Photoinactivation of Methicillin-Resistant *S. Aureus* Biofilm using a New Chlorin as Photosensitizer

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Keywords: Bacterial Biofilm, Membrane, Resistant Bacteria, Photoinactivation, Photosensitizer, Chlorin.

Abstract: Due to the increase in bacterial resistance to antibiotics, the development of new drugs and technologies for the eradication of microorganisms is a priority. Photodynamic Therapy (PDT) depends on the interaction between a light-sensitive compound (photosensitizer), light, and molecular oxygen. The reaction generates reactive oxygen species (ROS), which induce cell death by oxidative stress. Antimicrobial Photodynamic Therapy (A-PDT) may be a promising alternative for microbial infections since its action occurs by multiple targets, which hinders the development of resistance. The main goal of this study was the evaluation of the potential of a newly synthesized chlorin derivative sterically prevented from self-aggregation as a photosensitizer to photoinactivation Methicillin-Resistant *S. aureus* (MRSA) biofilm and to investigate the membrane integrity after the treatment. The results showed a high potential of this chlorin for photoinactivation of MRSA biofilms reducing the survival index more than 5 log CFU mL^{-1} leading to the unstructured membrane and consequent cell death by photooxidation of membrane components after A-PDT.

1 INTRODUCTION

Due to the increasing worldwide resistance of bacteria to antibiotics, the development of new drugs and technologies for the eradication of microorganisms is a priority (Sobotta *et al.*, 2019). The biggest problem with using antibiotics is that bacteria have different resistance mechanisms, which can result in the formation of biofilms that are even more refractory to treatments. Antimicrobial Photodynamic Therapy (A-PDT) may be a promising alternative for microbial infections since its action occurs by multiple targets, which hinders the development of resistance (Stanislaw *et al.*, 2018). PDT involves the combination of a photosensitizer (PS), molecular oxygen, and visible light of adequate wavelength to produce reactive oxygen species (ROS), causing the cell to die through the oxidation of its constituent biological molecules (Fig. 1).

PS is a substance that induces light sensitivity to chemical, physical, or both processes usually insensitive to light. Most photosensitizers have a heterocyclic ring similar to chlorophyll and the hemoglobin heme group. Photons are absorbed in the band of the electromagnetic absorption spectrum characteristic of photosensitizers and can transfer this energy to other molecules, especially to molecular oxygen, which will result in the release of short-lived energy species, leading to damage to the biological system involved (Hamblin *et al.*, 2008).

Chlorins are molecules of high abundance and importance in nature, present in most plants that make photosynthesis (De Oliveira *et al.*, 2014). CHL-Ph-A is a new chlorin derivative sterically prevented from aggregation due to the structural shape in “L”. Diels-Alder reaction was used to synthesize CHL-Ph-A from protoporphyrin IX (Linares *et al.*, 2017).

The objective of this study was to photoinactivate Methicillin-Resistant *S. aureus* (MRSA) biofilm using a new chlorin (CHL-Ph-A) with the aid of Full Factorial Design 2^3 and microscopy techniques to evaluate the integrity of the bacterial membrane.

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Amaral, L., Linares, I. and Perussi, J.
Photoinactivation of Methicillin-Resistant S. Aureus Biofilm using a New Chlorin as Photosensitizer.
DOI: 10.5220/0008938100920096
In *Proceedings of the 8th International Conference on Photonics, Optics and Laser Technology (PHOTOPTICS 2020)*, pages 92-96
ISBN: 978-989-758-401-5; ISSN: 2184-4364

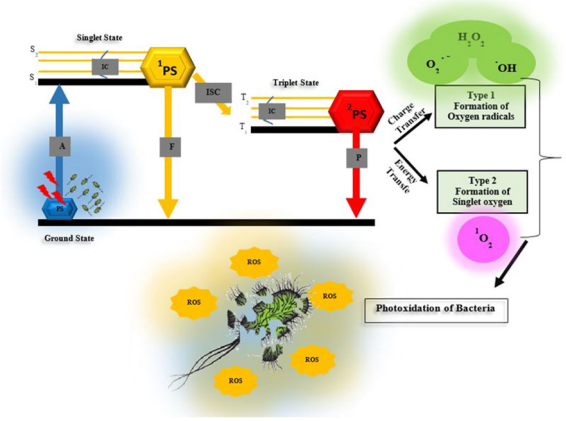

Figure 1: Mechanisms of oxygen reactive species production: when the photosensitizer (PS) is incubated with the bacteria, it absorbs energy from light and goes to the excited singlet state (S_0 → S_1 or S_2 decaying to S_1 by Internal Conversion – IC). The relaxation can occur by fluorescence (F) or by intersystem crossing (ISC). In the triplet state, the PS molecule may lose energy by phosphorescence (P) or energy transfer to molecular oxygen, generating singlet oxygen ($^1O_2$) by type II mechanism. When charge transfer occurs generating free radicals (type I), death of the bacteria may happen due to the oxidation of their cellular components.

2 METHODS

2.1 Bacterial Strain and Biofilm Culture Conditions

Methicillin-Resistant *Staphylococcus aureus* – MRSA (ATCC® 33591 ™) was grown of the planktonic form in Brain Heart Infusion (BHI) medium at 37° C for 18 h with orbital agitation at 250 rpm. After planktonic cultivation, the optical density of each bacterium was standardized to OD 600 in phosphate-buffered saline (PBS), using a HITACHI U-2800 spectrophotometer. For biofilm formation, 150 µL of the suspension was deposited on the 96-well plate and supplemented with 150 µL of the BHI medium. The plates were kept in an incubator for 48 h at 37° C.

2.2 Photosensitizer and Light Source

CHL-Ph-A (Fig. 2) was synthesized in our research group, and the procedure and full characterization are described in Linares *et al.*, 2018.

The light source used for photoinactivation of MRSA was an illumination platform called Biotable (Fig. 3) developed by the LAT at the Instituto de Física de São Carlos, Brazil, composed by 40 red LEDs (660 ± 10 nm).

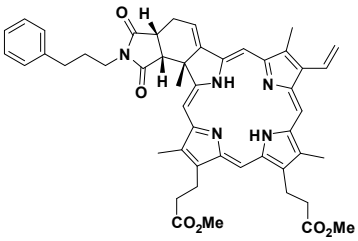

Figure 2: Molecular structure of chlorin CHL-Ph-A.

Figure 3: Red biotable 660 ± 10 nm used in irradiation procedures.

2.3 Photodynamic Inactivation of Bacteria in Biofilm Form

The MRSA biofilm photoinactivation was performed with the aid of Full Factorial Design 2^3 (FFD $2^3 = 8$ experiments), using three parameters with two levels and a central point allowing to perform a smaller number of experiments, less time consuming and lower expenses to obtain the results. The three parameters used were CHL-Ph-A concentration (C_{PS}: 5; 7.5 and 10 µmol L^{-1}) incubation time (IT: 20; 30 and 40 min), and light dose (LD: 15; 22 and 30 J cm^{-2}) at 660nm combined among them in a multivariate form, submitting the results to Two-way ANOVA.

2.4 Determination of the Bacterial Viability

MRSA biofilm was slowly homogenized, removing a 100 µL aliquot from each well of the 96-well plate (before and after A-PDT) and diluting from 10^{-1} to 10^{-8}. Four aliquots of 15 µL were taken from each dilution and deposited on BHI agar plates and incubated at 37° C for 18 h. Each BHI agar plate was divided into four quadrants, each assigned to a dilution. Quantification was performed by counting the colonies at the dilution in which they had 5 to 50 colony forming units (CFU). The number of survivors

present in the sample was determined by the average number of colonies, multiplied by the dilution, and the number of CFU per milliliter of the solution was obtained.

2.5 Membrane Integrity Analysis by Fluorescence Microscopy

MRSA Biofilm before and after photoinactivation was submitted to membrane integrity analysis. Biofilm was cultivated in microscopy slides and submitted to a mixture of SYTO®9 and Propidium Iodide (PI) (mixture of the LIVE/DEAD™ kit, Invitrogen Molecular Probes®) being finally analyzed by a Fluorescence Microscope (Olympus BX41) with 100X objective, 500 nm dichromatic filter, excitation at 460-490 nm and emission at 520 nm.

2.6 Morphostructural Analysis of Photoxidized MRSA

For the morpho-structural analysis by Scanning Electron Microscopy (SEM), the biofilm was cultivated under polystyrene slides 1x1 cm arranged in the bottom of 12 well plates. Then 1 mL of BHI broth was added, keeping them in the oven for 48 h at 37° C. Alternatively, in place of the BHI, 1 mL of the PS solution was placed in a previously defined concentration and irradiated. Then the biofilms were washed with PBS and fixed with 1 ml 2.5% glutaraldehyde for 1 hour. Then, the sample was dehydrated with ethyl alcohol in different concentrations: 10, 25, 50, 75, 90, and 100% for 20 min each. After drying, the slides were metalized and visualized in the LEO scanning electron microscope, model 440, with a magnitude of 60.00 KX.

3 RESULTS

3.1 Biofilm Photoinactivation

The inactivation obtained using CHL-Ph-A by the multivariate form presented in Table 1. The best photoinactivation obtained was 53 % for the more significant variation in the survival index ($\Delta \log_{10}$) of 5.13 corresponding to nine assays. This decrease in the survival index can be considered good (or enough) when dealing with biofilm, which is very difficult to inactivate. So, the best parameters were PS concentration of 5 µmol L^{-1}, IT of 40 min, and LD 30 J cm^{-2} reaching maximum photoinactivation of 4,52 ± 0,02 log CFU.

In a biofilm, bacteria have the same genetics as in planktonic culture, but their biochemical activities differ by 40%, presenting a greater difficulty to be eliminated due to acquired resistance (Wiesch *et al.*, 2011). Given this difficulty, antimicrobial photodynamic therapy can be employed as an option for indiscriminate use of antibiotics, thus reducing the problem related to bacterial resistance. The methodology does not entail resistance to bacteria due to the vast number of possible targets that ROS can act in preventing any bacterial adaptation/mutation. However, according to the American Society of Microbiology, the reduction must be more significant than required (> 3 log CFU mL^{-1}) for a new approach to be called antimicrobial (ASM, 2015). Fortunately, photodynamic therapy using CHL-Ph-A fulfills this requirement.

Table 1: Bacterial viability of MRSA biofilm after a-PDT with CHL-Ph-A. The results of the experiments are arranged according to the experimental matrix FFD 2^3 where IT: incubation time (min), LD: light dose (J cm^{-2}), and C: chlorin concentration (µmol L^{-1}). Nine different experiments were performed as described according to the values of the parameters used. After the procedures, the results of each assay (Colony Forming Unit – CFU) are described and presented as average ± standard deviation (SD) with n = 4 replicas.

Assay	IT (min)	LD (J cm^{-2})	C (µmol L^{-1})	CFU Average ± SD CHL-Ph-A
-	0	0	0	9,65 ± 0,03
1	20	15	5	7,78 ± 0,11
2	20	15	10	7,36 ± 0,14
3	20	30	5	7,46 ± 0,06
4	20	30	10	7,40 ± 0,04
5	40	15	5	6,11 ± 0,16
6	40	15	10	5,90 ± 0,12
7	40	30	5	4,52 ± 0,02
8	40	30	10	6,48 ± 0,03
9	30	22	7.5	6,48 ± 0,05

3.2 Membrane Rupture after Chlorin-PDT

The integrity of the bacteria membrane present in the biofilm was determined by Fluorescence Microscopy using the Live/Dead kit, which contains two markers, the fluorescent green SYTO®9 (S) and the fluorescent red propidium iodide (PI). The probe S penetrates both into intact cells or not because of its low molecular weight; however, the PI only penetrates cells with the damaged cytoplasmic membrane because of its high molecular weight resulting in the reduction of S intensity when both dyes coexist in the cell. Figure 4 shows the results. The CHL-Ph-A chlorin associated with photodynamic therapy enabled the cytoplasmic membrane disruption of MRSA in the biofilm, revealing the red color (Fig.4

B). On the other hand, the presence of membrane integrity is represented in green (Fig.4 A).

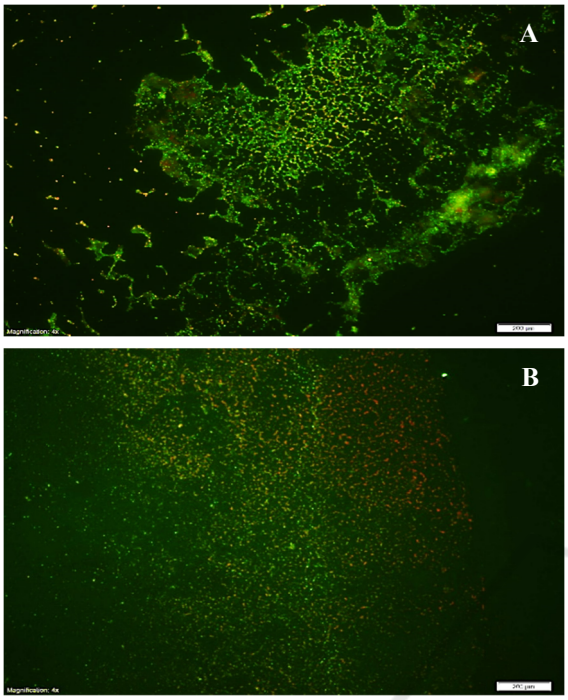

Figure 4: Fluorescence microscopy of MRSA biofilm: (A) Control and (B) after photoinactivation using CHL-Ph-A.

3.3 Analysis of MRSA Photoxidized after Chlorin-PDT

Scanning Electron Microscopy (SEM) was used to characterize the biofilm structure, bacterial morphology, as well as to evidence the photodynamic process. For this, *S. aureus* biofilm was submitted to SEM analysis in the best experimental conditions for CHL-Ph-A photooxidation using FFD 2^3. The parameters used were: C = 5 µmol L^{-1}; IT = 40 min, and LD = 30 J cm^{-2}.

Through Figure 5, it is possible to observe by Scanning Electron Microscopy the bacterial arrangements of *Staphylococci* colonies as well as the bacterium-bacterium interactions in biofilm presenting links between them through fimbria (highlighted in yellow). After A-PDT (Fig. 5B), a damaged structure was observed (highlighted in red), denoting photooxidation of cell membrane components in which cocci do not have a definite shape but a turgid structure as a deformation.

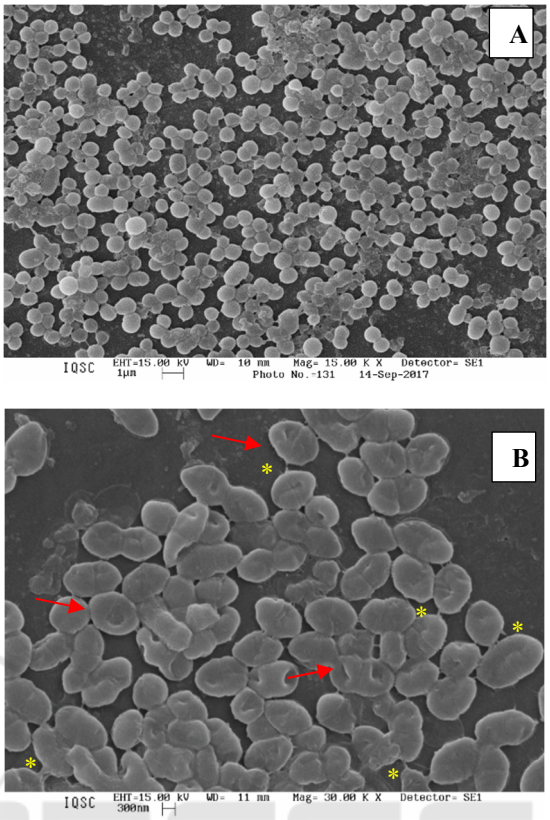

Figure 5: Scanning Electron Microscopy of MRSA Biofilm: (A) Control and (B) After photoinactivation using CHL-Ph-A.

4 CONCLUSIONS

The results suggest that A-PDT of MRSA biofilm using CHL-Ph-A has excellent potential to combat MRSA biofilm since reductions up to 5.13 $\Delta\log_{10}$ were reached. The efficiency of photooxidation and the potential to eradicate biofilm was observed and proven by SEM and Fluorescence Microscopy. The results suggest that antimicrobial photodynamic therapy using this new chlorin may be a good alternative for the treatment of antibiotic-resistant bacterial infections.

Overall, A-PDT is expected to be implemented to ensure a broad bacterial inactivation. It is also expected that the use of multivariate models, such as complete factorial design 2^3 be usefull in future experiments aiming to reduce costs and experimental time in order to search for better responses that enable even greater photoinactivation.

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo/Centro de Pesquisas de Ótica e Fotônica (FAPESP/CEPOF) 2013/07276-1.

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