuminated with the 50 J/cm^2 light fluence. For both strains suspensions, the UFC/mL values were obtained after plating aliquots of only 25 μL on agar plates.

Effect of aPDT on XTT absorbance values of planktonic suspensions

The XTT absorbance values for MSSA are depicted in Fig. 3 and they were directly influenced by the light fluence and delivery system. The combination of ClAlPc and light reduced cell metabolism regardless of the delivery system. Considering the respective dark control of the anionic NE (C−NE) as having 100 % of cell metabolism, the anionic NE-ClAlPc reduced MSSA metabolism by 63.5 and 93.7 % when combined with light fluences of 15 and 25 J/cm^2, respectively ($p<0.05$). For the cationic NE-ClAlPc, reductions of 72.5 and 86.5 % in MSSA metabolism were observed with light fluences of 15 and 25 J/cm^2, respectively, considering the respective dark control of the cationic NE (C+NE) as having as 100 % of cell metabolism. Illumination of free ClAlPc with light fluences of 15 and 25 J/cm^2 reduced MSSA metabolism by 91 and 92 %, respectively. For cationic NE-ClAlPc and free ClAlPc, the increase of light fluence did not result in significantly greater reduction in MSSA metabolism ($p>0.05$) and both of them had potential to cause a significant decrease in cell metabolism in dark conditions. However, the reduction in XTT absorbance values observed in the controls of these delivery systems was not sufficient to cause a significant reduction in the growth of MSSA (Fig. 1).

For MRSA, the delivery system and light fluence were decisive in the antimicrobial effect of ClAlPc (Fig. 4). Free ClAlPc presented the best results, reducing MRSA metabolism

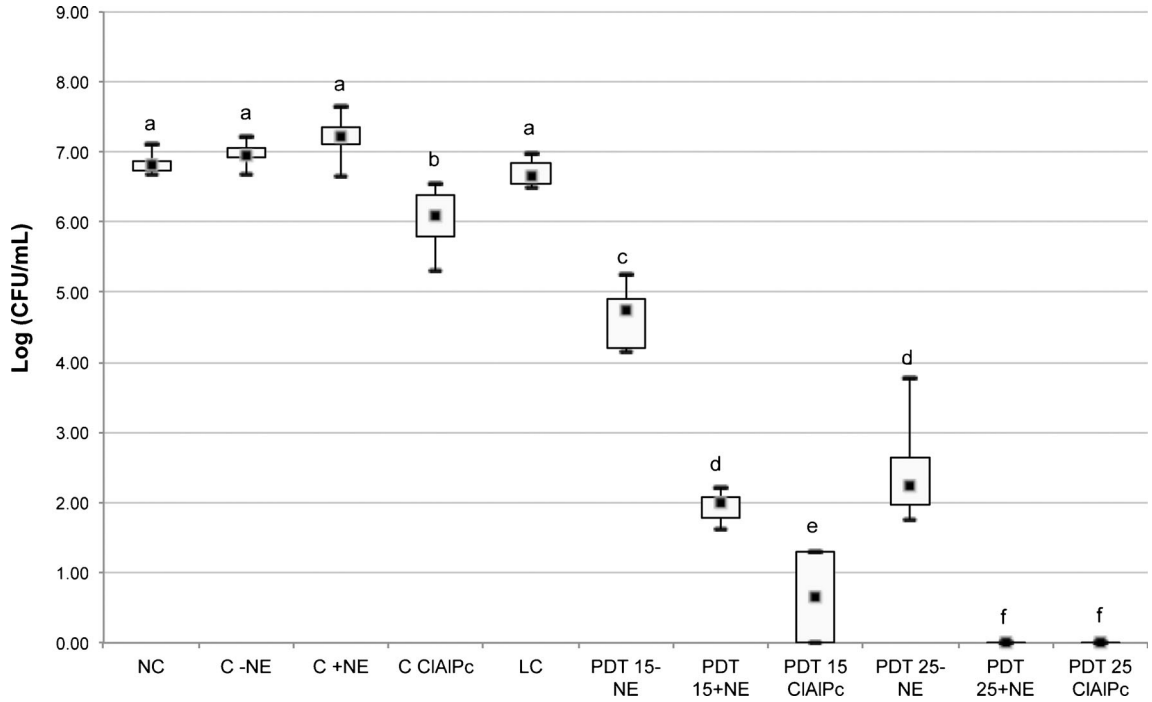

Fig. 1 Box-plot graphic representation of mean values and standard deviation of logarithmic of survival counts (CFU/mL) of MSSA planktonic suspensions according to the light fluence (0, 15, or 25 J/cm^2) and drug delivery system (free, anionic, or cationic NE-ClAlPc). The volume used to determine the CFU per milliliter value was 25 μL plated on agar plates, $n=10$

by approximately 96 and 92 % when illuminated with light fluences de 25 and 50 J/cm^2, respectively, without significant difference ($p>0.05$) between them. For the cationic NE-ClAlPc, significant differences ($p<0.05$) were found between the light fluences; while no reduction in cell metabolism was observed with illumination at 25 J/cm^2, the 50 J/cm^2 light fluence reduced cell metabolism by 70 %. Anionic NE-ClAlPc also presented a light fluence-dependent behavior, causing a significant reduction in MRSA metabolism (around 45 %) only in combination with the higher light fluence.

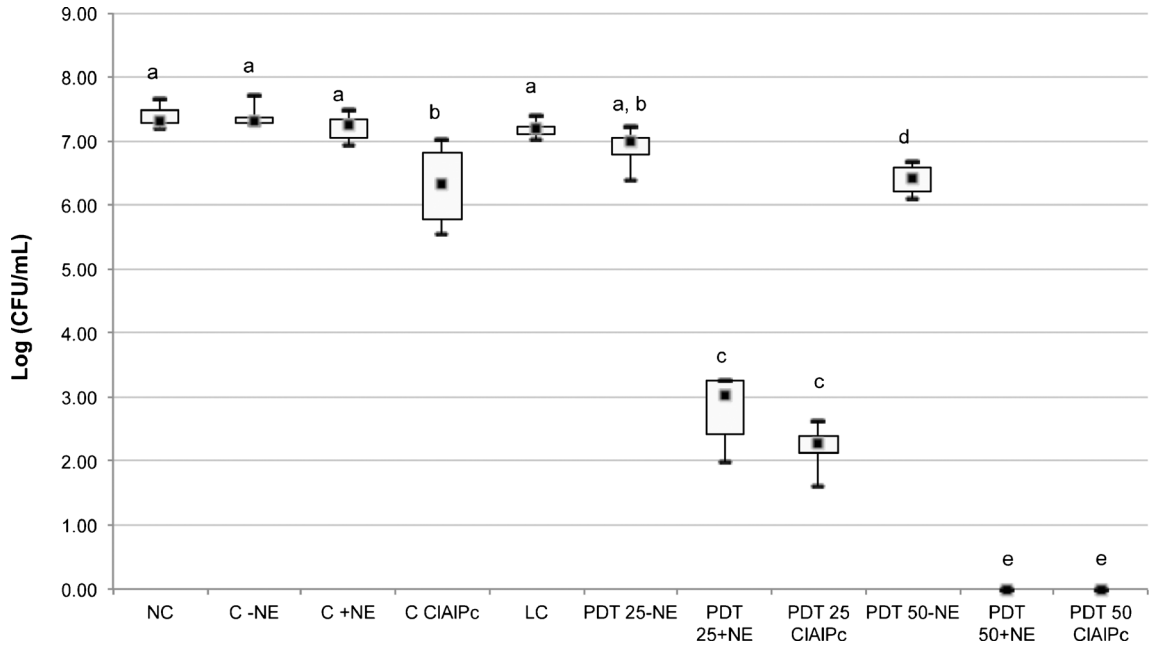

Fig. 2 Box-plot graphic representation of mean values and standard deviation of logarithmic of survival counts (CFU/mL) of MRSA planktonic suspensions according to the light fluence (0, 25, or 50 J/cm^2) and drug delivery system (free, anionic, or cationic NE-ClAlPc). The volume used to determine the CFU per milliliter value was 25 μL plated on agar plates. $n=10$

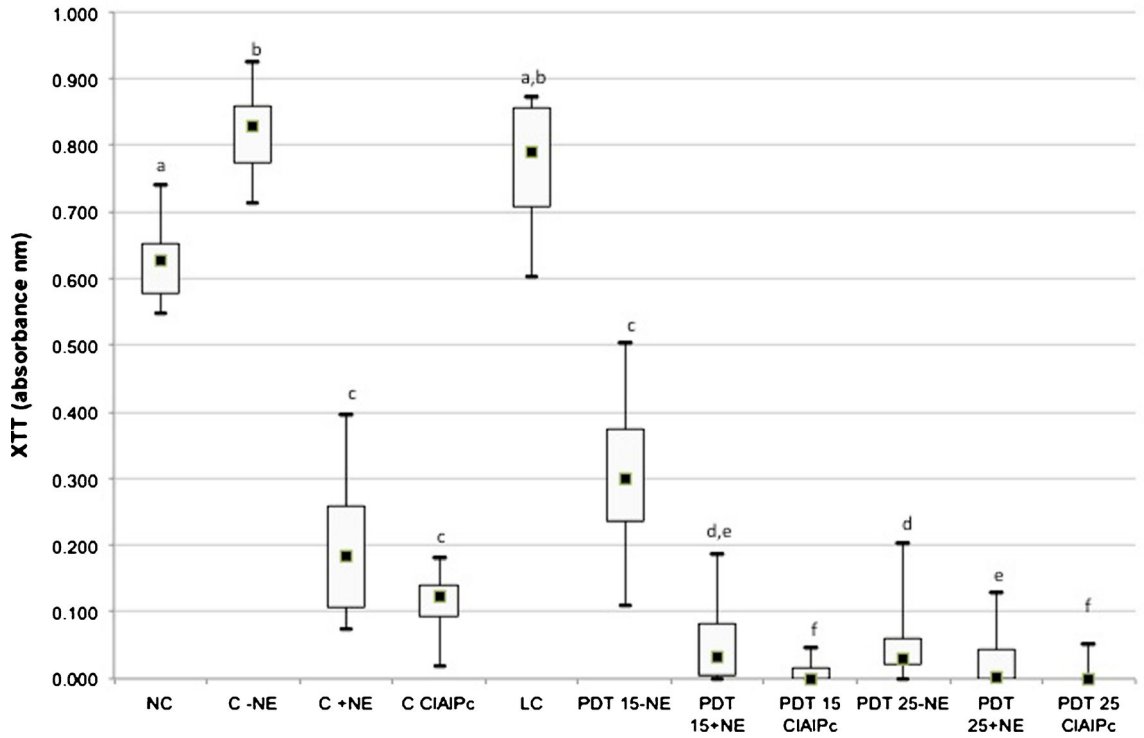

Fig. 3 Box-plot graphic representation of mean values and standard deviation of absorbance values (XTT assay) obtained for the MSSA planktonic cultures subjected to the different experimental conditions. Groups identified with the *same letter* do not differ statistically (Multiple Comparison based on pairwise ranking test; $p>0.05$). $n=10$

Regarding the dark controls (C−NE, C+NE, C ClAlPc), only the cationic NE-ClAlPc (C+NE) presented significant toxicity when compared with the negative control ($p<0.05$); the other controls did not affect significantly ($p>0.05$) the metabolism of MRSA biofilms.

Effect of aPDT on XTT absorbance values of bacteria biofilms

The MSSA biofilms used in this study were developed during 48 h and were treated with ClAlPc encapsulated in different

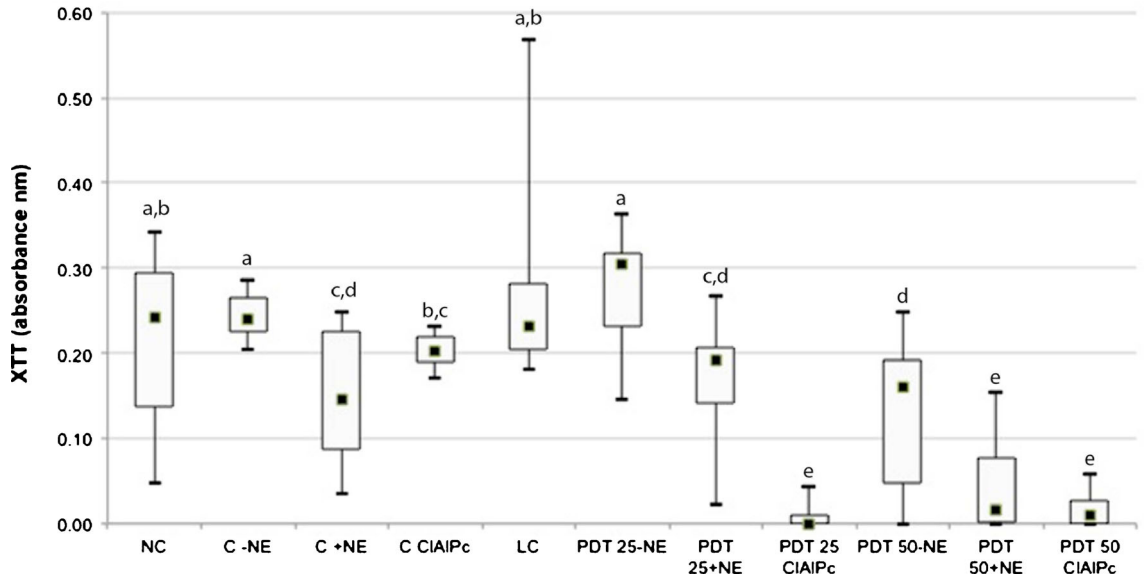

Fig. 4 Box-plot graphic representation of mean values and standard deviation of absorbance values (XTT assay) obtained for the MRSA planktonic cultures subjected to the different experimental conditions. Groups identified with the *same letter* do not differ statistically (Multiple Comparison based on pairwise ranking test; $p>0.05$). $n=10$

delivery systems for an incubation period of 30 min. The same light regimen as for the planktonic suspensions (660 nm LED, 15 and 25 J/cm^2) was used. The absorbance values (XTT assay) for MSSA are presented in Fig. 5 and they were influenced by the type of delivery system, superficial charge, and light fluence. Cationic NE-ClAlPc was the only delivery system that presented significant dark toxicity to the biofilms ($p<0.05$). However, considering the respective dark control of the cationic NE (C+NE) as having 100 % of metabolism, cationic NE-ClAlPc in combination with light fluences of 25 and 50 J/cm^2 decreased biofilm metabolism by 58 and 80 %, respectively. Anionic NE-ClAlPc and free ClAlPc caused a significant decrease in the metabolism of MSSA biofilms only when irradiated with the higher light fluence—42 and 43 %, respectively.

MRSA biofilms were obtained in the same way as described for MSSA biofilms and their absorbance values (XTT assay) are depicted in Fig. 6. Regardless of the light fluence, the cationic NE-ClAlPc was the most effective against MRSA biofilms, reducing their metabolism by 76.5 and 73.5 % when illuminated with light fluences of 25 and 50 J/cm^2. Free-ClAlPc presented an intermediate behavior and caused a significant decrease in the metabolism of MRSA biofilms only when irradiated with the higher light fluence (55 %). The anionic NE-ClAlPc was not capable of reducing MRSA biofilm metabolism, regardless of light fluence. The dark (C−NE, C+NE, C ClAlPc) and light (LC) did not affect negatively the absorbance values when compared with the negative control (not exposed to ClAlPc or light), which indicates that the reductions in the metabolism of MRSA biofilms resulted from the photodynamic action of ClAlPc.

Discussion

The incidence of staphylococcal infections caused by antibiotic-resistant strains has led to the development of new therapeutic modalities, such as aPDT. To increase the success of this therapy, research has been directed on light-sensitive drugs with photodynamic efficiency to kill microorganisms and new delivery systems to these drugs, which are hydrophobic compounds in most cases.

Among the second-generation PSs, the phthalocyanines are noteworthy because of their more effective production of singlet oxygen, with higher oxidative power compared with hematoporphyrin and phenothiazine derivatives. Their chemical structure permits the association of several metals, which increases the phototoxicity and ionic characteristics of these drugs [14]. In addition to their high photodynamic activity, phthalocyanines are easy to synthesize, have rapid elimination with minimal adverse effects and are commercially available [15]. In spite of these advantages, the highly

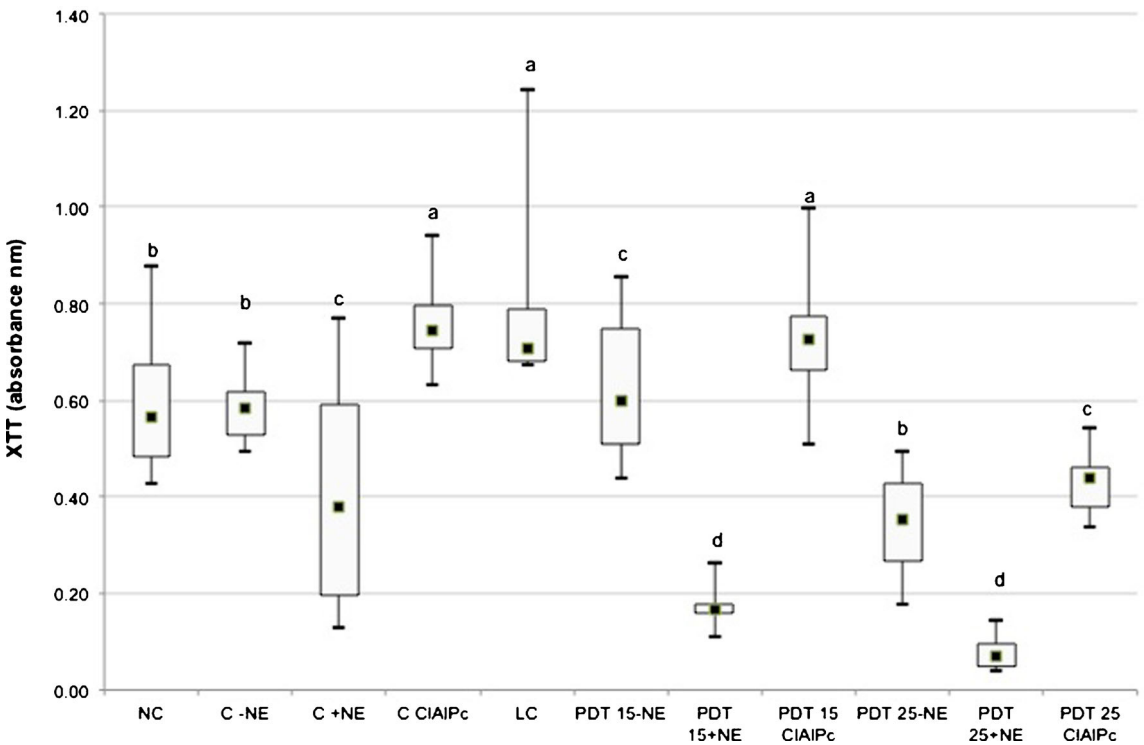

Fig. 5 Box-plot graphic representation of mean values and standard deviation of absorbance values (XTT assay) obtained for the MSSA biofilms subjected to the different experimental conditions. Groups identified with the *same letter* do not differ statistically (Multiple Comparison based on pairwise ranking test; $p>0.05$). $n=10$

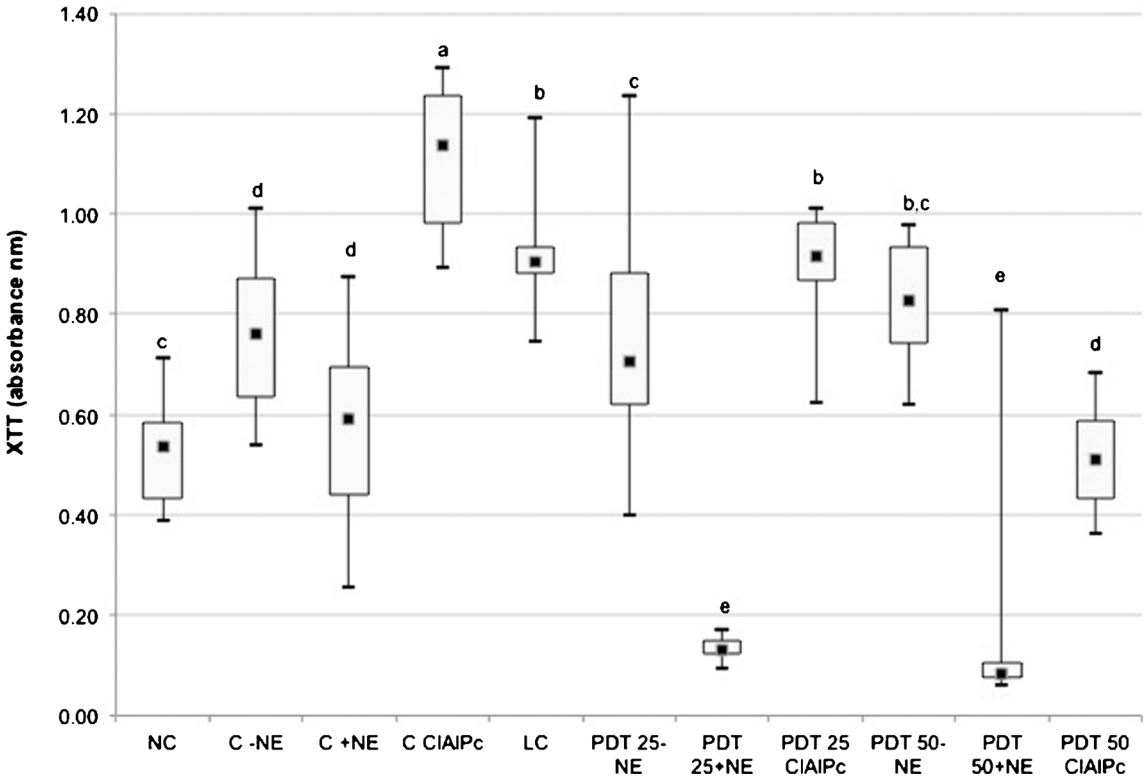

Fig. 6 Box-plot graphic representation of mean values and standard deviation of absorbance values (XTT assay) obtained for the MRSA biofilms subjected to the different experimental conditions. Groups identified with the *same letter* do not differ statistically (Multiple Comparison based on pairwise ranking test; $p>0.05$). $n=10$

hydrophobic nature of phthalocyanines impairs their circulation in aqueous medium and increases the possibility of aggregate formation, making necessary the use of these drugs with organic solvents or delivery systems, such as polymeric micelles, liposomes, nanoparticles, and NE, which are mainly employed in antitumor PDT [11, 13, 16, 17]. Most aPDT studies have used PS in aqueous solutions either diluted directly in saline or initially dissolved in an organic solvent, such as DMSO, and then diluted in aqueous medium, if necessary [18]. Although this is a viable procedure for in vitro experiments, clinical application of phthalocyanines using this form of delivery is difficult because saline and organic solvents are instable media, which would require preparation of the drug at the moment of use to ensure minimal alterations of its physical properties. Furthermore, DMSO is not an excellent delivery system for in vivo application because it increases the permeability of the host cells to the drug, possibly causing tissue damage and systemic effects. Among the drug delivery systems for aPDT, NE represent an excellent option as a topical delivery system with high capacity of penetration into the tissues [13, 19], better biodistribution and greater drug stability, reducing the possibility of aggregate formation and loss of photodynamic efficiency [20]. Although the use of drug delivery system may reduce the singlet oxygen production, the absolute value of this production alone should not be the only parameter to evaluate the photodynamic activity of a photosensitizer. For AlPC, the reduction of its aggregation properties and changes in excited state lifetime, lead to the activity of other reactive oxygen species production, such as hydroxyls radical and superoxide anion. Therefore, the present study evaluated the phototoxic properties of ClAlPc encapsulated in cationic and anionic NE and compared with ClAlPc diluted in organic solvent in the photokilling of different planktonic suspensions and biofilms of *S. aureus* (MSSA and MRSA).

The results obtained for the planktonic suspensions revealed photokilling of both *S. aureus* strains (MSSA and MRSA) by ClAlPc encapsulated in cationic NE-ClAlPc or diluted in DMSO. On the other hand, anionic NE-ClAlPc caused only a 4 $\log_{10}$ reduction of MSSA cell survival and did not cause any reduction in MRSA CFU/mL. It is known that Gram-positive bacteria have a relatively thick cell wall, composed basically by peptidoglycans and negatively charged due to the residues of teichoic acid [21]. In this way, cationic molecules have greater affinity by the cell wall, facilitating the active transport of these drugs into the bacterial cells [6, 22–24]. Mantareva et al. [22] evaluated the photodynamic effect of cationic and anionic hydrosoluble zinc-phthalocyanines against MSSA and MRSA and found

that both strains were photokilled by the cationic phthalocyanine in concentrations up to 6 μM, while the anionic phthalocyanine needed higher light fluences to cause 3 to 4 $\log_{10}$ reduction in *S. aureus* growth. A previous study evaluating the photodynamic efficiency of a porphyrin encapsulated in neutral and cationic liposomes showed a significant inhibition of MRSA growth only when the cationic phospholipid carrier was used, resulting in 4.5 and 6 $\log_{10}$ reduction when the porphyrin was illuminated with a halogen light source for 5 and 10 min, respectively [6]. Therefore, the superficial charge of the delivery system or even the drug itself is determinant on the photodynamic efficiency.

In addition, the lower affinity of the anionic delivery system by bacterial cells was influenced by removal of the excess of extracellular drug. Due to the optical characteristics of NE, after the pre-incubation period, the excess of extracellular NE was removed because their opaque characteristics made difficult light distribution in the bacterial suspensions and biofilms. This means that the antimicrobial phototoxic damages were caused exclusively by the drug that penetrated into the bacterial cells. The influence of absorption of light-sensitive drugs by target microorganisms on the efficiency of aPDT has been investigated in experiments with and without the drug present during irradiation. Grinholc et al. [25] evaluated the photokilling potential of a protoporphyrin against MSSA and MRSA planktonic cultures and found that washout of the PS before illumination resulted in minimal bactericidal effect. A recent study, however, did not find significant difference in the phototoxic potential of a chlorine to *S. aureus* cultures with and without washout of the PS before illumination [26]. These results indicate that the type of delivery system and PS determine the capacity of the drug to penetrate into bacterial cell more rapidly than host cells. The capacity of cationic NE-ClAlPc to be readily absorbed by the target cells, as demonstrated in the present study, is important because it limits the phototoxic damage to the cells that absorbed this PS, without causing damage to the subjacent normal cells.

Another interesting finding of the present study was that free ClAlPc and cationic NE-ClAlPc presented similar results in reducing CFU/mL of MSSA and MRSA planktonic suspensions. Tsai et al. [27] compared two forms of encapsulation of hematoporphyrin (liposomes and polymeric micelles) and its free form (dilution in saline) in the photoinactivation of Gram-positive pathogens. The authors reported that photokilling was more effective when liposomes and polymeric micelles were used with porphyrin at lower concentrations. However, the three delivery systems had the same behavior when higher concentrations of porphyrin were used. Therefore, it may be speculated that the concentration used in the present study was sufficiently high to cause photoinactivation of the bacteria, as similar results were obtained for free ClAlPc and cationic NE-ClAlPc.

In the present study, in addition to the delivery system and superficial charge, the light fluence also influenced the aPDT efficiency. For both *S. aureus* strains, only the higher light fluence caused photokilling of the planktonic suspensions. For MSSA, photokilling of the microorganisms was observed when the cationic NE-ClAlPc and free ClAlPc were illuminated with a light fluence of 25 J/cm^2. Even for the anionic NE-ClAlPc, illumination with the higher light fluence resulted in a 4 $\log_{10}$ reduction of bacterial cell survival, while a reduction of only 2 $\log_{10}$ was observed with the lower light fluence. For MRSA, twice the light fluence (50 J/cm^2) was necessary to a full photodynamic inactivation of this strain compared with MSSA. This higher resistance of MRSA to aPDT has been demonstrated [27–29]. Grinholc et al. [28] reported that although no association was established between antibiotic resistance and photoinactivation, the results demonstrated that the different MRSA strains were less susceptible to aPDT than the MSSA strains. Tsai et al. [27] also found that the methicillin-resistant strain was more resistant to aPDT than the MSSA, requiring a fourfold higher concentration of hematoporphyrin to be inactivated. The greater resistance of MRSA strains to aPDT has been related to the capsular polysaccharide structure on the bacterial cell surface, which would limit the penetration of the PS into the MRSA cells, reducing the toxic effects of the ROS generated during photodynamic inactivation [30].

In addition to the photodynamic effect of ClAlPc on survival counts (CFU/mL) of MSSA and MRSA planktonic suspensions, the metabolism of the bacterial suspensions after aPDT was also evaluated using the XTT assay, which is based on MTT salt reduction by the active electron transport system of bacteria [31]. For MSSA, both forms of NE encapsulation and free ClAlPc resulted in accentuated reduction of cell metabolism, regardless of the light fluence used for irradiation of the drug. For MRSA, however, a significant reduction of bacterial cell metabolism was observed only when the higher light fluence (50 J/cm^2), except for free ClAlPc, which also reduced cell metabolism by approximately 96 % when illuminated with the light fluence of 25 J/cm^2. These results suggest that aPDT caused an initial alteration in the active electron transport of cell membrane responsible for bacterial respiration, which represents the basic principle of XTT assay. The results of the XTT assay showed that the cationic NE-ClAlPc and free ClAlPc presented a significant dark toxicity to MSSA planktonic suspensions, and only the cationic NE-ClAlPc was toxic in the dark to the MRSA planktonic suspensions.

Higher toxicity of cationic molecules, either the PS or the delivery system, has been previously reported [17, 32, 33]. Kussovski et al. [32] evaluated the effect of cationic phthalocyanines against Gram-negative bacteria, and found photokilling of *Aeromonas* when one of the phthalocyanines was used at a concentration above 2 μM. Bourré et al. [33] reported that

porphyrin at concentrations above 1 μM was also toxic when conjugated with a peptide, causing a ~3 to 6 $\log_{10}$ reduction in CFU/mL counts of Gram-positive species, such as *S. aureus*. This higher toxicity of cationic molecules is related to their potential to bind more rapidly to microorganisms and alter the properties of their plasma membrane.

Regarding the biofilms, cationic NE-ClAlPc produced the best results, reducing the cell metabolism by around 80 and 76.5 %, for MSSA and MRSA, respectively. Biofilms are structures formed by microorganisms involved by an extracellular polymer matrix and represent the most common form of bacterial organization in the nature [34]. This polymer matrix reduced the permeation of antibacterial agents, increasing bacterial resistance and persistence of infections, and therefore, a number of strategies have been proposed to increase the permeability of biofilms to antimicrobial agents and PSs used in aPDT [12]. Sharma et al. [9] evaluated the effect of pretreatment with the chelating agent EDTA on the photoinactivation of staphylococcal biofilms, and found that this agent increased aPDT efficiency against *Staphylococcus epidermidis* by disrupting the biofilm structure and reducing bacterial cell counts, but it did not improve the photodynamic action against *S. aureus* biofilms [9]. A recent study evaluated the antimicrobial photodynamic potential of different types of cationic gallium-phthalocyanines (GaPc) against biofilms of Gram-positive and Gram-negative bacteria and fungi [23]. The time of formation of the biofilms was only 18 h, representing an intermediate stage and they were treated with GaPc at 6 μM and LED light at 50 J/cm^2. The aPDT caused photokilling of the fungal biofilms and maximum 1 to 2 $\log_{10}$ reduction on in the counts of viable colonies of the bacterial biofilms. The authors suggested that because of the longer time required for replication of fungal cells, the period of 18 h resulted in fungal biofilms with a smaller number of microorganisms when compared with the bacterial biofilms, which were more resistant. In our study, the biofilms were formed during 48 h, period that allowed bacterial growth rate higher than the planktonic suspension (standard suspension of 1×10^7 cells mL^{-1}). Therefore, at the PDT treatment, the number of cells for biofilms was greater than for planktonic suspensions, turning the effectiveness of this therapy for biofilms a challenge. Although complete reduction of biofilm metabolism was not achieved, aPDT with cationic NE-ClAlPc appeared as a good option as it reduced the metabolism of both *S. aureus* biofilms. In addition, it is known that singlet oxygen, a product of the photodynamic reaction, also acts on the extracellular matrix polysaccharides [12], causing disruption of this structure and facilitating the action of other therapies used in combination with aPDT. Regarding the limitations of this in vitro study that includes the non-detection of extracellular polymeric substance and only one method of evaluating the effects of PDT on biofilms, the cationic NE-ClAlPc presented promising results for the photokilling of planktonic and biofilm cultures of MSSA and MRSA, offering greater stability to the drug for clinical application and greater permeation of biofilms with a more efficient action against the microorganisms.

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