Photodynamic inactivation of Staphylococcus aureus and Escherichia coli using a new bacteriochlorin as photosensitizer

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ABSTRACT

In this study, we used bacteriochlorin as a photosensitizer, characterized by their low toxicity in the absence of light, presenting absorption around 780 nm, with the objective of evaluating their photodynamic inactivation potential on *Staphylococcus aureus* and *Escherichia coli*. Bacteriochlorins were synthesized from the extraction of bacteriochlorophylls from non-sulfurous purple bacteria and were then converted to bacteriochlorins. *S. aureus* and *E. coli* microorganisms were used in the planktonic and biofilm forms. For the formation of biofilms on glass coverslips, suspensions of the microorganisms at the concentration of 10^6 CFU/mL were inoculated into each well of a microplate. There was an exchange of culture medium (Tryptic Soy Broth - TSB) every 24 hours for 7 days, pre-washing the coverslips with a phosphate-buffered saline (PBS), to ensure that only adhered microorganisms were grown and then incubated at (36 ± 1)°C between the middle exchanges. After 7 days of induction, the biofilm was mature, like those normally found in nature, and then it was applied different treatments (light doses associated with FS concentrations). At the end of the treatment, the coverslips underwent an ultrasonic disintegration, and the quantitative evaluation of viable cells was performed by plate counting using the plate method in Tryptic Soy Agar (TSA), incubating at (36 ± 1)°C for 24 hours. The results showed that the PDI for *E. coli* was not successful even when it was more susceptible to the planktonic form, whereas for *S. aureus* the results showed a reduction in cell viability 6 logs for the planktonic forms, but lower to 1 log in biofilms. Therefore, novel studies using bacteriochlorins and surfactants will be performed to verify the potential of this alternative treatment method.

Keywords: photodynamic inactivation; bacteriochlorin; *Staphylococcus aureus*; *Escherichia coli*

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1. INTRODUCTION

Although advances in the treatment of diseases caused by bacteria, fungi, viruses and protozoa, and despite the existence of several methods of microbial control, microorganisms, mainly the bacteria, acquire resistance to these methods, being a challenge for a medical area the development of new methodologies for its control and treatment. For example, methicillin-resistant *Staphylococcus aureus* (MRSA), which defies modern therapy, becomes a severe hospital infection problem.[1]

Bacterial biofilms can be defined as associations of microorganisms and their extracellular products, which are adhered to biotic or abiotic surfaces.[2] Although bacteria may have a free existence (planktonic lifestyle) in the environment, most of them are associated with biofilm. Biofilm is a complex, highly structured, and highly dynamic microbial ecosystem that facilitates the uptake of nutrients and the maintenance of an environment with suitable physical and chemical conditions.[3] In a bacterial biofilm, the microorganisms that compose it presents higher resistance to antibiotics than planktonic lifestyle, although these mechanisms have not been elucidated yet. Also the use of antibiotics in the biofilm, microorganisms become more resistant to chemical and physical agents, as used in the cleaning procedure.[4]

Photodynamic inactivation (PDI) is a combination of a photosensitizer agent, light, and molecular oxygen to cause cell death. During the irradiation, the photosensitizer present in the target area is excited and induces a local toxicity that can cause oxidative cellular damage.[5] Cell death can be induced through two types of mechanisms: type I, when a photo

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induced electron transfer from the excited photosensitizer to biomolecules is observed, producing active free radicals; and type II, when the triplet oxygen is excited and results in the production of highly reactive singlet oxygen species.[6,7] Bacteriochlorins are the primary candidates as photosensitizers. They belong to a class of sensitizers characterized by their low toxicity in the absence of light since these compounds exhibit relatively intense absorptions in the region of 750-800 nm. This region, which has a large wavelength, is advantageous due to the high penetration that the red light has.[8,9]

Bacteriochlorophylls can be found naturally in some microorganisms, including purple bacteria, such as *Rhodopseudomonas palustris*. These microorganisms use Mg^{2+} complexes present in the bacteriochlorophyll-A, as light absorptive chromophores, necessary for photosynthesis under anaerobic conditions.

2. MATERIAL AND METHODS

2.1. Photosensitizer

Bacteriochlorins were synthesized from the extraction of bacteriochlorophylls from non-sulfurous purple bacteria and were then converted to bacteriochlorins. Initially 0.5 mg of dry bacteriochlorin in trisma form was diluted in 50 µL of Dimethyl Sulfoxide (DMSO) until its total solubilization, followed then by the addition of 450 µL of Phosphate Buffered Saline (PBS: NaCl 8 g; KCl 0.2 g; Na_2HPO_4 1.15 g; KH_2PO_4 0.2 g; distilled water 1 L), to a concentration of 1402 µM. From this stock, the photosensitizer was then adjusted to the desired concentrations being diluted in PBS. Further, for the treatments with surfactants, 0.5 mg of dry bacteriochlorin was diluted in 500 µL of DMSO, still at 1402 µM, followed by adjustment to the desired concentrations being diluted in the respective surfactant.

2.2. Surfactants

Were tested three surfactants at different concentrations, which two of them were non-ionic (Pluronic F-127 and Polyoxyethylene sorbitan monolaurate - Tween 20) and one were anionic (Sodium Dodecyl Sulfate - SDS). For treatments at concentrations of 0.1% (m/m), 0.01g of the respective surfactant was weight and filled with 0.9% saline solution up to the 10g total. For treatments at concentrations of 0.3% (m/m), 0.03g of the respective surfactant was weight and filled with 0.9% saline solution up to the 10g total. For treatments at concentrations of 0.5% (m/m), 0.05g of the respective surfactant was weight and filled with 0.9% saline solution up to the 10g total. For treatments at concentrations of 1.0% (m/m), 0.1g of the respective surfactant was weight and filled with 0.9% saline solution up to the 10g total. The absorbance and fluorescence of 25 µM bacteriochlorin in the presence of surfactants can be seen in figure 1.

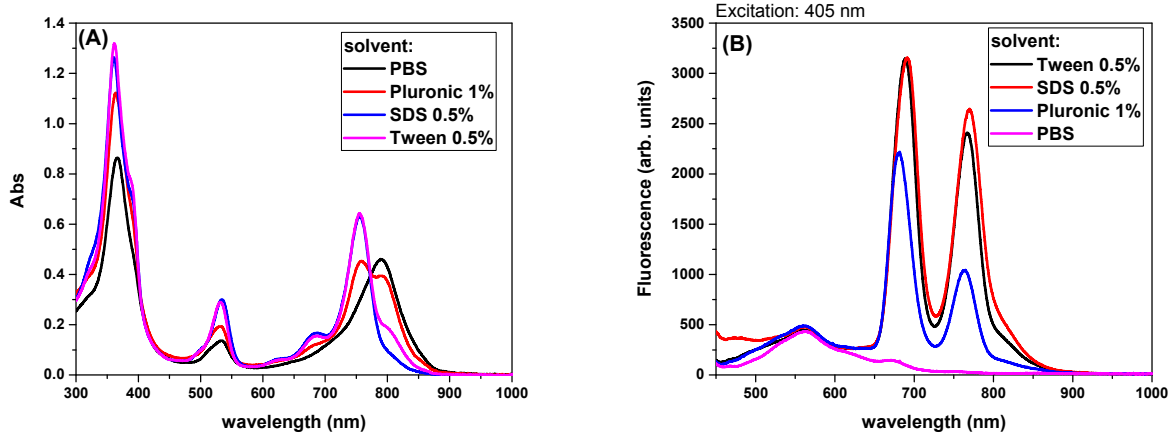

Figure 1: Absorbance (A) and fluorescence (B) of 25 µM bacteriochlorin in different solvents.

2.3. Culture Standardization

The culture of planktonic lifestyle microorganisms was obtained through its growth in culture medium Tryptic Soy Broth (TSB) for 24 hours. After that, the inoculum was adjusted in PBS using 0.5 McFarland standards. From this, the suspensions were diluted 1:100 in TSB, obtaining a culture at proximally at 10^6 CFU/mL of *Staphylococcus aureus* (gram-positive) or *Escherichia coli* (gram-negative) in planktonic lifestyle.

2.4. Bacterial Biofilm Formation

For the bacterial biofilm formation, pre-sterilized 13 mm diameter glass coverslips were placed in a 24-wells microplate with the aid of a sterilized forceps, followed by the addition of one milliliter of the bacterial suspension in TSB broth in each well. The microplates were incubated at (36 ± 1)° C for 7 days. The culture medium was changed every 24 hours. To prioritize the growth of the cells adhered to the coverslips, before each exchange of culture medium, the wells were washed with 1 ml of PBS three times before adding the culture medium.

2.5. Photodynamic inactivation

To the analysis of planktonic bacteria and biofilms in the absence of surfactant, experimental groups were obtained using different light doses and photosensitizer concentrations. The planktonic experimental groups and biofilms without surfactants were incubated for 15 minutes with the photosensitizer so that it could penetrate through the cell walls. Experimental groups using surfactants were incubated with the photosensitizer for 30 minutes. The microplate wells were irradiated with a homogeneous home-made system of LEDs of 30 mW/cm^2 at 780 nm.[10]

2.6. Disaggregation of the formed bacterial biofilm

After the biofilms were submitted to the different treatments, the coverslips were placed in Falcon conical tubes with a sterilized forceps, and 4 mL of PBS and 1 mL of the corresponding treatment was added in each of the tubes. The tubes were conditioned in an ultrasonic vessel for 16 minutes at a power of 150 W and 50 Hz for the disaggregation of biofilm microorganisms.

2.7. Quantitative evaluation of viable bacterial cells of biofilms

To evaluate quantitatively, the viable bacterial cells was used the spread plate method. Then, serial dilutions up to 10^{-5} were performed in PBS from the disrupted suspension. Subsequently, 25 μL of the dilutions and the original suspension were plated in tryptic soybean agar (TSA) with the aid of sterile Drigalsky handles. Afterwards, the plates were incubated in a (36 ± 1)° C incubator for 24 h, for the growth of the bacterial cells that were alive in the biofilm and then the counting of colony forming units (CFU) in each experimental condition.

3. RESULTS AND DISCUSSION

3.1. *Staphylococcus aureus* in planktonic lifestyle

Photodynamic inactivation procedure was applied to *S. aureus* in planktonic lifestyle. The results are showed in figure 2. Thus, in planktonic lifestyle is expected a greater reduction in viability than when in the biofilm.

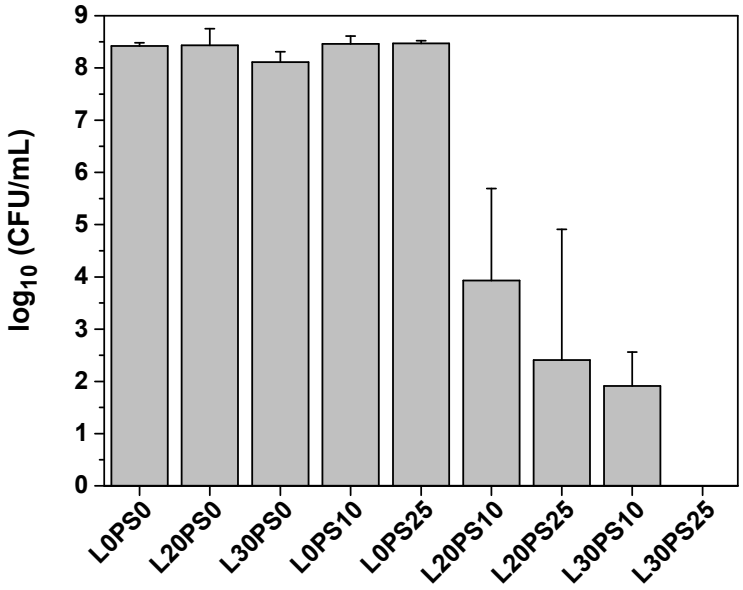

Figure 2: *S. aureus* planktonic lifestyle exposed to 0, 20 and 30 J/cm^2 of light (L), plus 0, 10 and 25 μM of photosensitizer (PS).

In this case, all treatments combining infrared light irradiation and photosensitizer presented a significant result of viability reducing of *S. aureus* in its planktonic lifestyle.

3.2. *Escherichia Coli* in planktonic lifestyle

The treatment of *E. coli* was performed in its planktonic lifestyle, and the results can be seen in figure 3.

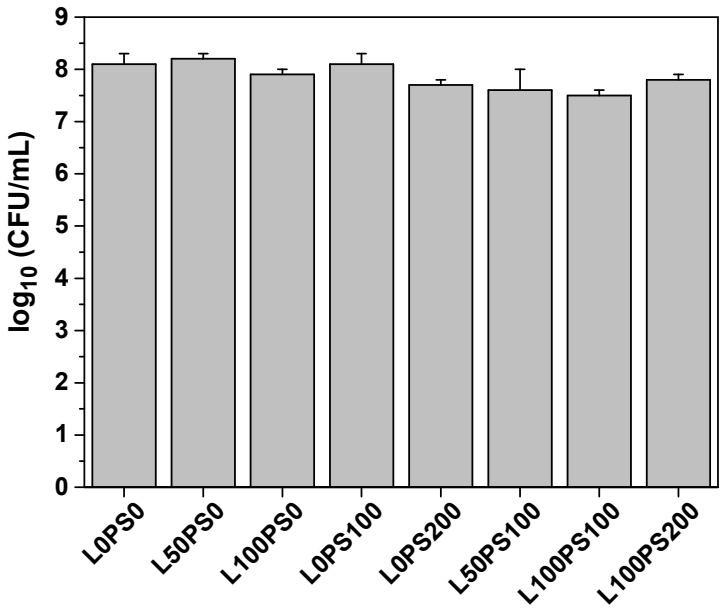

Figure 3: *E. coli* planktonic lifestyle exposed to 0, 50 and 100 J/cm^2 of light (L), with 0, 100 and 200 μM of photosensitizer (PS)

As well as in other studies with gram-negative microorganisms treatment with *E. coli*, the planktonic form did not provide satisfactory results.[11,12] This fact can be related to the difficulty of the photosensitizer in penetrating the gram-negative microorganism cell wall.

3.3. *Staphylococcus aureus* in biofilms

Figure 4 shows the results of PDI of *Staphylococcus aureus* in biofilms.

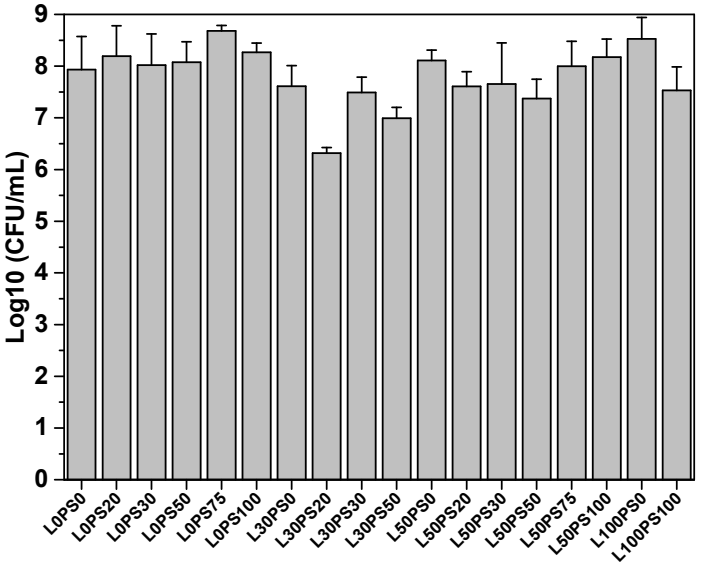

Figure 4: *S. aureus* biofilms exposed to 0, 30, 50 and 100 J/cm^2 of light (L), plus 0, 20, 30, 50, 75, 100 μM of Photosensitizer(PS).

In general, it is notable that practically all the treatments that used only near infrared light or only the treatment with the photosensitizer obtained an increase of the viability, occurring a biostimulation by light as observed in the literature.[13,14] No reports were found in the literature on the induction of microbial growth by the addition of photosensitizer. The existing reports are that these because they are not toxic when not illuminated, would not be expected to reduce any. About the treatments with near infrared light and photosensitizer, all obtained a positive result, some better than others.
As discussed by Dunne et al., 2002[15] the process of biofilm formation is complicated, and requires several steps until its irreversible fixation. Thus, there are several influences for a biofilm formation, existing biofilm variations despite the same culture conditions.

3.4. *Staphylococcus aureus* in biofilms using surfactants

3.4.1. Pluronic F-127

The results of PDI using Pluronic F-127 in biofilm of *S. aureus* are presented in figure 5.

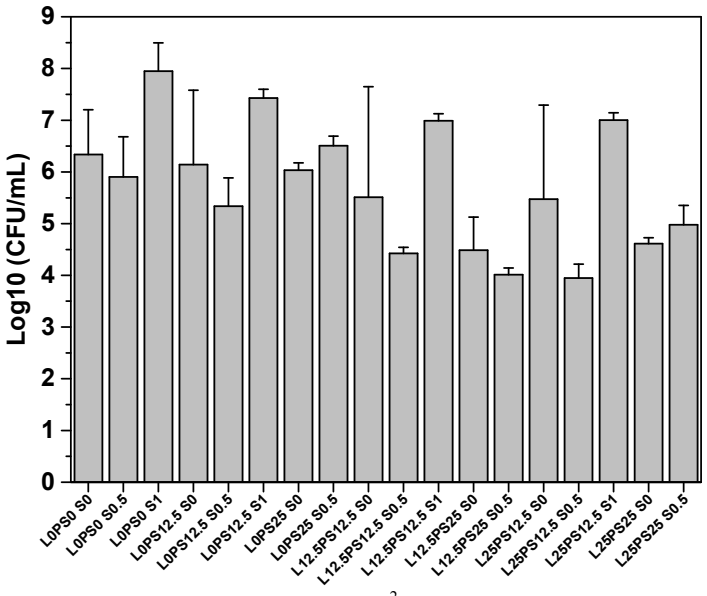

Figure 5: *S. aureus* biofilms exposed to 0, 12.5 and 25 and 50 J/cm^2 of light (L), plus 0 and 50 μM of photosensitizer (PS), with 0, 0.5 and 1 % of Pluronic F127 (S).

In this treatment the increase in the concentration of Pluronic F-127 caused an antagonistic effect to the desired, indicating the ability of the surfactant to promote bacterial growth as occurred with Leszczyńska et. al. (2010)[16] with another type of bacterial cells. As reported by Rauprich et. al. (2010)[17], surfactants in certain circumstances can serve as a nutritional source for microorganisms, thus promoting their growth.

3.4.2. Sodium Dodecyl Sulfate (SDS)

Figure 6 shows the results of PDI in *Staphylococcus aureus* biofilm using SDS.

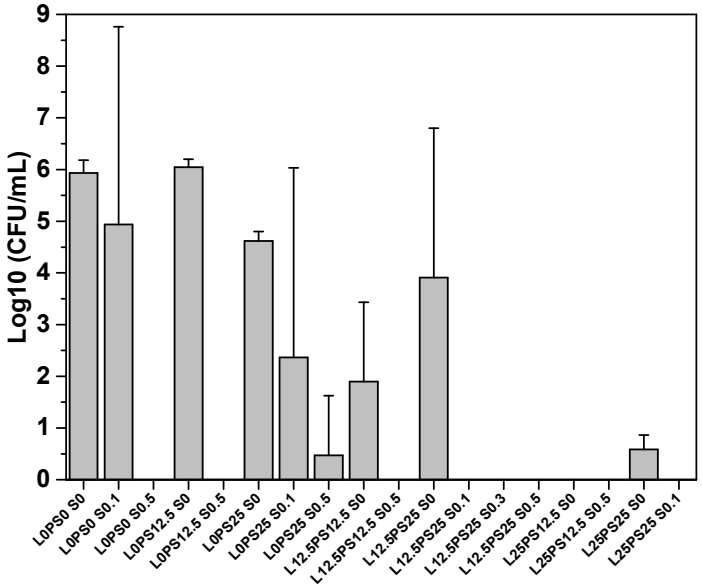

Figure 6: *S. aureus* biofilms exposed to 0, 12.5 and 25 J/cm^2 of light (L), plus 0, 12.5 and 25 μM of Photosensitizer (PS), plus 0, 0.1%, 0.3% 0.5% of SDS (S).

Even at different concentrations, SDS appears to cause cell death without needing the photosensitizer. As reported in the literature by Makgotlho et. al. (2013)[18], sub-inhibitory concentrations of SDS interfere in the synthesis of the gene called *sae* related to the virulence of the microorganism. This interference in gene synthesis may be responsible for causing cell death. As noted by Panhóca et. al. (2014)[19], SDS even at a concentration of 0.1% (v/v), causes a cytotoxic effect on *Streptococcus mutans* biofilms leading to a viability reduction above 90%, and virtually complete inactivation when combined with photodynamic therapy. Anionic surfactants not only alter the surface characteristics of the solids by adsorption, but they can also increase the solubility of discretely soluble compounds in water and can reduce the resistance to mass transfer.[20]

3.4.3. **Polyoxyethylene sorbitan monolaurate (Tween 20*)***

In figure 7 is presented the results of the PDI using Tween 20 with bacteriochlorin.

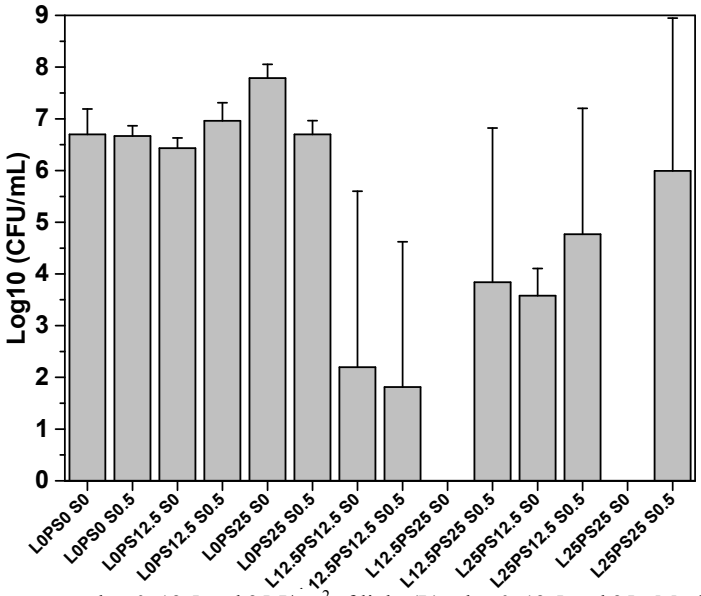

Figure 7: *S. aureus* biofilms exposed to 0, 12.5 and 25 J/cm^2 of light (L), plus 0, 12.5 and 25 μM of Photosensitizer (PS), plus 0, and 0.5% of Tween 20 (S).

Apparently, the concentration of surfactant used stimulated microbial growth, contrary to the desired effect. In the absence of Tween 20, the results of the PDT were more efficient in the control of growth of microorganisms than using Tween 20. ISHII et al. (2014) observed that when S. aureus is exposed to surfactants such as Tween 20 some genes have their expression altered in some way, mostly being highly expressed. They also found the NWMN_0246 gene, which they called *psyA* (pulmonary surfactant-inducible factor A), which synthesizes a protein of unknown function, nevertheless which possesses an NfeD-like C-terminus domain. Many NfeD-like C-terminates proteins are found in prokaryotes, and some of them contribute to lipid metabolism. Thus, there is a possibility that the *psyA* gene product may be involved in membrane repair in cells that have been damaged by fatty acids and detergents. Therefore, Tween 20 has in some ways developed an activity in the expression of genes that caused the repair of cell membrane damage, preventing a greater efficacy of the photodynamic therapy.

4. CONCLUSION

Regarding the susceptibility to inactivation by PDI, gram-positive bacteria were more susceptible than gram-negative species. As observed, PDI seems to not affect gram-negative, due to the mentioned motifs of the cell wall. Comparing the susceptibility of *S. aureus* when in planktonic form and when in biofilm, we noticed that the planktonic form is less resistant than the biofilm, even in several concentrations of treatments with photosensitizer and near-infrared light. Surfactants were used to enhance the photosensitizer penetration efficiency leading to an increase in bacterial cell death. However, the results obtained were dependent on the surfactant used. Nonionic surfactants such as Pluronic F-127 and Tween 20 promoted stimulation of microbial growth rather than inhibiting it. Sodium Dodecyl Sulfate, on the other hand, appears to have promoted large microbial control independent of PDI.

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