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Photodynamic inactivation induced by carboxypterin: a novel non-toxic bactericidal strategy against planktonic cells and biofilms of *Staphylococcus aureus*

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Microbial related contamination is of major concern and can cause substantial economic losses. Photodynamic inactivation (PDI) has emerged as a suitable approach to inhibit microorganism proliferation. In this work, PDI induced by 6-carboxypterin (Cap), a biocompatible photosensitizer (PS), was analyzed. The growth inhibition of *Staphylococcus aureus* exposed to artificial UV-A radiation and sunlight in the presence of Cap was investigated. After UV-A irradiation, 50 µM Cap was able to decrease by three orders (with respect to the initial value) the number of *S. aureus* cells in early biofilms. However, this concentration was 500 times higher than that needed for eradicating planktonic cells. Importantly, under solar exposure, 100 µM Cap was able to suppress sessile bacterial growth. Thus, this strategy is able to exert a bactericidal effect on sessile bacteria and to eradicate planktonic cells by exposing the Cap-containing sample to sunlight.

Keywords: photodynamic inactivation; *Staphylococcus aureus*; carboxypterin; biofilm

Introduction

Microbial contamination is of major concern and the cause of significant economic losses (Ashraf et al. 2014) in fields related to human health, drinking water supply, food processing and storage and several other industries. In paper mills for instance, damage caused by microbes may include acidification, raw material degradation or the discoloration of pigments (Flemming et al. 2013). Moreover, during food processing, the presence of antimicrobial resistant bacteria owing to cross-contamination or the use of antibiotics during agricultural production or livestock breeding represents a direct risk to human health (Verraes et al. 2013). The adhesion of bacteria to surfaces results in the formation of biofilms, which are known to increase the resistance of the cells within them to environmental stress and antimicrobial compounds, when compared with free-living or planktonic cells. Bacteria living in biofilms can be a threat to human health (Tan et al. 2014) as they may cause infections associated with indwelling devices, such as catheters and implants (Gomes et al. 2014). Bacteria within biofilms may also cause a reduction in the efficiency of cooling water systems (Nagai et al. 2013), spoil food in the food industry, reducing its shelf-life and facilitating the transmission of disease (Giaouris et al. 2014) or reduce water quality in drinking water distribution systems (Liu et al. 2013).

Among biofilm-forming bacteria, the Gram-positive pathogen *Staphylococcus aureus* is associated with several pathologies such as pustules, pneumonia, meningitis and arthritis (Madigan et al. 2010). This species is usually found in the normal flora of the respiratory tract and skin (Lina et al. 1999) and is able to synthesize extracellular polymeric substances (EPS), which facilitate cell attachment to surfaces and the formation of biofilms (Götz 2002). Most infection-related complications arising from orthopedic and indwelling devices as well as dental biomaterials (Gueorgieva 2010) are related to *S. aureus* biofilms (Arciola et al. 2012). Moreover, methicillin-resistant *S. aureus* (MRSA) has been identified in wastewater exiting from treatment plants, indicating that this bacterium is able to survive in environments different from clinical ones, and even to grow under nutrient-poor conditions (Börjesson et al. 2010; Rosenberg Goldstein et al. 2012).

Since conventional disinfection and cleaning methods may contribute to increasing microbial resistance and can be an inefficient control of biofilm formation, new strategies are continuously under development in order to reduce the formation of biofilms and/or to enhance the eradication of sessile cells (Simões et al. 2010). Novel methods to control the growth and proliferation of bacteria, either as planktonic or sessile cells, should include innocuous, biocompatible and/or biodegradable

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compounds that do not have adverse or harmful secondary effects in a broad variety of environments.

Photodynamic inactivation (PDI) involves the use of non-toxic compounds known as photosensitizers (PS), which in their electronically excited states (PS*) may interact with molecular oxygen or yield free organic radicals, both reaction pathways producing reactive oxygen species (ROS) and/or free organic radicals that are toxic to cells (Dougherty et al. 1998). These processes take place through mechanisms involving energy and/or electron transfer reactions. Some PS molecules interact with bacterial membranes and cause damage that results in cell death (Demidova & Hamblin 2004).

Pterins are a family of heterocyclic compounds widespread in biological systems and derived from 2-aminopteridin-4(3*H*)-one, or pterin (Ptr) (Figure 1). Most common Ptr derivatives present substituents at position 6 and are water soluble. Several Ptr derivatives participate in relevant biological processes such as the synthesis of amino acids (Nichol et al. 1985) and nucleobases (Blakley 1969), nitric oxide metabolism (Hevel & Marletta 1992) and the activation of cell-mediated immune responses (Fuchs et al. 1988). The photochemical behavior of Ptr derivatives in aqueous solution and the consequent formation of ROS have been previously reported (Lorente & Thomas 2006; Oliveros et al. 2010). Upon UV-A (320–400 nm) excitation, these compounds form triplet excited states with high quantum yields, and generate organic radicals and ROS. It has been previously demonstrated that Ptr derivatives do not affect the viability of Hela cells under dark conditions (Denofrio et al. 2011; Alvarez et al. 2013). Conversely, upon UV-A irradiation, Ptr-mediated processes cause mitochondrial failure and membrane damage, leading to cell death (Denofrio et al. 2011).

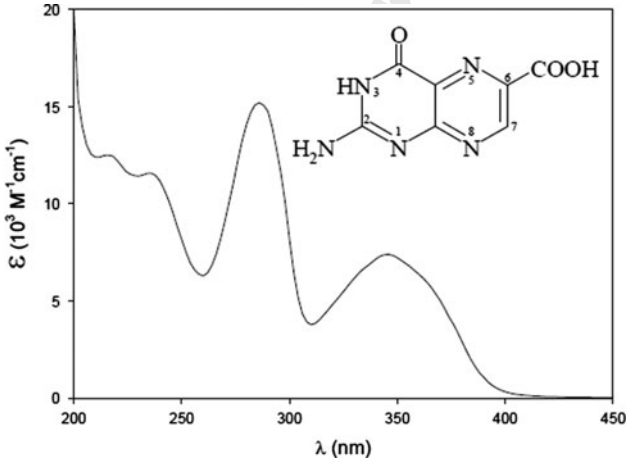

Figure 1. Molecular structure of Cap and its absorption spectrum in neutral and slightly acid aqueous solutions.

Ptr derivatives are biocompatible, water-soluble and efficient PS when excited within the UV-A spectral domain and are therefore good candidates for PDI. The aim of the current study was to assess the capacity of Ptr derivatives as PS to reduce the viability of both planktonic and sessile bacteria, under artificial UV-A radiation and sunlight. *S. aureus* was chosen as a model bacterium due to its implication in human infections and its capability to form biofilms, particularly in hospital environments and wastewaters (Thompson et al. 2013). The Ptr used as PS was 6-carboxypterin (Cap), because its carboxylic group confers high solubility in water and exhibits a satisfactory photostability, ie the quantum yield of its photodegradation is rather low (Suárez et al. 2000).

Materials and methods

General

Reagents

Cap (Shircks Laboratories) was of the highest purity available (99%) and was used without further purification. Other chemicals were from the Sigma Chemical Co. The pH of the aqueous solutions of Cap was adjusted to pH = 6.7 by adding drops of a HCl or NaOH solution from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 M to 2.0 M. As a control, the electronic absorption spectrum of each solution was recorded on a Shimadzu UV-1800 spectrophotometer.

Superoxide consumption

In order to evaluate the consumption of superoxide anion by the nutrient broth (standard manufacturer composition or diluted broth) or the EPS within the biofilm matrix, quantitative assays were performed following the procedure described by Beauchamp and Fridovich (1971). Thus, the diminution of superoxide anion was determined by the inhibition of nitro blue tetrazolium reduction using 100 µl of sample (standard manufacturer composition or diluted broth, EPS) per assay. A triplicate series of experiments was carried out in each case.

Steady-state irradiation

UV irradiance quantification

Irradiance (E) is defined as the radiant power, incident on a small element of surface containing the point under consideration, divided by the area of the element. Spectral irradiance (E_λ) is the derivative of E with respect to wavelength (λ, W m^{-2} nm^{-1}). Measurements of E_λ for both sources (*vide infra*) were carried out with a high-resolution Avantes spectrometer (AvaSpec-ULS3648 model) which acquires spectral irradiance from 250 nm

to 810 nm). The UV irradiance (E_{UV}) was obtained by integrating E_λ values in the 250–400 nm range.

UV-A irradiation

Samples were irradiated with an artificial UV-A source (UV-A lamp, Rayonet RPR lamps, Southern N.E. Ultraviolet Co.). The wavelength of the maximum intensity was λ_{max} = 350 nm with a bandwidth of 20 nm. The spectral irradiance was measured at 1.05 cm from the source resulting in E_{UV} = 31 ± 4 W m^{-2}.

Solar exposure

The equipment and samples were mounted on a horizontal platform in La Plata city, Argentina (34.90°S, 57.92°W; 25 m asl) during the spring period. The E_λ of the sun on the exposed surface and sample was simultaneously measured at around solar noon (at 12:39 h; local time = Universal Time – 3 h). All the trials were performed under clear sky conditions.

Bacterial cultures and biofilm formation

S. aureus ATCC 25923 was grown in Nutrient Broth (Merck, Darmstadt, Germany) at 28°C with shaking (250 rpm) overnight. After incubation, the bacterial suspension was adjusted to 10^6 colony-forming units (CFU) ml^{-1} (initial planktonic cells) in fresh growth medium. The initial number of cells present was confirmed by the viable count method.

Bacterial adhesion experiments on microtiter plates were performed using a procedure previously described by O'Toole and Kolter (1998). Briefly, aliquots (200 µl) of cell suspension (10^6 CFU ml^{-1}) were added to the wells of sterile flat-bottom 96-well polystyrene microtiter tissue culture test plates (96-well, Greiner Bio-One, Germany) and incubated for 2 h at 28°C to allow the bacterial cells to adhere (2 h-adhesion). The wells were then washed with sterile phosphate buffered saline solution (PBS, 10 mM, pH = 7.4) in order to remove cells which were not tightly attached to the surface.

Quantification of the bacteria attached to the surfaces of the wells was performed by the serial dilution method and plate counting after their detachment. The surfaces of the wells were scraped with sterile cotton swabs (three wells per dilution). Swabs transferred into tubes containing 2 ml of PBS were sonicated for 15 min with a Testlab sonicator (40 kHz with power output of 160 W). Experiments with and without ultrasound treatment were carried out and a significant difference in viable cells was not found between the results obtained under both conditions. Later, the number of bacteria in the sonicated suspension was determined by serial dilution followed by plating onto nutrient agar for 48 h at 28°C. A triplicate series of experiments and two replicates were carried out in each case. These values were used as the 'initial number of sessile cells' to which the reduction of viable cells after antimicrobial treatment was referred.

In order to isolate EPS from *S. aureus* biofilms, the procedure previously described by Sadovskaya et al. (2005) was adapted. Biofilm formation was carried out as described in the previous paragraphs, except that the 96-wells microtiter plates were replaced by 24-well microtiter tissue culture test plates (Greiner Bio-One), to increase the amount of biomass. The biofilm formed on the walls and the bottom of each well was washed with sterile PBS. Harvesting the biofilm biomass was done by adding 0.5 ml of PBS (pH 7.4) into the well and scraping away the biofilm with a pipette tip. Aliquots of the biofilm suspensions were transferred into Eppendorf tubes, and sonicated for 20 min. The sonicated biofilm suspensions were subsequently centrifuged (14,000 rpm, 6 min, centrifuge Eppendorf 5415C) to remove the cells and the supernatants were exposed to a second cycle of sonication and centrifugation. Finally, the EPS-containing supernatant (free from bacteria) was obtained.

Antimicrobial activity of carboxypterin (Cap) against* S. aureus *kept in the dark

The antimicrobial effect of Cap kept in the dark was evaluated against planktonic and sessile cells of *S. aureus*. First, the minimum inhibitory concentration (MIC) of Cap against the *S. aureus* strain was determined by the microtiter method as described in the CLSI guidelines (CLSI 2009), except for replacing Müller-Hinton broth by nutrient broth. The MIC was defined as the lowest concentration of Cap at which bacterial growth was not detected after growth for 20 h. The assays were performed in triplicate from independent bacterial cultures. Wells containing only fresh diluted nutrient broth (1:10 dilution) served as the negative controls. The positive controls consisted of wells inoculated with bacterial suspension and fresh diluted nutrient broth.

A serial two-fold dilution of Cap was prepared in 1:10 diluted nutrient broth. Cap solutions for antimicrobial assays ranged from 0.1 µM to 200 µM. The culture containing planktonic cells was exposed to Cap for 2 h before viable cells were enumerated. For sessile cells, the Cap was added to wells containing pre-existing biofilms (2-h exposure to culture, see above). The cultures/biofilms were kept in darkness after the addition of Cap (radiation-free experiment, dark control).

Photodynamic inactivation (PDI) of planktonic and sessile bacteria

The PDI of bacteria was firstly evaluated against planktonic cells. Briefly, aliquots (100 µl) of cell suspensions

(10^6 CFU ml^{-1}) were added to the wells of sterile 96-well microtiter plates containing 100 µl of serial two-fold dilutions of Cap (see above). The wells were exposed to UV-A radiation for 2 h and incubated at 28°C. Afterwards, the number of bacteria in the wells was determined using the plate count method. The plates were incubated at 28°C for 48 h. A triplicate series of experiments and two replicates were carried out in each case.

The second step was to evaluate PDI against sessile bacteria. With this aim, *S. aureus* biofilms were formed for 2 h at 28°C in 96-well culture plates. The biofilms formed on the surface of the wells were gently washed twice with PBS and then incubated with 200 µl of serial two-fold dilutions of Cap ranging from 0.1 to 200 µM in diluted nutrient broth at 28°C. After incubation for 2 h under UV-A irradiation, the Cap solutions were removed and the biofilms were washed twice with PBS. Subsequently, the biofilms were detached and bacteria were enumerated as described previously. Control assays were carried out with biofilms grown in diluted nutrient broth as follows: (i) dark control (see above): the biofilms were kept in darkness after the addition of Cap (radiation-free experiment); (ii) UV-radiation control: the biofilms were incubated in fresh diluted nutrient broth and irradiated with UV-A for 2 h (Cap-free experiment); and (iii) growth control: the biofilms were incubated in fresh diluted nutrient broth for 2 h (UV-A and Cap free experiment). The growth control represents the maximum number of sessile cells expected in the biofilms after growth for 2 h.

Photodynamic inactivation of sessile cells under solar radiation

The effectiveness of PDI on sessile cells under solar radiation was evaluated. Assays were performed following the protocol described above but using the sun as a natural UV source. Previous analysis indicates that in the spring period (according to location and climatic conditions in the region) E_{UV} values can reach ~ 45 W m^{-2} (Luccini et al. 2006; Ipiña et al. 2012, 2014). Comparing E_{UV} values obtained at solar noon and the E_{UV} value corresponding to the UV-A lamp, a suitable exposure time was estimated. Briefly, biofilms grown for 2 h in 96-well culture plates were incubated in 200 µl of serial two-fold dilutions of nutrient broth containing Cap in the concentration range 0.1–200 µM. In order to achieve a uniform intensity of the natural source (relative difference barely 2%), the plates were exposed on a clear sky day for 80 min centered at solar noon. A container with water was used as a heat sink. The solar spectral irradiance and the tray transmittance were measured and it was found that the total mean transmittance, ie the sunlight that passed through this container, exceeded 86% irradiance, ie 36.9 ± 4 W m^{-2}. In all the experiments, the solar UV dose was 17.3 ± 3 J cm^{-2}. After treatment the Cap-containing solutions were removed from the wells and the biofilms were washed twice with PBS solution and enumerated by the plate count method. All assays were performed in triplicate series.

Statistical analysis

Experimental assays were performed in triplicate and numerical data are presented as means with error bars representing SDs. The data were statistically analyzed using a one-way ANOVA to evaluate differences between groups. Differences between means were considered statistically significant when $p \leq 0.05$.

Results and discussion

Simulating the organic charge of the water sources to be treated

PDI may be a suitable strategy for the suppression of bacterial contamination in water flows of practical importance such as wastewater or drinking water, characterized by a total organic carbon content of approximately 140 mg l^{-1} and 2.4 mg mg l^{-1} average values for wastewater and drinking water, respectively, depending on the origin of the water sample. Commercially available nutrient broths are characterized by a carbon content much higher (~3 g l^{-1}) than that of wastewater. As such a high carbon content may hinder the action of PDI, for the assays in the current study the organic content of the bacterial culture medium was reduced to be closer to the level found in water flows, while ensuring the minimum carbon content needed for bacterial growth was present. Thus, several dilutions of the culture broth, prepared according to manufacturer's recommendations, were made and a 1:10 dilution was selected because it was close to the organic content of wastewater flows and did not affect the growth of the cells in the time elapsed for the assays. In addition, as the organic content in the 1:10 diluted broth was higher than that found in wastewater the experimental conditions represented a more unfavorable situation for PDI application, ensuring its potential utility for wastewater treatments.

Planktonic bacteria

Toxicity of Cap

The MIC of Cap showed that this PS had no inhibitory effect without irradiation, since bacteria grew normally in Cap-containing culture media in the 0.1–200 µM concentration range. The number of viable cells after 0.1 and 200 µM Cap treatment were 2.9 ± 1.1 × 10^9 and 2.8 ± 1.9 × 10^9 CFU ml^{-1}, respectively, similar to the numbers obtained for the positive control (2.3 ± 1.0 × 10^9 CFU ml^{-1}).

Control assays: growth control, dark control and radiation control

A set of controls was carried out in order to assess (1) the growth of bacteria without any treatment (Growth control), and the antibacterial effect of (2) Cap (UV-A free, dark) and (3) UV-A radiation (Cap free) as single factors (Figure 2). The initial bacterial number was also included in the figure for comparison purposes. The 'Dark control' (Cap, UV-A free) showed that 2 h treatment with Cap in the 0.1–200 μM range under dark conditions did not affect the cell viability. It is worth noting that the dark control values at each concentration was close (no significant difference, $p < 0.05$) to those of the growth control. In contrast, 2 h exposure of planktonic bacteria to UV-A radiation ('Radiation control', Cap-free) decreased the cell viability by 1 log10 unit with respect to the initial bacterial number. In agreement with this result, it has been reported previously that blue light (405 nm) irradiation was successful for the inactivation of both Gram-positive bacteria, including *S. aureus*, and Gram-negative bacteria (Maclean et al. 2009).

Eradication of planktonic bacteria

PDI was assessed by exposing a cell suspension containing Cap to UV-A irradiation for 2 h. The number of viable planktonic cells was determined after treatment with increasing concentrations of Cap (Figure 2). Using 0.1 μM and 0.2 μM Cap + 2-h UV-A radiation the number of viable bacteria decreased > 4 log10 units when compared to the initial CFU ($5.9 \times 10^6 \pm 4.7 \times 10^6$ CFU ml^{-1}), showing a good bactericidal effect. Subsequent additions of Cap increased the bactericidal effect so that viable bacteria were not detected after exposure to 0.4 μM + UV-A. Therefore, in the case of planktonic cells, the combination Cap (in the 0.1–0.4 μM concentration range) + UV-A radiation, but not the single factors (Cap or UV-A), is suitable as an antimicrobial strategy to inhibit the growth or to eradicate the cells.

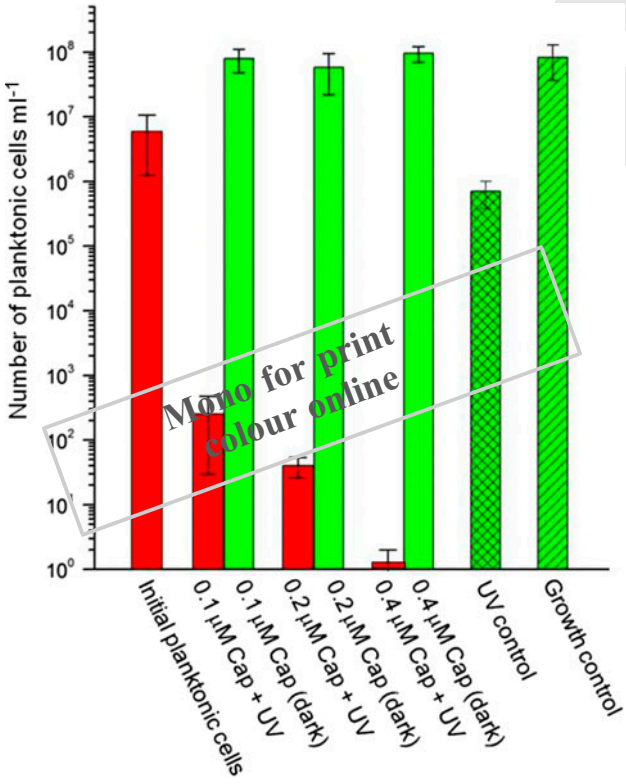

Figure 2. CFU for planktonic cells in the dark and after UV-A irradiation (λ_{max} = 350 nm) for 2 h.

Sessile bacteria

Inhibition of biofilm formation

The initial stage of biofilm formation on a particular surface is the transport of bacteria towards the surface followed by the attachment of cells. This is a crucial step in biofilm development that includes the initial interaction of bacteria with abiotic or biotic surfaces, which can ultimately lead to colonization. Reducing the number of attached cells is therefore a strategy of choice to prevent biofilm formation (Beloin et al. 2014).

Planktonic cells in culture, similar to those previously used (see above), were able to attach and form a young biofilm with an average cell density of $3.5 \times 10^6 \pm 2.2 \times 10^6$ CFU well^{-1} during a 2-h exposure period. This value corresponds to 17.6% of the total cells in the well. It was hypothesized that if the planktonic cells were exposed to PDI during this initial 2-h period of biofilm formation, the number of cells able to reach the surface and attach to it would be substantially reduced. The results confirmed this assumption. After PDI treatment for 2 h with 0.1 μM Cap (able to reduce the number of planktonic cells by 4 log10 units), no viable sessile bacteria were found on the surface. Consequently, the surviving cells were unable to attach to the surface or were killed after 0.1 μM Cap PDI. Assays using Cap concentrations 10 times lower (0.01 μM) (data not shown) showed that cells could attach to the surface under these conditions. Consequently, the PDI treatment proposed in this work is effective as a preventive treatment for inhibiting biofilm formation with Cap concentrations ≥ 0.1 μM.

Inhibition of pre-existing biofilm proliferation

It was also of interest to test whether PDI induced by Cap on *S. aureus* was able to eradicate pre-existing biofilms. The *S. aureus* strain exhibited good biofilm formation on microtiter plates. As mentioned above, after 2-h exposure to culture, the mean viable count of sessile cells recovered from nine wells was $3.53 \times 10^6 \pm 2.23 \times 10^6$ CFU well^{-1}. Non-treated biofilms (growth control)

showed mean viable counts = 4.20 × 10^7 ± 2.45 × 10^7 CFU $well^{-1}$ following a 2-h growth period. After PDI treatment, the viability of sessile cells decreased > 3 log10 and 4 log10 units at 50 μM of Cap with respect to the initial sessile cells and growth controls, respectively (Figure 3). Thus, this concentration was sufficient to exert a bactericidal effect.

When the antimicrobial action of PDI against planktonic and sessile cells is compared, it can be seen that the minimum concentration showing a bactericidal effect is 500 times greater in the case of sessile cells forming a biofilm (50 μM *vs* 0.1 μM), highlighting the marked increase in resistance of the cells to the antimicrobial treatment.

Biofilm resistance

It is well known that bacteria living in biofilms are more resistant to aggressive environments as well as antimicrobial compounds (Beloin et al. 2014). This increase in resistance has been attributed to several factors: (1) the EPS secreted by sessile cells might work as a protective and reactive layer, reducing the amount of foreign agents able to interact with cells, as well as forming a physical barrier limiting the penetration of antimicrobials; (2) the reduction in metabolism and growth rates, especially of those cells located deeply in the biofilm; (3) the phenotype of sessile cells differs from planktonic ones, which implies the expression of specific protective factors (persister cells) (Davies 2003).

It should be noted that the reduction in the number of viable sessile cells after PDI treatment in the range 50–200 μM of Cap reaches a plateau (Figure 3). Thus, a constant number of cells was able to resist the adverse conditions and remain alive even after more aggressive conditions. It is speculated that there are at least two causes of this resistant behavior: the presence of persister cells and the difficulty of PS penetrating into the biofilm. Persisters comprise a population of cells displaying tolerance to prolonged treatment with antibiotics. They are phenotypic variants of wild bacteria that are genetically identical to susceptible bacteria. Although they are generally non-growing, their phenotypic tolerance allows them to remain viable in the presence of bactericidal antibiotics. These particular bacteria seem to establish active defense systems towards oxidative stress (Kint et al. 2012). It is believed that the presence of persisters, rather than the biofilm architecture, is the main factor responsible for the resistance of cells within biofilms to antimicrobial agents (Spoering & Lewis 2001). Interestingly, the origin of persisters is varied: they may pre-exist in the biofilm (Balaban et al. 2004; Amato et al. 2014) or may be formed in response to a metabolic or oxidative stress (Amato et al. 2014), or during a biocidal treatment (Dörr et al. 2009; Orman & Brynildsen 2013).

Several reports have demonstrated that biofilms are more resistant to PDI than planktonic cells (Mantareva et al. 2011). It was shown that the resistance was mainly related to difficulties in PS penetration within the biofilm matrix. Accordingly, it is more difficult for highly effective polycationic PS to penetrate the anionic biofilm matrix because it binds too strongly to it compared with the less effective PS with fewer cationic charges (Vatansever et al. 2013). Notwithstanding the increase in PS concentration, penetration within the biofilms may be limited by the modified matrix after interaction with PS. Thus, the increase in Cap concentration is not necessarily associated with the increase in effectiveness. Both persisters and/or the difficulty in PS penetration could explain the presence of a plateau in Figure 3.

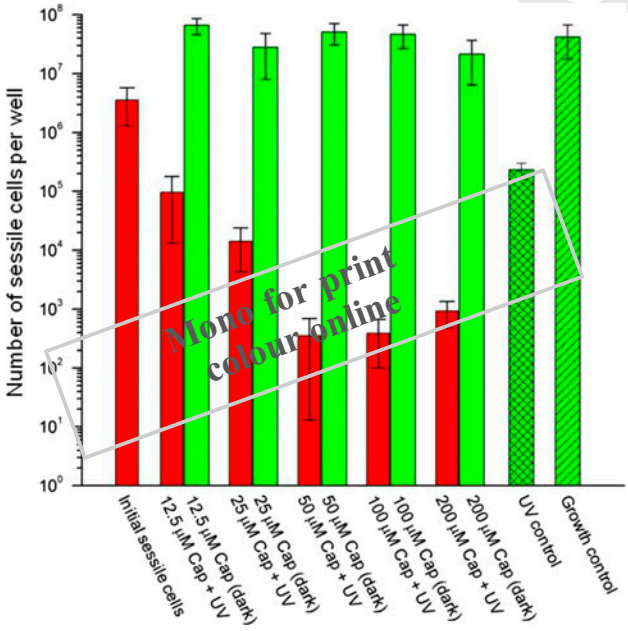

Figure 3. Number of viable sessile *S. aureus* in the dark and after 2-UV-A irradiation (λ_{max} = 350 nm) for 2 h.

Use of sunlight as a UV source

Sessile cells treated with 100 μM Cap and solar exposure showed a 3 log10-unit reduction in viable cells (Figure 4), indicating that the PDI treatment is also successful when sunlight is used as the UV radiation source. The inactivation of both planktonic and biofilm-forming bacteria was achieved using the advantage of the high availability of sunlight which results in reduced costs for the treatment.

Possible inactivation mechanism

Generation of ROS and cell damage

Bactericidal action under PDI treatment is attributed to the effect of ROS species and organic radicals generated

in situ by the UV-A excitation of Cap that leads to the formation of its triplet excited state (^{3}Pt*). Previous studies performed with different Ptr derivatives (Oliveros et al. 2010; Thomas et al. 2013; Castaño et al. 2014) demonstrated that three major reaction pathways compete for the deactivation of the triplet excited states of Ptrs: intersystem crossing to the singlet ground state (Reaction 1, see below); energy transfer to O_2 leading to the regeneration of Pt and the production of singlet molecular oxygen ($^1O_2$) (Reaction 2); and reaction with an electron donor (Reaction 3), which can be a biological substrate (S) such as proteins, DNA or their components, to form the corresponding pair of radicals ($Ptr^{\cdot-}$ and $S^{\cdot+}$). In the following step, the electron transfer from $Pt^{\cdot-}$ to O_2 regenerates Ptr and forms the superoxide anion radical ($O_2^{\cdot-}$) (Reaction 4), which undergoes disproportionation into H_2O_2 and O_2 (Reaction 5). Finally a group of processes that might include the reactions of $S^{\bullet+}$ (and/or its deprotonated form $S(-H)^{\bullet}$) with $O_2^{\cdot-}$, O_2 and H_2O, leads to the formation of products (PR) (Reaction 6) and, hence, damaged macromolecules (Oliveros et al. 2010; Thomas et al. 2013; Castaño et al. 2014). The reduction in the number of viable planktonic *S. aureus* observed in the present experiments might be the result of a combination of mechanisms, ie upon irradiation, oxidation of cellular components by ROS ($^1O_2$, $O_2^{\cdot-}$, H_2O_2) and the triplet excited of Cap might contribute to cell damage.

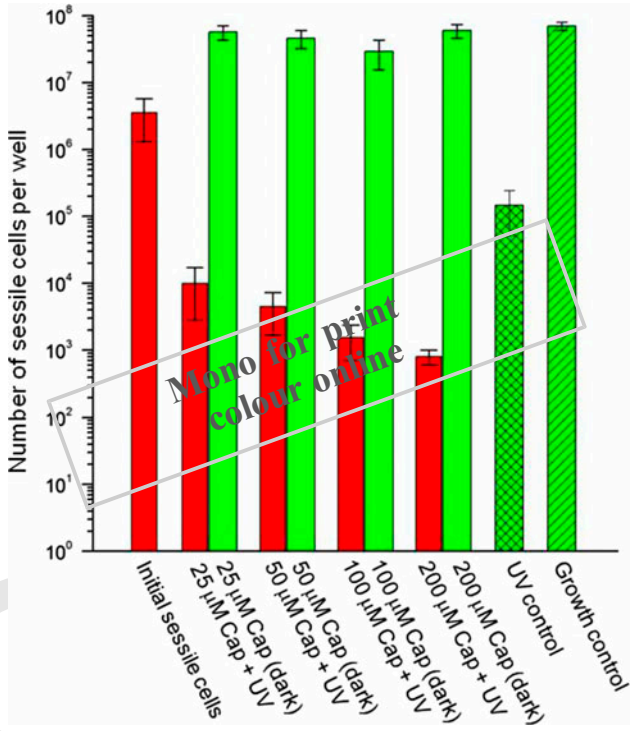

Figure 4. Photodynamic inactivation of biofilms using sunlight. The figure shows the number of viable sessile bacteria after PDI treatment (25–200 μM) using solar radiation as a UV source.

$$^3Pt^* \longrightarrow Pt \quad (1)$$

$$^3Pt^* + O_2 \longrightarrow Pt + {}^1O_2 \quad (2)$$

$$^3Pt^* + S \longrightarrow Pt^{\bullet-} + S^{\bullet+} \quad (3)$$

$$Pt^{\bullet-} + O_2 \longrightarrow Pt + O_2^{\bullet-} \quad (4)$$

$$2\,H^+ + 2\,O_2^{\bullet-} \longrightarrow H_2O_2 + O_2 \quad (5)$$

$$S^{\bullet+} \longrightarrow PR \quad (6)$$

Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are able to damage the activity of metalloenzymes and the integrity of DNA in microorganisms, inducing self-protection mechanisms involving enzymes and repair systems (Imlay 2013). However, environmental conditions may increase the amount of such species, either by increasing the endogenous production or by the presence in the surroundings of compounds able to produce $O_2^{\cdot-}$ and H_2O_2 that eventually may be incorporated to the cell. As H_2O_2 is able to cross the bacterial membrane of Gram-negative bacteria in similar amounts to water, oxidative stress is likely to be produced in the H_2O_2-rich atmosphere (Imlay 2013). On the other hand, it has been proposed that lipids and proteins present in the staphylococcal cell wall and/or membrane can be damaged by outer superoxide (Karavolos 2003). These external $O_2^{\cdot-}$ radicals may enter into the cell through anion channels, disrupting the internal superoxide equilibrium and therefore leading to a loss of bacterial viability due to external oxidative stress (Karavolos 2003).

Organic substances and ROS depletion

The lower susceptibility of sessile cells to PDI can be explained by the factors that enhance biofilm resistance mentioned above and also in terms of the localization of the PS inducing the generation of ROS. Since most ROS are highly reactive, their concentration might decrease as they diffuse from the generation site to the cells, probably due to their reaction with organic molecules present in the liquid medium as well as in the biofilm and particularly associated with the EPS. Indeed, Planchon et al. (2013) found that the killing capacity of hydroxyl radicals and superoxide is exacerbated in *Synechocystis* mutant strains with depleted EPS production. Additionally, it has been demonstrated that the damage to *Sinorhizobium meliloti* by H_2O_2 was mitigated when mutants expressing exopolysaccharides were challenged, as succinoglycan was able to decrease the *in vitro* levels of oxygen peroxide (Lehman & Long 2013). In order to investigate the effect of broth and EPS components on the concentration of ROS a qualitative analysis of $O_2^{\cdot-}$ consumption in the bacterial growth environment was carried out. It was found that the superoxide anion in the broth either at the concentration recommended by the manufacturer or at a 1:10 dilution decreased, by ~50% or 25%, respectively, compared to that in PBS. Also, the $O_2^{\cdot-}$ concentration was reduced by ~ 35% by EPS isolated from *S. aureus* used in this work. Importantly, notwithstanding that the ROS concentration is reduced by the organic content of the system, the results indicated that PDI induced by Cap (Cap-PDI) is appropriate for the inhibition of *S. aureus* biofilm growth when 50 or 100 μM are used with UV-A or sunlight as UV sources, respectively.

Potential applications of Cap-PDI

Ptr derivatives participate in relevant biological processes and are not cytotoxic in the dark; therefore, Cap-PDI could be used to kill planktonic cells in drinking water using sunlight. Further, since the method is also efficient in preventing the early stages of biofilm growth, it could be effective in medical applications and hospital environments, replacing the use of other *in situ* applications of antimicrobial treatments. Several clinical applications of photodynamic therapy (PDT) have emerged for the treatment of localized infections. For instance, an improvement in bleeding and plaque problems in chronic periodontal disease has been achieved after the use of PDT (Berakdar et al. 2012). Blood products have been successfully challenged with PDT as an antimicrobial, antifungal and antiprotozooal treatment (Wainwright 2000). It has been also demonstrated that nasal bacterial colonization in patients with chronic sinusitis is reduced when antibiotic resistant biofilms are treated with PDT (Wainwright 2000). In this regard, Cap would be a suitable PS for PDT not only for these potential medical-related applications, but also for disinfection of indwelling devices, which could be achieved by immersing the device into a Cap solution at the appropriate concentration and irradiating with an artificial UV-A source or sunlight. Importantly, it can be implemented in outdoor conditions, which is a great advantage due to the availability of the light source that results in a lower cost of the treatment.

Conclusions

It has been demonstrated that Cap-PDI is a suitable strategy for the prevention of *S. aureus* growth, either as 'free cells' (planktonic) or as adherent (sessile) bacteria forming an early pre-existing biofilm (10^6 cells well^{-1}). The efficacy of the PDI in killing planktonic cells facilitates the prevention of biofilm formation. The susceptibility to Cap is lower for pre-existing biofilms (≥50 μM Cap + UV-A) than for their planktonic counterparts (≥0.1 μM Cap + UV-A), mainly due to the fact that the PS must reach the inner sessile cells, overcoming the eventual trapping and quenching of Cap by organic substances, including EPS. The presence of persisters may be another cause of the absence of an additional biocidal effect when the PS concentration was increased.

Importantly, the method is also efficient in preventing biofilm growth using sunlight as the UV source (+ ≥ 100 μM Cap). As sunlight is readily available this could be a low cost strategy suitable for use in developing countries for the treatment of water and devices.

Conflict of interest disclosure statement

No potential conflict of interest was reported by the authors. AQ15

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