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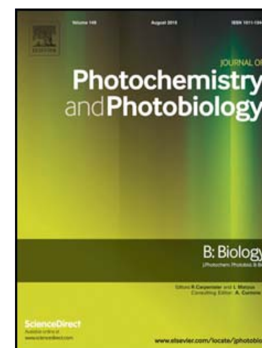

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Photodynamic inactivation of planktonic and biofilm growing bacteria mediated by a *meso*-substituted porphyrin bearing four basic amino groups

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Abbreviations
CFU: colony forming units; CSLM: confocal scanning laser microscopy; DMF: dimethylformamide; PDI: Photodynamic Inactivation, PS: photosensitizer; SEM: scanning electronic microscopy; TAPP: 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylammoniumpropoxy)phenyl]porphyrin; TAPP-PDI: TAPP mediated photodynamic inactivation; Tetra-Py+-Me: 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetraiodide; $TMAP^{4+}$: 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl) porphyrin; TB: Toluidine blue; TMPyP: 5,10,15,20-tetrakis(1-methyl-4-pyridinium)porphyrin tetra-(*p*-toluenesulfonate); TSA: Trypticase Soy agar; TSB: Trypticase Soy Broth.

Keywords
Photodynamic inactivation, porphyrin, bacteria, *Staphylococcus aureus*, biofilm, planktonic.

ABSTRACT

Biofilm-associated diseases account for 80% of all infections in humans. Due to the emergence of antibiotic resistances, alternative therapies such as Photodynamic Inactivation (PDI) of microorganisms have emerged. Porphyrins with intrinsic positive charges have been proposed as successful photosensitizers (PSs) against microorganisms. We have recently designed the new synthetic porphyrin 5,10,15,20-tetrakis[4-(3-*N*,*N*dimethylammoniumpropoxy)phenyl]porphyrin (TAPP) containing four basic amine groups in the periphery of the tetrapyrrolic macrocycle, which can acquire positive charges at physiological pH, thus favouring the interaction with biomembranes.
Illumination of planktonic cultures of *Staphylococcus aureus* at 180 J/cm^2 in the presence of 2.5 μM TAPP induced complete bacteria eradication.

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For the TAPP-PDI treatment of *S. aureus* biofilms, higher light fluences and PS concentrations were needed. Employing 20 µM TAPP and 180 J/cm^2, around 3-log CFU reduction were obtained.
In order to determine the efficacy of TAPP-PDI on Gram-negative bacteria, we performed planktonic and biofilm assays employing *Pseudomonas aeruginosa*. Much higher TAPP doses as compared to *S. aureus* were needed to achieve planktonic bacteria photosensitization (3-log CFU reduction at 20 µM TAPP and 180 J/cm^2). On the other hand, high concentrations of TAPP were non toxic to *P. aeruginosa* growing on biofilms, and employing 30 µM TAPP and 180 J/cm^2 we obtained 3-log CFU reduction.
The main conclusion of the present work is that TAPP is a promising and efficient PS capable of promoting photodynamic killing of both Gram -negative and -positive in planktonic bacteria, though more effectively in the latter. In addition, TAPP-PDI induces similar photoinactivation rates in both bacteria types growing on biofilms, with lower dark toxicity in the Gram-negative one.

INTRODUCTION

Staphylococcus aureus are Gram -positive cocci whose natural reservoirs are human skin and mucous membranes [1]. The rupture of any of those barriers can favour the development of *S. aureus*-induced human diseases, including severe chronic or acute infections both in hospitalized patients and community individuals [2]. The eradication of *S. aureus* is difficult not only due to the emergence of antibiotic resistances (such as methicillin, vancomycin) but also to their ability to form highly organized communities, known as biofilms [3]. Infections caused by bacterial biofilms are an immediate problem for public health as, according to the National Institutes of Health, biofilm-associated diseases account for 80% of all infections in humans. For this reason, alternative therapies are necessary to control the Staphylococcal infections. Photodynamic Inactivation (PDI) or antimicrobial photodynamic therapy utilizes the ability of photosensitizers (PSs), in combination with visible light, to produce reactive oxygen species that are lethal to the target pathogen [4-6].

The discovery of natural photosensitizers as well as the design of new synthetic ones is a key point in the successful treatment of infections, especially of those caused by biofilms. Phenothiazinium dyes, porphyrins, chlorins, xanthene dyes, natural products such as curcumin, bacteriochlorophyll derivatives and functionalized fullerenes are among the most widely used agents employed in the photosensitization of planktonic and biofilm bacteria [5,7-9].

The broad range of porphyrin derivatives employed as PSs in the PDI of microorganisms includes the chemical modified natural porphyrins as well as the synthetic porphyrins, which can be neutral, anionic or cationic [10]. The number of positive charges, the charge distribution in the porphyrin structure and the meso-substituent groups seem to have different effects on the photoinactivation of bacteria [11].

Several porphyrins with intrinsic positive charges have been successfully tested as photoinactivating agents against microorganisms [12, 13]. In particular, the positive charges on the tetrapyrrolic macrocycle appear to promote a tight electrostatic interaction with negatively charged sites at the outer surface of bacteria, increasing the efficiency of the photoinactivation processes, especially in the Gram-negative species [12]. A wide range of substituted cationic porphyrins have been used to mediate PDI of diverse species of pathogens [14]. It has also been reported that cationic PSs are particularly efficient at eradicating biofilms developed by Gram-positive bacteria [15].

In previous studies, we have investigated the photodynamic activity of cationic porphyrin derivatives with different patterns of substitution as PSs to eradicate specially Gram-negative bacteria [16, 17]. Porphyrins bearing three or four cationic charges showed to be active PSs to inactivate *Escherichia coli* [18]. Moreover, other authors have shown that non-cationic porphyrins conjugated with polylysine oligomers, which are positively charged at physiological pH values, can efficiently promote the

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photoinactivation of bacteria [19]. Thus, intrinsically non-charged molecules which are eventually charged at physiological pH are promising PSs to be used in the photoinactivation of microorganisms.

Many compounds have been described as potential PSs for PDI against bacteria biofilms so far, but conflicting results have been found. Biofilms of several species growing both in plastic and natural surfaces have been treated with PDI employing a broad range of PSs, achieving results ranging from less than 1 log colony forming units (CFU) reduction to 5 logs [15, 20, 21]. Notwithstanding, few studies have been carried out employing porphyrins for the photodynamic treatment of biofilm infections. PDI employing TMPyP [5,10,15,20-tetrakis(1-methyl-4-pyridinium)porphyrin] induced 1 to 2 fold reduction of *S. aureus* [22] and 4-fold reduction in *Pseudomonas aeruginosa* biofilms [23]. However, Cieplik et al [7] did not find any effects applying TMPyP-PDI to *E. faecalis* biofilms.

We have recently designed the new synthetic porphyrin 5,10,15,20-tetrakis[4-(3-*N*,*N*dimethylammoniumpropoxy)phenyl]porphyrin (TAPP) containing four basic amine groups in the periphery of the tetrapyrrolic macrocycle, which can acquire -depending on the external pH- positive charges, thus favouring the interaction with biomembranes. We have also studied the spectroscopic and photodynamic properties of TAPP in different media [24].

Even without having intrinsic cationic charges, TAPP showed a higher photodynamic activity in comparison with its cationic counterpart $TAPP^{4+}$ in a simple biomimetic medium formed by n-heptane/sodium bis(2-ethylhexyl)sulfosuccinate/water (reverse micelles), [24], suggesting that this intrinsically non-charged porphyrin can be a promising agent for the photodynamic inactivation of microorganisms.

Since PDI of bacteria is nowadays considered to be an important and efficient alternative to other conventional approaches of bacteria treatment, the aim of this study was to evaluate the photosensitizing ability of TAPP for the treatment of planktonic and biofilm cultures of *S. aureus* as a model of Gram-positive bacteria and *Pseudomonas aeruginosa* as a model of Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains

S. aureus strain RN6390 and a clinical isolation of *P. aeruginosa* were employed. Bacteria were stored in Trypticase Soy Broth (TSB) (Difco, USA) with 20% glycerol at -20ºC until use.

Photosensitizer

TAPP was synthesized as previously described by Caminos et al [25]. Its structure is depicted in Figure 1 and absorbance spectrum in Figure 2. Stock solutions were prepared in dimethylformamide (DMF), and then diluted in water before use.

Photoinactivation of S. aureus planktonic cultures

An aliquot from overnight cultures of bacteria was grown on TSB at 37ºC under constant shaking until an optical density of 0.7 at 600 nm equivalent to *ca.* 10^8 CFU/ml. was obtained. The bacterial suspensions were centrifuged at 13,000 rpm for 10 min at 4ºC, washed and suspended in sterile PBS to be utilized in PDI assays. An aliquot of 0.5 ml of each bacteria inoculum was added into each well of a 24-multiwell plate and incubated in presence of TAPP. After 10 min of incubation in the dark at room temperature, the plates containing the bacterial suspensions were illuminated in the presence of TAPP employing a non-coherent light source. Viable bacteria were quantified by plating an aliquot of serial dilutions on TSA. To assess total bacteria eradication, the whole 0.5 ml bacteria inoculum was plated. After 24 h incubation of the TSA plates at 37°C, the number of CFU/ml was determined. The following

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treatments were employed as controls: i) bacteria treated with TAPP but not exposed to light; ii) non TAPP-treated bacteria exposed to light; and iii) non TAPP-treated bacteria not exposed to light.

Biofilm formation and Photodynamic inactivation

The assessment of biofilm formation was performed as previously described, with some modifications [26]. Briefly, bacteria cultures were grown overnight and diluted 1:1000 in 0.25 % glucose supplemented TSB. An aliquot of this cell suspension was inoculated into sterile 24-well polystyrene microtiter plates (Greiner Bio-One). After 24 h of static incubation at 37°C, the medium in each well was replaced by 0.25 % glucose supplemented sterile PBS. Afterwards, TAPP was added onto biofilms and statically incubated for 10 min at 37ºC. After being washed with PBS, biofilms were irradiated as described below. Following irradiation, the biofilms were scrapped and the resulting suspensions were homogenized by vortex shaking. Bacterial viability (CFU/ml) was determined by plating on TSA as previously described.

Light source

Multiwell plates containing bacterial suspensions or biofilms were placed on a glass slide and exposed to the light source from above and below at 20°C with water filters and air-cooling. The radiation source was a set of two ELH tungsten halogen GE Quartzline lamps with a reflector (500 W, General Electric Co., Cleveland, Oh, USA) placed at 25 cm distance from the sample, which provided a homogeneous total fluence rate of about 25.2 mW/cm^2 on the surface of the sample measured with a FieldMaster power meter and a LM3 HTP sensor (Coherent Inc., USA). Emission spectrum of the lamp was recorded on a Photon Technology International Quanta Master fluorimeter (USA). Corrected spectrum is depicted in Figure 2. Fluence measurements and spectra were determined by interposing the same water filters in order to avoid IR and UV contribution. The light dose was modified by switching the exposition time, which resulted in fluences between 22.5 and 180 J/cm^2 respectively.

Bacterial TAPP binding/uptake assay

For the planktonic bining assay, overnight cultures of *S. aureus* were diluted in fresh medium and grown to mid-exponential phase, and 3 x 10^8 CFU/ml were employed. For the biofilm binding assay 24 h old biofilms were formed as explained above. TAPP was added to 1 ml of either bacteria suspension or biofilm culture to produce final concentrations of 2.5, 10, 20 and 50 µM. Cultures were incubated for 10, 20 and 60 min at 37 °C and subsequently, bacteria suspensions or scrapped biofilms were centrifuged for 10 min at 13,000 g. The supernatants were removed and the remaining pellets were washed twice with PBS. The washed pellets were then mixed with DMSO at room temperature and sonicated. The mixtures were centrifuged for 10 min at 13,000 g and the DMSO supernatants were removed for fluorescence analysis. Fluorescence was determined at excitation wavelength set to 419 nm and the emission wavelength set to 660 nm after analysis of the emission spectrum (Perkin Elmer LS55 fluorimeter, UK). The concentration of TAPP was determined through the use of standard curves of fluorescence versus concentration of TAPP dissolved in DMSO. This value was then divided by the CFU of each culture, to determine the number of fmoles of TAPP per CFU.

Biofilm microscopy analysis of viability

S. aureus biofilms were developed on sterile coverslips placed in multiwell plates, and were treated as indicated previously. To determine the viability of bacteria within the biofilms after the different treatments, a BacLight Live/Dead viability kit (Molecular Probes, Eugine, OR) was used. The kit includes two fluorescent nucleic acid stains: SYTO9 and propidium iodide. SYTO9 penetrates both viable and non viable bacteria, while propidium iodide penetrates bacteria with damaged membranes and quenches SYTO9 fluorescence. Dead cells, which take up propidium iodide, fluoresce red whereas fluorescing green cells are considered viable. To assess viability, 1 µl of each stock solution from each stain was added to 3 ml of PBS and, after mixing, 500 µl of the solution was dispensed into 24-well microplates containing the biofilms and incubated at room temperature for 15 min in the dark. Stained

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biofilms were examined by confocal scanning laser microscopy (CSLM) employing a Zeiss LSM 510 Meta Confocal Microscope. The excitation and emission wavelengths used for detecting SYTO9 were 488 and 525 nm and propidium iodide was excited at 520 nm, and its emission was monitored at 620 nm. Optical sections of 0.56 µm were collected, and for each sample, images from three randomly selected positions were acquired. The images were processed using ImageJ software (Wayne Rasband, National Institutes of Health). ImageJ was also employed to obtain quantitative values by measuring fluorescence intensity for each channel. Briefly, a fluorescence value was obtained for every optical section of the analysed image, and then the values from the different sections were added up in order to obtain a single value for each image. Thus, considering that no significant colocalization of both signals was observed, the relative intensity in each channel was used as indicator of the biofilm viability condition.

Scanning electron microscopy (SEM)

S. aureus biofilms were grown on coverslips as described above. Following the appropriate treatments, samples were then fixed for scanning electron microscopy (SEM) with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h at 4°C. After additional washing, the samples were incubated using increasing concentrations of ethanol (25, 50, 75, and 96%) for 10 min and dried at room temperature. Finally, the specimens were coated with Au-Pd in a Thermo VG Scientific SC 7620 Sputter Coater and examined under a SEM (Philips model XL30 TMP, Eindhoven, The Netherlands).

Statistical analysis

Data with normal distribution were analysed using the paired *t*-test. Statistical tests were carried out using the GraphPad Software (version 5.0; GraphPad Prism). P values < 0.05 were considered significant. Means ± standard deviations were depicted.

RESULTS

TAPP-PDI of planktonic S. aureus cultures

Planktonic cultures of the *S. aureus* RN6390 strain were illuminated at a fixed light dose of 180 J/cm^2 in the presence of increasing concentrations of TAPP (Figure 3). Upon illumination, CFU counts decreased as a function of TAPP concentration. Employing a concentration of TAPP as low as 0.1 µM, we obtained a slight but significant photodynamic effect (6.8 x 10^7 CFU/ml) with respect to the non-irradiated control treated with TAPP (4.2 x 10^8 CFU/ml, $p<0.05$) and with only 1 µM, more than 5-log reduction is achieved. In addition, using the highest TAPP concentration of 2.5 µM, complete bacteria eradication was achieved.

We observed a decay in bacterial viability of *S. aureus* exposed to 2.5 µM TAPP as a function of the increasing light dose (Figure 4). The lowest light dose employed (22.5 J/cm^2) induced a 1.5-log decrease of CFU counts (4.5 x 10^6 CFU/ml of TAPP-PDI vs 3.0 x 10^8 CFU/ml of light treated control). At the highest dose employed of 180 J/cm^2, a 8-log reduction in bacterial viability was obtained, that is, complete bacteria eradication was achieved. It is worth noting that bacteria exposed to the maximum light dose without TAPP did not show any noticeable differences in colony counts as compared to the control.

TAPP-PDI of S. aureus biofilms

For the TAPP-PDI treatment of *S. aureus* biofilms, higher light fluences as well as higher PS concentrations were needed to achieve photoinactivation (Figure 5). Dark toxicity of TAPP is only noticed when employing high TAPP concentrations (from 30 µM onwards). On the other hand, TAPP mediated PDI begins to be significantly effective from 15 µM onwards (1.5x10^6 CFU/ml of TAPP-PDI

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vs 1.9×10^8 CFU/ml of TAPP alone, $p < 0.05$), inducing around a 2-log CFU reduction. Employing higher TAPP concentrations of 20 µM, around 3 logs of CFU reduction were obtained (2.8×10^5 CFU/ml of TAPP-PDI vs 1.3×10^8 of TAPP treatment, $p < 0.05$). Higher non-toxic TAPP concentrations did not further increase the PDI-mediated effect, except for the 50 µM concentration, dose at which dark toxicity per se induces a 1.5 log reduction, whereas TAPP-PDI phototoxicity induces an additional 3-log reduction.

PDI as a function of the light dose employing 20 µM TAPP shows that significant photodynamic inactivation of bacteria biofilms was obtained even at 22.5 J/cm^2 (6.8×10^7 of TAPP-PDI vs 3.4×10^8 of the light control, $p<0.05$) (Figure 6). An impairment of CFU counts upon increasing the light dose was noticed. However, the slope of the CFU decrease was much less pronounced than the light dose dependence curve of planktonic treated bacteria.

TAPP uptake/binding by S. aureus growing in planktonic and biofilm states

Figure 7 depicts the binding/uptake of TAPP both in planktonic and biofilm growing bacteria as a funtion of the incubation time and the porphyrin concentration. Whereas the binding/uptake of TAPP by planktonic bacteria does not increase with the incubation time, it does slightly increase in the case of bacteria forming biofilm. In both forms of bacteria growth, the amount of TAPP bound/incorporated to the bacteria augments as a function of the TAPP concentration, though such increase is much more marked in planktonic bacteria. In terms of porphyrin uptake, the bacteria suspensions bind/incorporate three orders of magnitude more TAPP than bacteria growing in biofilms under equal conditions of TAPP exposure.

Microscopic observation of S. aureus biofilms treated with TAPP-PDI

The effect of TAPP-PDI treatment on biofilms was also assayed by scanning electron microscopy (SEM). These images (Figure 8) reveal that after treatment, many bacteria detached from the surface, showing less dense bacterial clusters without noticeable changes in bacteria appearance. However, viability in these remaining clusters was also impaired as evidenced by confocal laser scanning microscopy (CLSM) analysis. Whilst TAPP-only treated *S. aureus* biofilms showed clumps of mostly live bacteria (fluorescing green), biofilms treated with TAPP-PDI showed cells fluorescing both green (live) and red (dead). Quantification of green and red signals suggests that most TAPP-PDT treated dead cells have detached from the surface and that the proportion of live cells dropped from 80 to 54%. Thus, both the reduction on bacterial density and the impairment on viability of the remaining cells account for the drop in colony counts induced by TAPP-PDI observed in Figures 5 and 6.

TAPP-PDI treatment of P. aeruginosa growing in planktonic and biofilm states

In order to determine the efficacy of TAPP-PDI on Gram-negative bacteria, we performed planktonic and biofilm assays employing *Pseudomonas aeruginosa*. Firstly, we subjected planktonic suspensions of the bacteria to different concentrations of TAPP and applied a light dose of 180 J/cm^2 (Figure 9). To achieve bacteria photosensitization, TAPP concentrations inducing dark toxicity were needed. We obtained a significant degree of photoinactivation employing 10 µM TAPP and light (1.5-log CFU reduction respect to the TAPP control), and from 20 µM onwards the level of CFU reduction was around 3 logs as compared to the TAPP control. On the other hand, high concentrations of TAPP were non toxic to *P. aeruginosa* growing on biofilms, and significant CFU reductions were obtained employing 30 µM TAPP and light (3 logs of CFU reduction as compared to the TAPP control) (Figure 10).

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DISCUSSION

Among other features, it has been proposed that the cationic character of the PS influences the PDI outcome. It has been reported that cationic porphyrin derivatives are able to induce the photoinactivation of Gram-positive and -negative bacteria and some studies have compared the efficiency of synthetic *meso*-substituted cationic porphyrins with different charge distribution (tetra-, tri-, di- or monocationic). Some studies have demonstrated that tetracationic porphyrins are efficient PSs against both Gram-positive and -negative bacteria, and some others have reported that di- and tricationic porphyrins are more efficient PSs than tetracationic ones against both bacteria types [11, 27-29].

Porphyrins containing intrinsic or precursors of cationic substituents have attracted considerable interest because of their notable ability as phototherapeutic agents [30]. In particular, TAPP is highly efficient in the production of singlet molecular oxygen with a quantum yield of about 0.5 in DMF [24]. These photodynamic properties together with a high affinity for bacteria make this porphyrin a promising efficient photosensitizing agent.

The TAPP molecule consists of a porphyrin substituted by four aliphatic amine groups in the periphery of the macrocycle, which can at physiological pH acquire positive charges. The substitutent on the TAPP molecule, which is a *N*,*N*-dimethyl-*n*-propylamine, present a pKa~10 in water. Therefore, ~99.8% of amine groups are protonated at a physiological pH of 7.2 and thus, TAPP should have their amine groups similary protonated **[**24**].** Moreover, the amino groups of TAPP are spaced from the porphyrin ring by an aliphatic chain, having therefore a minimal influence on the photophysical properties of the porphyrin [17]. In addition, the propoxy spacer provides a higher mobility of the charges, thus promoting the binding to microbial cells.

Planktonic *S. aureus* bacteria treated with 1 µM TAPP and 180 J/cm^2 light dose suffered a photoinactivation of ~5 log decrease, and comparable results of photoinactivation were obtained using 2.5 µM TAPP and a light dose of 90 J/cm^2. Similarly but at a lower extent of bacteria photosensitization, 15 µM TAPP and 180 J/cm^2 light induce a 2-log CFU reduction of biofilm growing bacteria, and the same degree of bacteria cell death was obtained applying 20 µM TAPP and 120 J/cm^2, thus suggesting that lower TAPP concentrations and higher light doses could be employed achieving equal levels of photosensitization.

Some reports on the efficiency of other cationic PSs on the PDI of planktonic growing bacteria are difficult to compare with ours mainly due to different bacteria densities and the irradiation systems used. For instance, the cationic porphyrins 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl) porphyrin ($TMAP^{4+}$) and $TMPyP^{4+}$ are known to act as photosensitizers in eradication of microorganisms [31, 32], but the light doses are not comparable to ours.

However, employing similar light doses to the ones used in the present work (133 J/cm^2) and 4 µM porphyrin, $TMPyP^{4+}$ induced less than 1-log unit reduction of CFU counts, whereas $TMAP^{4+}$ produced a 3-log unit decrease in *S. aureus* cell survival [33]. Moreover, photoinactivation mediated by 5,10,15,20-tetrakis[3-(*N*-ethyl-N-methylcarbazoyl)]porphyrin (TCP^{4+}) showed less than a 1-log unit reduction of CFU when cells were treated with 1 µM photosensitizer [34]. On the other hand, a degree of photosensitization similar to that attained in the present work was obtained using 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetraiodide (Tetra-Py+-Me) and other different tricationic porphyrin derivatives against the Gram-positive bacteria *Enterococcus faecalis* and *S. aureus* (~7-log CFU reduction employing 5.0 µM and low light doses) [11, 32].

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In the present work, to achieve photoinactivation of biofilms, both higher concentrations of TAPP and light doses were needed as compared to those used in planktonic cultures. Whereas 2.5 µM TAPP was enough to induce total planktonic bacteria eradication (8 logs CFU reduction), a concentration 10 times higher was needed to induce a 3-log CFU reduction of biofilm growing bacteria. Both hindrance of the penetration of light within the biofilms [35] and the inability of the PS to diffuse to the inner regions of the structure [20, 36] could be producing lower photoinactivation levels. In addition, the genetic diversity within the biofilm and bacteria communication via quorum sensing contribute to the higher resistance of biofilm structures to PDI treatment [37].

We obtained *S. aureus* CFU biofilms reductions of 3 log steps employing 20 µM TAPP and a 180 J/cm^2 light dose. Higher TAPP doses induced slightly higher bacteria viability reduction at the expense of dark toxic effects. The presence of dead cells throughout the biofilm revealed by CLSM analysis implies that there was no hindrance for the PSs to diffuse into the biofilms. In addition, the detachment of parts of the biofilm leading therefore to a disruption of its architecture was found for TAPP-PDI treatments, as previously reported for photodynamic action of other PSs [22].

Regarding the efficacy of PDI on bacteria biofilm photosensitization, a good number of varying results have been reported. However, very few studies have been carried out employing porphyrins as PSs on PDI of *S. aureus*, and they have showed very different results. Di Poto et al [22] treated biofilms from three distinct strains of *S. aureus* with 10 µM TMPyP and irradiated them with white light (200 mW/cm^2), obtaining a rate of bacteria killing of 1 to 2 log steps at the highest light dose depending on the strain. In addition, the same tetra-substituted cationic porphyrin induced a significant reduction in bacterial survival of *S. epidermidis* after illumination [38]. On the other hand, PDI employing the tetracationic porphyrin Tetra-Py+-Me (5,10,15,20-tetrakis(1-methylpyridinium-4-yl) porphyrin tetra-iodide) [39] induced maximum reductions of 6.3 logs on *S. aureus* biofilms, which was achieved by employing 20 µM of the porphyrin and applying a low energy light dose of 4 mW/cm^2 for a long time period of 270 min (64.8 J/cm^2). Not only the total energy delivered but also the fluence employed may have an impact on the outcome of PDI treatment.

PDI-killing of Gram-positive bacteria is definitely much easier to accomplish than that of Gram-negative bacteria. While Gram-positive bacteria present a thick and porous cell wall of inter-connected peptidoglycan layers that surround a cytoplasmic membrane, Gram-negative bacteria have an outer membrane, a thinner peptidoglycan layer and a cytoplasmic membrane that contains endotoxins and blocks antibiotics, dyes, and detergents, protecting the sensitive inner membrane and cell wall. Accordingly, to perform PDI, the PS employed needs to penetrate the cell walls of the bacteria and end up in the plasma membrane or in the cytoplasm; however, the membrane barriers of the Gram-negative bacterial cell limit the simple diffusion of the PSs into the bacterial cytosol [40].

In the present work we extended our studies to the Gram-negative bacteria *P. aeruginosa.* Much higher concentrations of TAPP were required to photoinactivate planktonic cultures of *P. aeruginosa* as compared to *S. aureus*. Whereas 6-log CFU reduction of *S. aureus* suspensions was achieved upon irradiation using 1 µm TAPP, only 3-log CFU reduction of *P. aeruginosa* was obtained employing 20 µM TAPP, which is a high concentration that induces dark toxicity. On the other hand, quite a similar degree of efficacy (3-log CFU reduction) was shown after TAPP-PDI treatment of *S. aureus* and *P. aeruginosa* biofilms. Moreover, dark toxicity was seen at high concentrations of TAPP in *S. aureus* biofilms, whereas the porphyrin was not toxic at all in *P. aeruginosa* biofilms.

Considering the response to TAPP-PDI of the Gram-positive and Gram-negative strains employed in this work, we hypothesize that the complexity of the biofilm architecture surpasses its differences of TAPP penetration. This feature becomes clear if we analyze the amount of TAPP bound/incorporated to

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S. aureus bacteria growing in biolfilms, which is 3 orders of magnitude lower than the amount bound to planktonic bacteria.

Bacteria growing on biofilms are embedded into a slime composed mainly of extracellular DNA, proteins, and polysaccharides. The biofilm matrix—also referred to as EPS (extracellular polymeric substance)—itself may slow drug-diffusion by its higher viscosity or can even act as a barrier. Positively charged agents may bind to negatively charged EPS-molecules and π-π- interactions of aromatic surfaces are possible, preventing penetration of the respective drug in deeper parts of the biofilm [41]. Considering our results, TAPP molecules may cross the EPS biofilm barrier equally in the Gram-positive and -negative bacteria analyzed, though their slime may present different composition.

The main conclusion of the present work is that TAPP is a promising and efficient PS capable of promoting photodynamic killing of both Gram-negative and -positive in planktonic bacteria, though more effectively in the latter. In addition, TAPP-PDI induces similar photoinactivation rates in both bacteria types growing on biofilms, with lower dark toxicity in the Gram-negative one.

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FIGURE LEGENDS

Figure 1: TAPP structure

Figure 2: Absorbance spectrum of TAPP in PBS and lamp emission spectrum

Figure 3: Photodynamic inactivation of *S. aureus* planktonic cultures as a function of TAPP concentration
S. aureus planktonic cultures were treated with different TAPP concentrations for 10 min, previous to irradiation at 180 J/cm^2. Viability of post-treated cultures was established by plating on TSA followed by colony counts. Darkness: bacteria exposed to different TAPP concentrations.

Figure 4: TAPP-PDI of *S. aureus* planktonic cultures as a function of light dose
After 10 min of dark incubation with 2.5 µM TAPP, *S. aureus* cultures were irradiated employing several light doses ranging from 22.5 to 180 J/cm^2. Viability of post-treated cultures, expressed as CFU/ml, was established by plating on TSA followed by colony counts. Control: bacteria exposed to different light doses. The results represent the means ± SD of three independent assays performed in duplicates.

Figure 5: *S. aureus* biofilm inactivation as a function of TAPP concentration
S. aureus biofilms were treated with different TAPP concentrations for 10 min, previous to irradiation at 180 J/cm^2. Viability of suspensions derived from post-treated biofilms, expressed as CFU/ml, was established by colony counts. *$p<0.05$ relative to non-TAPP treated biofilms. Darkness: bacteria exposed to different TAPP concentrations. The results represent the means ± SD of four independent assays performed in duplicates.

Figure 6: TAPP-PDI of *S. aureus* biofilms as a function of light dose
After 10 min of dark incubation with 20 µM TAPP, *S. aureus* cultures were irradiated employing several and light doses ranging from 22.5 to 180 J/cm^2. Viability of suspensions derived from post-treated biofilms, expressed as CFU/ml, was established by colony counts. Control: bacteria exposed to different light doses. *$p<0.05$ relative to non-TAPP treated biofilms. The results represent the means ± SD of four independent assays performed in duplicates.

Figure 7: Binding and/or uptake of TAPP to *S. aureus* bacteria
Suspensions (A) or biofilms (B) of *S. aureus* were incubated in presence of different concentrations of TAPP for diferent time periods, and the amount of TAPP bound and/or incorporated to the bacteria was quantified by fluorescence and normalized to the CFU number. The results represent the means ± SD of two independent assays performed in duplicates.

Figure 8: CLSM and SEM imaging analysis of TAPP-PDI treated *S. aureus* biofilms
Biofilms treated with 20 µM TAPP and non-illuminated (A, C) or TAPP (20 µM) treated and illuminated at 180 J/cm^2 (B, D). Following treatment, biofilms were stained with the BacLight Live/Dead kit before CLSM analysis (A, B) or fixed and observed on SEM (C, D). Quanification of red and green fluorescence signals from A and B images (E).

Figure 9: Photodynamic inactivation of *P. aeruginosa* planktonic cultures as a function of TAPP concentration
P. aeruginosa planktonic cultures were treated with different TAPP concentrations for 10 min, previous to irradiation at 180 J/cm^2. Viability of post-treated cultures was established by plating on TSA followed by colony counts. Darkness: bacteria exposed to different TAPP concentrations. *$p<0.05$

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relative to dark TAPP-treated biofilms. The results represent the means ± SD of three independent assays performed in duplicates.

Figure 10: *P. aeruginosa* biofilm inactivation as a function of TAPP concentration
P. aeruginosa biofilms were treated with different TAPP concentrations for 10 min, previous to irradiation at 180 J/cm^2. Viability of suspensions derived from post-treated biofilms, expressed as CFU/ml, was established by colony counts. *$p<0.05$ relative to dark TAPP-treated biofilms. Darkness: bacteria exposed to different TAPP concentrations. The results represent the means ± SD of four independent assays performed in duplicates.

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FIGURES

Figure 1

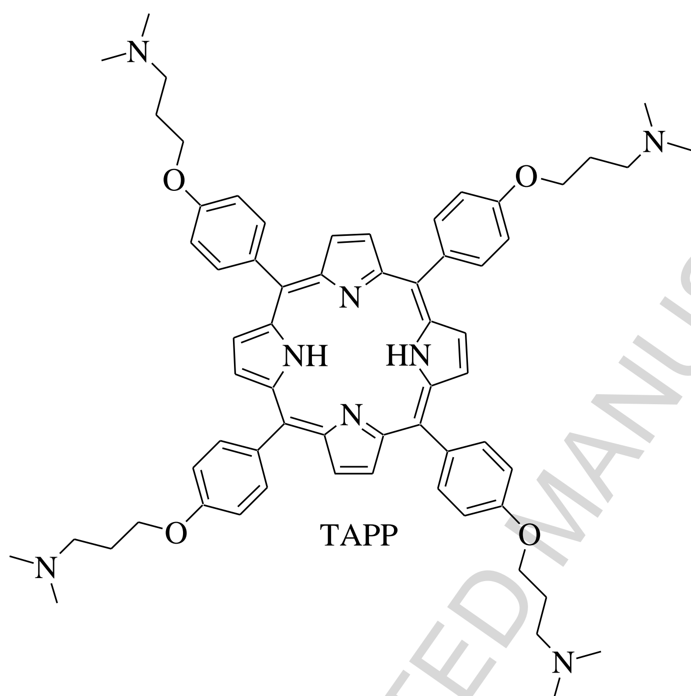

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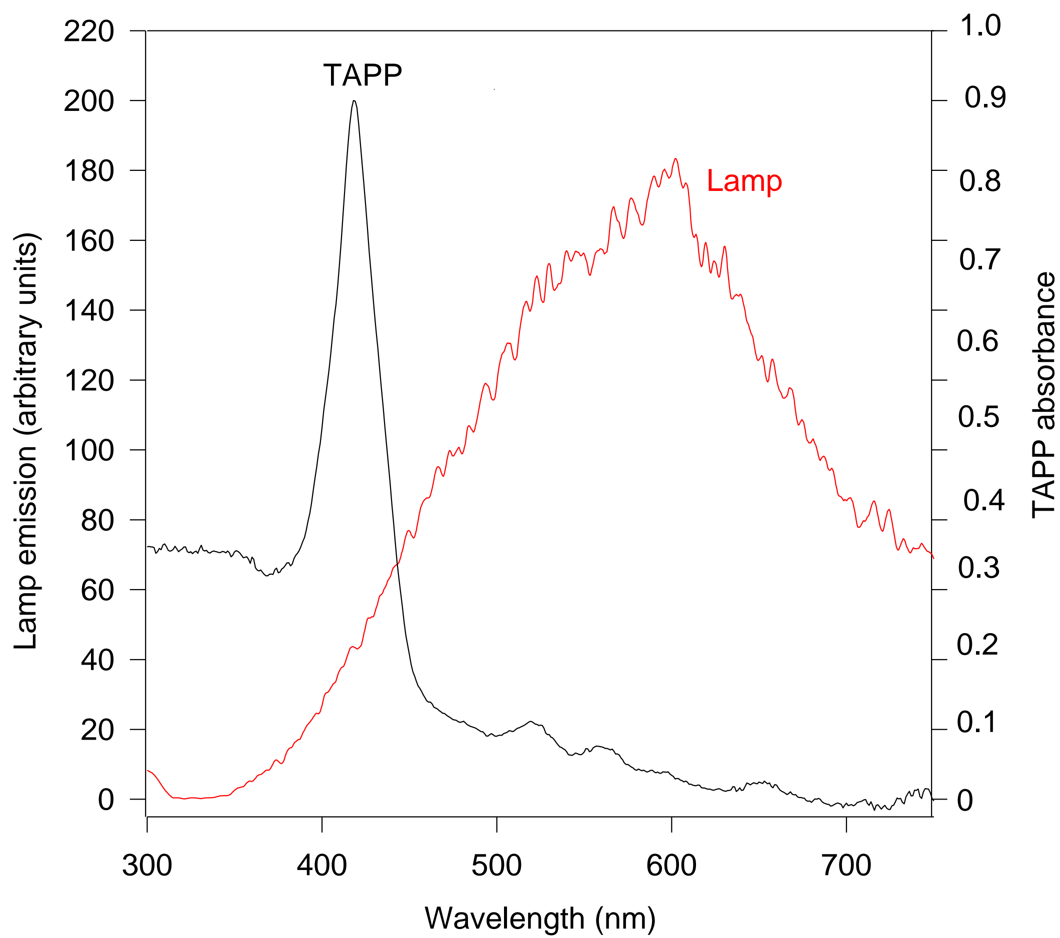
Figure 2



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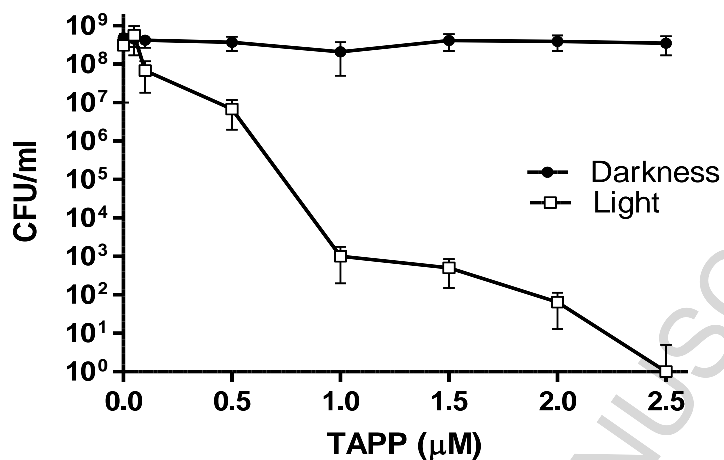

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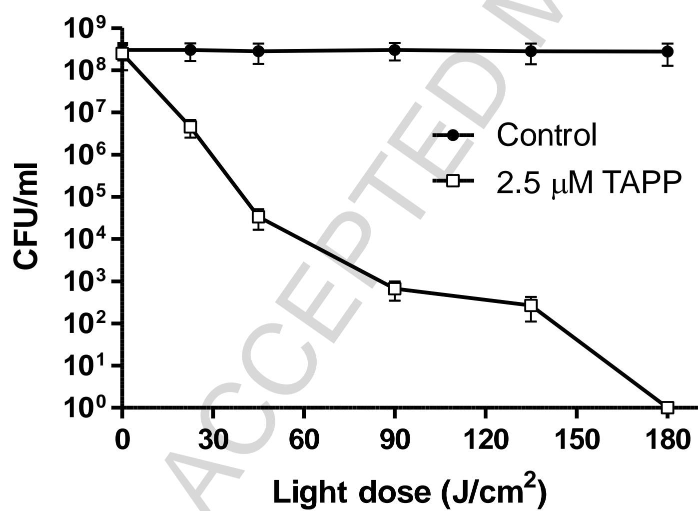

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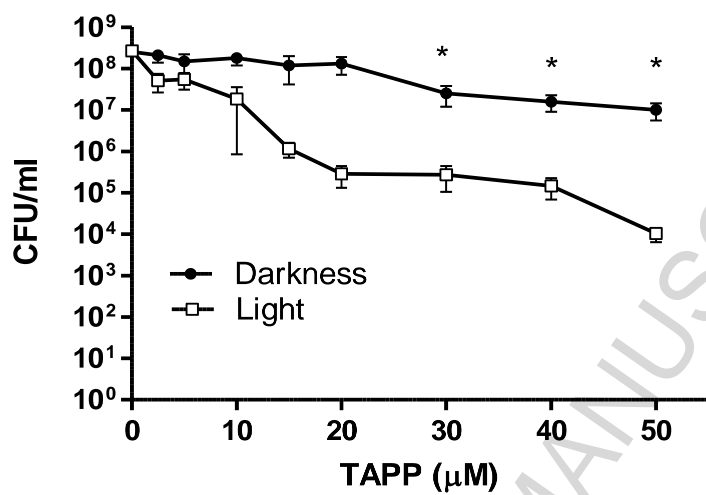
Figure 5



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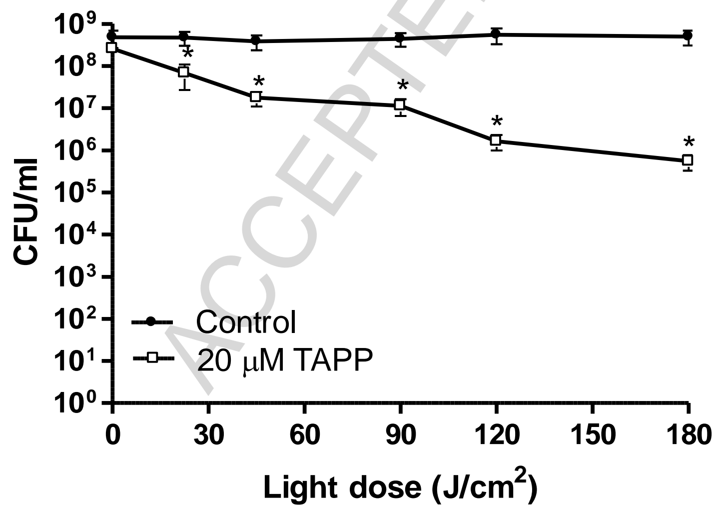

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Figure 7

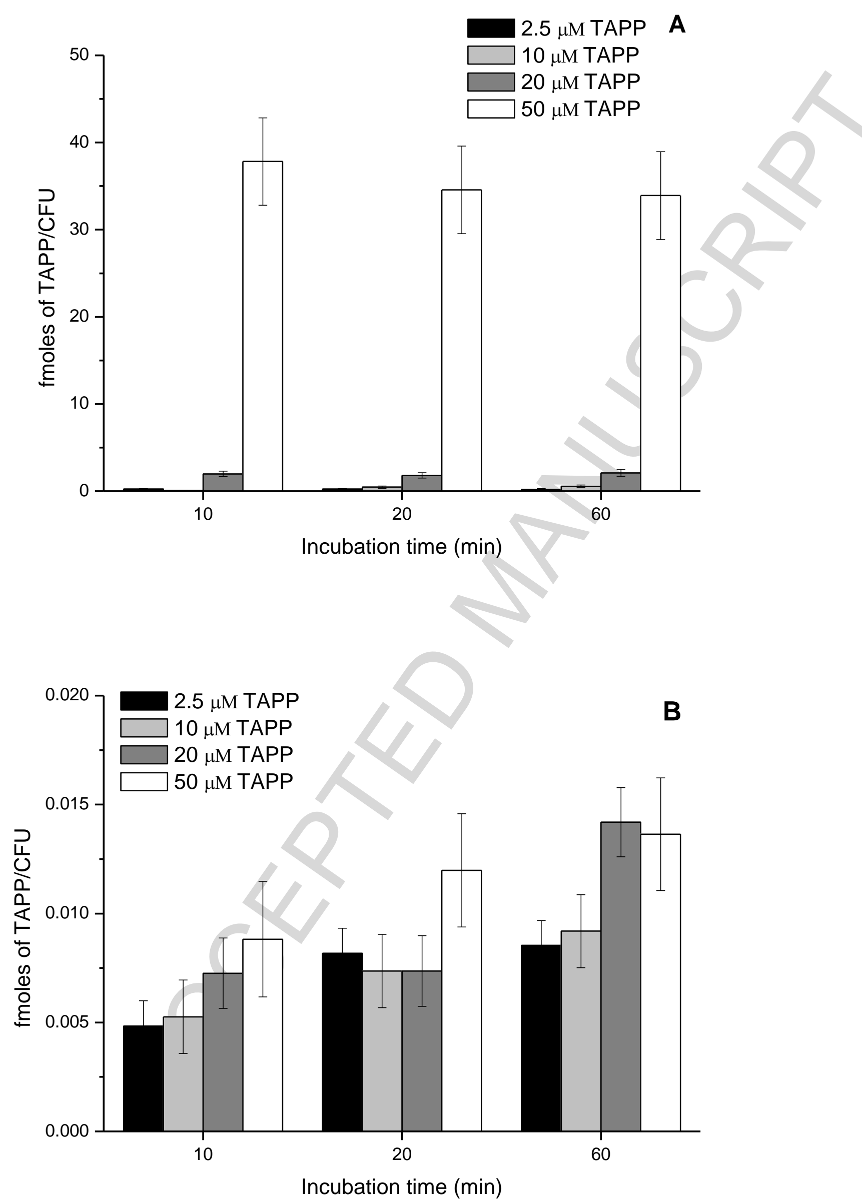

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Figure8

TAPP TAPP-PDI

CLSM

A B

SEM

C D

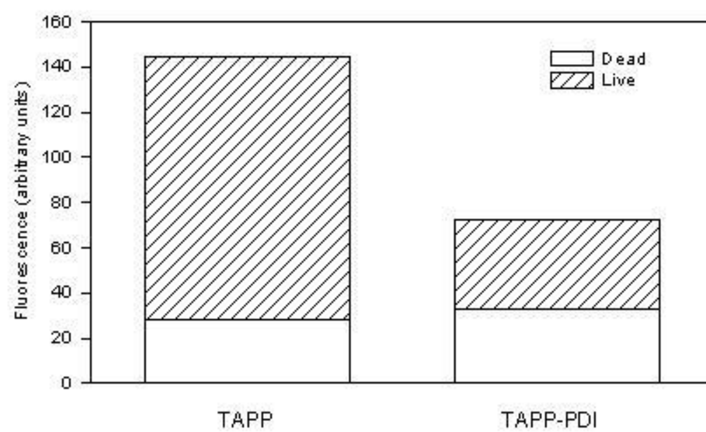

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Figure 9

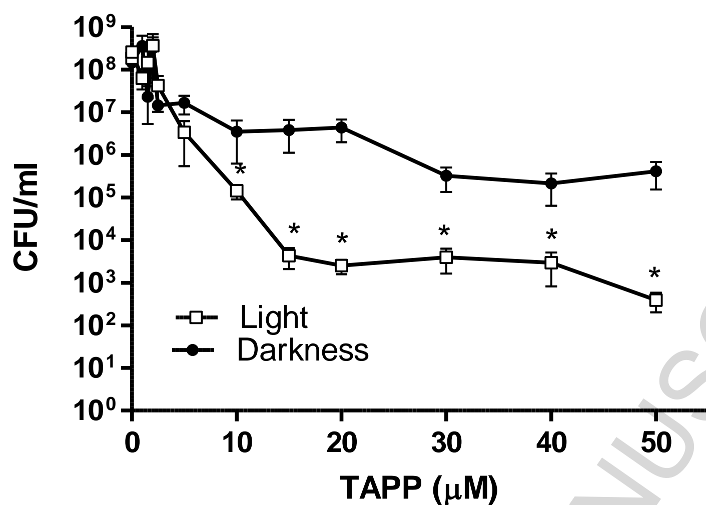
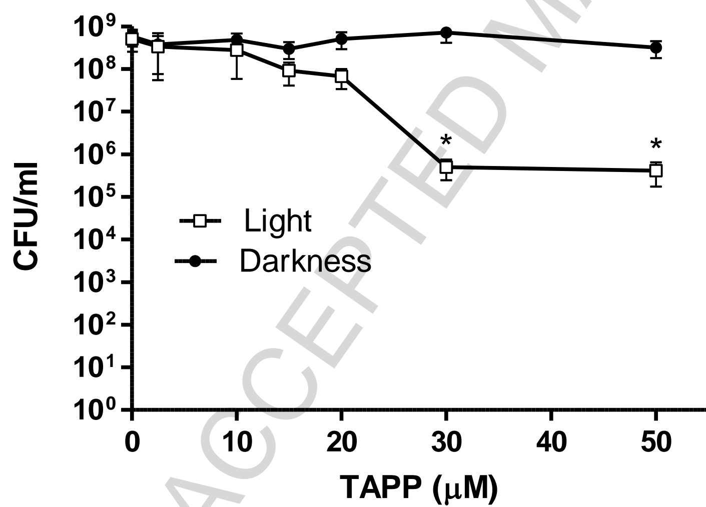


Figure 10



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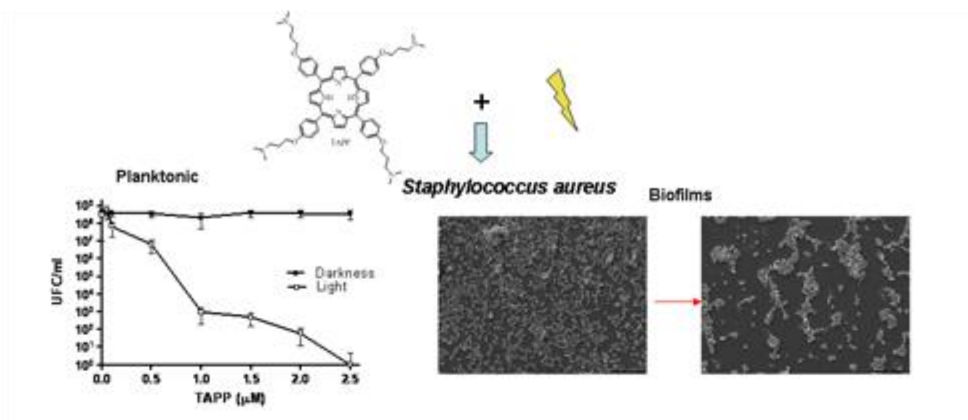
Graphical Abstract



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Highlights

A porphyrin which is charged at physiological pH was used for *S. aureus* inactivation.

Photodynamic treatment induced complete eradication of *S. aureus* in planktonic state.

The same treatment induced 3 logs of CFU reduction of *S. aureus* biofilms.

Gram -positive and -negative bacteria biofilms were equally photosensitized.

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