Cationic, anionic and neutral dyes: effects of photosensitizing properties and experimental conditions on the photodynamic inactivation of pathogenic bacteria

Sonia Sabbahi, Layla Ben Ayed and Abdellatif Boudabbous

ABSTRACT

The aim of this study was to evaluate the photobactericidal effect of four photosensitizers (PSs) with different structural and physico-photochemical properties, namely mesotetracationic porphyrin (T_4MPyP), dianionic rose Bengal (RB), monocationic methylene blue (MB) and neutral red (NR). Their photokilling activity was tested in vitro on pathogenic bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) suspended in nutrient broth (NB) and in phosphate buffered saline (PBS) through following their influence on the PSs antimicrobial efficacy. Photodynamic inactivation (PDI) experiments were performed using visible light (L) and different PSs concentrations (20–70 μM). The ability of these PSs to mediate bacterial photodynamic inactivation was investigated as a function of type of PS and its concentrations, spectral and physico-chemical properties, bacterial strain, irradiation time and suspending medium. Indeed, they showed antibacterial effects against *S. aureus* and *P. aeruginosa* with significant difference in potency. *Staphylococcus aureus* suspended in NB showed 0.92 log units reduction in viable count in the presence of T_4MPyP at 20 μM. Changing the suspending medium from NB to PBS, *S. aureus* was successfully photoinactivated by T_4MPyP (20 μM) when suspended in PBS at least time exposure (10 and 30 min), followed by MB and RB.

Key words | photodynamic inactivation, photosensitizers, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, visible light

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INTRODUCTION

The first experience with photodynamic therapy (PDT) dates back approximately 110 years when Raab (1900) observed that exposure to the acridine hypochloride dye and visible light is lethal to *Paramecia caudatum* and reported its principle. Nowadays, this PDT is used for the treatment of cancer and infectious diseases (Maischt 2006; Baptista & Wainwright 2011). On the other hand, the increasing interest in photodynamic antimicrobial chemotherapy as a promising technology for wastewater treatment demands more efficient photosensitizers (PSs) and faster methods for the monitoring of the photoinactivation process (Carvalho *et al.* 2007; Costa *et al.* 2008). Photodynamic therapy is a clinical treatment that uses light and a photosensitizer (dye). In this way, PDT or photosensitization combines a non-toxic photoactive dye-photosensitizer with harmful light source (sunlight or artificial light) to generate singlet oxygen and free radicals that kill microbial cells. The two oxidative mechanisms of photosensitization are named Type I and Type II (Donnelly *et al.* 2008; Calin & Parasca 2009). The reactive oxygen species (ROS) such as $^1O_2$, $OH^{\cdot}$, O_2^{-}, H_2O_2 are toxic to pathogens with very limited damage to the host tissues (Costa *et al.* 2008). As a result of the ROS-based mode of action, which is nonspecific in terms of its site of action in the microbial cell, the chance of resistance generation is very low, and photoantimicrobials are effective against conventional-resistant bacteria (Wainwright *et al.* 1998, 2012). They have been shown to be effective in vitro against bacteria (including antibiotic-resistant strains of

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nosocomial pathogens in particular, is resulting in increased human morbidity and mortality and is escalating health costs) (Dahl *et al.* 1989; Tome *et al.* 2004; Maragakis *et al.* 2008; Huang *et al.* 2010; Nakonechny *et al.* 2010), viruses (Costa *et al.* 2008), fungi (Gonzales *et al.* 2010; Souza *et al.* 2010), yeasts (Lambrechts *et al.* 2005; Calin & Ion 2010; Oriel & Nitzan 2012) and parasites (Sabbahi *et al.* 2008a). Many PSs possess antimicrobial properties such as: (i) high quantum yield of generating singlet oxygen, (ii) minimal or no dark toxicity, and (iii) good specificity or selectivity towards the target(s) (Guo *et al.* 2010). The assessment of the effect of a panel of nine PSs, eight synthetic (porphyrin, rose Bengal, erythrosine, eosin Y, methylene blue, methylene violet, neutral red (NR), safranine O) and one natural (chlorophyll) previously tested in vitro experiments against pathogenic microorganisms has been accomplished (Sabbahi 2010). Three of them (porphyrin, rose Bengal and methylene blue) were effective in killing environmental samples such as faecal bacteria in wastewater (Jemli *et al.* 2002, 2003). Moreover, several studies of methylene blue (MB) photodynamic action on *Staphylococcus aureus* have been performed. In summary, not only the reactive species antioxidants, but also whether the dye monomer or dimer was reacting and the ratio of their concentrations were important characteristics in determining the photobactericidal potential of the dye (Sabbahi *et al.* 2008b). The high inactivation of faecal indicators in Tunisian secondary wastewater, using a combination of the phenothiazinium dye with natural sunlight or artificial visible light determined on a small scale, was dependent mainly on the MB concentration, its application process and pH. In order to avoid primarily leaching of the compound into the environment and to further understand the MB photosensitization mechanisms, MB has been properly immobilized within resin. The faecal coliforms and faecal streptococci were found to be susceptible to the photodynamic action of MB fixed, seemingly a combination of Type I and Type II processes, and the relative efficiency of each depends notably on the experimental conditions (Sabbahi *et al.* 2010). The present study complements our previous work on the search for these PSs to be considered as good candidates for the photoinactivation of a large spectrum of environmental microorganisms suspended in nutrient broth, a requisite for an effective antimicrobial treatment for wastewater is its effectiveness in the inevitable presence of organic matter, firstly. Secondly, this study aimed to compare the efficiency of four PSs and chemical substituents differing in structures parent molecule, substituent types, ionic charges on the photodynamic inactivation of a Gram (+) (*S. aureus*) and a Gram (–) bacterium (*Pseudomonas aeruginosa*), suspended in nutrient broth. To our knowledge, the photodynamic inactivation of these bacteria by the four PSs including NR which is less used for bacteria photodynamic inactivation, under our experimental conditions (nutrient broth as suspending medium used without bacteria washing for its release from medium residual substances, dye concentrations used, PSs functional groups and irradiation time) has not been reported. In this context, we tested also the effect of the phosphate buffered saline (PBS) on the photoinactivation of *S. aureus*. In fact, the T_4MPyP was chosen as photosensitizer since it has proven to be a successful antimicrobial compound.

METHODS

Bacteria

Microorganisms isolated derive from surgical samples collected from the microbiological department in Tunis (Tunisia) of the Charles-Nicolle hospital for *P. aeruginosa* A1 and from the Children's Hospital for *S. aureus* ATCC 25923. The selection of bacteria models tested here is based, firstly, on their frequency in wastewater samples and secondly on their pathogenicity and resistance to conventional disinfection treatments. The two bacterial strains were grown aerobically overnight at 37 °C in liquid culture (nutrient broth; 1% tryptone, 0.5% yeast extract, 0.5% NaCl w/v). After 18 h in the stationary phase, microorganisms were harvested by centrifugation at 1,050 g for 10 min, washed twice with nutrient broth (NB) or 10 mM phosphate-buffered saline (PBS, pH = 7.3, 2.7 mM KCl, 137 mM NaCl) and suspended in 2.5 ml NB or PBS.

Photosensitizers

The stock solution of the four PSs mesotetracationic porphyrin (T_4MPyP) (Porphyrins Products Logan, UT, Alfa

Inorganics, Karbruha, German); methylene blue (COOPER-Cooperation pharmaceutique Française 77000 Melum); rose Bengal (RB) (Prolabo) and neutral red (RPE Analyticals Farmi Italica Carloerba) were prepared, with different dilutions, in sterile distillated water at a concentration of 1 g l^{-1} and stored at 4 °C in darkness until use. Before use, the stock solution was allowed to warm up to room temperature. The chemical structures and physicochemical properties of the four PSs used (Merchat 1995; Wagner *et al.* 1998; Redmond & Gamlin 1999; Romanova *et al.* 2003) are reported in Figure 1 and Table 1, respectively.

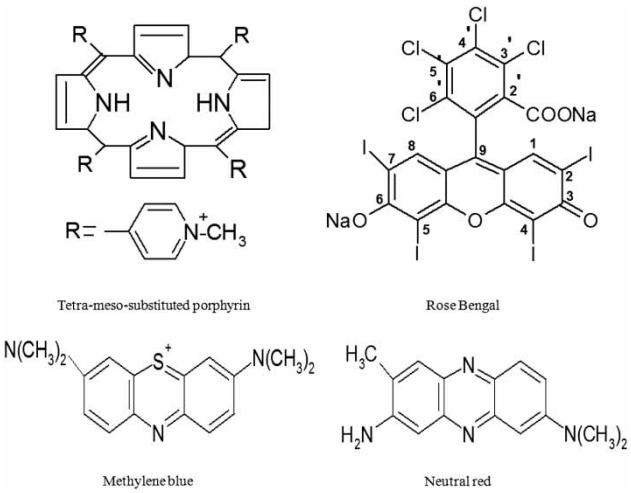

Figure 1 | Chemical structures of the four photosensitizers examined here. The core structure of each is a polycyclic heteroaromatic ring system. These molecules are generally planar with different charges.

Table 1 | Spectral, photophysical and photochemical properties of photosensitizers

PS	Mol wt (g mol^{-1})	ε (M^{-1} cm^{-1})[a]	φ^1O_2[b]	λ_{max} (nm)	Dye groups
T_4MPyP	1,363.36	1.97×10^5	0.74	422	Porphyrin
RB	1,017.65	1.136×10^5	0.76	549	Xanthene
MB	373.9	7.1089×10^4	0.5–0.6	670	Thiazine
NR	288.87	$1–2.33 \times 10^4$	–	540	Phenazine

Mol wt, Molecular weight.
[a]ε, Extinction coefficient.
[b]φ^1O_2, Quantum yield of singlet oxygen production.
λ_{max}, Absorption wavelength maximum measured in water.

Light source

All illuminations were carried out with visible light from a 500 W halogen lamp light source (OSRAM) which emits in the range of 500–750 nm with peak at 650 nm. To avoid heating of the samples, the photocontainers (with a final volume of 2.5 ml) were run at 28 °C covered by ice to maintain the temperature constant. The irradiance at the level of microorganism samples was 50 mW cm^{-2} as measured with solarmeter-pyranometer (Instruments HEANNS messger.A.TE, solar 118).

PHOTOSENSITIZATION PROCEDURE

Experimental setup

Bacterial cultures, grown overnight, were diluted in NB or PBS to a final concentration of approximately 10^7 CFU ml^{-1}. These bacteria suspensions were equally distributed into a 96-well flat-bottomed microliter plate (Sterilin, Stafford, UK) and incubated for 10 min with 100 μL of PS which were added to achieve final concentrations of 20, 50 and 70 μM corresponding to a total volume of 2.5 ml per beaker. The samples were protected from light and were incubated for 10 min in the dark, at 25–30 °C, with different concentrations of sensitizers. Light and dark controls were carried out during the experiments. In the light control, no PS was added, but the beaker was exposed to the same irradiation protocol. In the dark control, the PSs at different concentrations used (20, 50 and 70 μM) were added to the beaker and they were covered.

Photodynamic inactivation in NB

The microorganisms were harvested as described above and suspended in fresh NB. Survival was determined after 30, 60, 120, 180, 240, 300 and 360 min of illumination.

Photodynamic inactivation in PBS

Staphylococcus aureus was chosen for the experiments carried in saline solution, harvested as described above and suspended in PBS by centrifugation at 1,050 g for 10 min. A 20 μM of T_4MPyP was applied for these experiments.

Survival was determined after 10 and 30 min of illumination which corresponds to light doses of 30 and 90 J cm^{-2}, respectively.

Statistics

Values are expressed as mean percent change from starting control ± standard deviation. The CFU ml^{-1} results where survival fractions were transformed and analyzed by analysis of variance (ANOVA-MANOVA) and the Duncan test. Statistical analysis was performed using Statistica program with the statistical significance assumed to be at the $p < 0.05$ level.

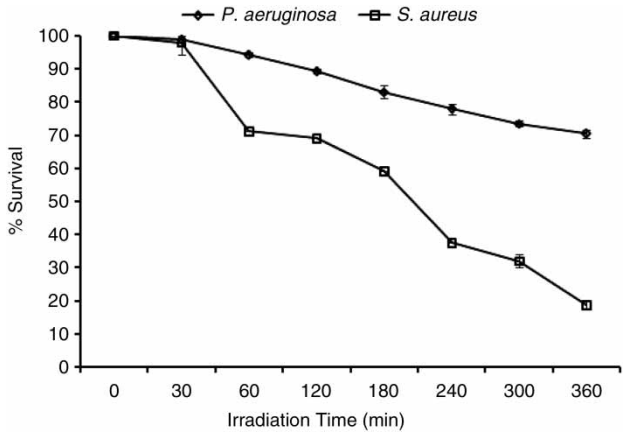

Figure 2 | Effect of continuous irradiation on (◇) *P. aeruginosa* and (□) *S. aureus* cell survival in the absence of PSs (light control).

RESULTS

Photodynamic inactivation in NB

With some exceptions, in most in vitro studies, buffer or broth (sometimes diluted) is used as suspending medium (Banfi *et al.* 2006; Tavares *et al.* 2010). It is known that the consistency of the suspending medium strongly influences the efficacy of antimicrobial photodynamic inactivation (Nitzan *et al.* 1998; Lambrechts *et al.* 2003).

The photosensitization experiments against pathogenic bacteria of this study were conducted in a non-diluted liquid culture medium. So, to prevent the maximum possibility of fixing a portion of the selective culture media protein by PS, a complex nonselective medium (nutrient broth) with low concentration of proteins was chosen (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Mean and standard deviation values of the survival fractions obtained in the photosensitization of both bacterial species by T_4MPyP, RB, MB and NR are evaluated. For the two bacteria strains studied, the photodynamic inactivation (PDI) using the different PSs significantly (ANOVA, $p < 0.05$) reduced the numbers of colony forming units (CFUs) per milliliter comparing to the control group (L-PS-). Control experiments showed that in the absence of PSs, illumination alone had a small effect on *S. aureus* and *P. aeruginosa*, so the use of only visible light did not cause a significant microbial reduction (Figure 2). The differences observed between the light and PS (L + PS+) and without light and PS (L−PS−) groups were statistically significant (ANOVA, $p < 0.05$) for all exposure times (Figures 2 and 3). Without illumination, the PSs used showed no inherent dark effect to the two microorganisms tested under the concentrations as less or equal to 50 μM. Upon illumination, the PSs showed obvious photosensitizing effect against either bacterium under the conditions of our experiments.

The results obtained with mesotetracationic porphyrin (T_4MPyP) at a concentration of 20 μM are shown in Figure 3(a). There was *P. aeruginosa* (5.2×10^7 CFU ml^{-1}) reduction of 3.3 log units corresponding to a percentage of survivors of 0.05%, when the artificial visible light (50 mW cm^{-2}; 540 J cm^{-2}) was applied for 180 min. Under the same conditions, *S. aureus* (1.3×10^7 CFU ml^{-1}) showed 0.92 log units reduction in viable count (percentage of survivors of 11.9%). Photodynamic action with RB promoted a reduction in the number of CFU ml^{-1} of *P. aeruginosa* and *S. aureus*, as shown in Figure 3. The rate reduction for these two strains observed in the xanthene dye (PS + L+) compared with PS−L− was higher when RB was used as a PS. However, there was no significant statistical difference (ANOVA, $p > 0.05$) between the three concentrations tested (20, 50 and 70 μM) in the photodynamic inactivation against *S. aureus* for the first 30 min of time exposure to visible light. In addition, the extent of microbial reduction increased with the increase of time exposure to visible light. For RB, at the lowest concentration studied (20 μM) and at 60 min of illumination, *S. aureus* was photoinactivated to a 95.5% (survival fraction of 3.46 ± 1.92%, reduction of 1.46 log units). Using the same irradiation time, *P. aeruginosa* culture showed a survival fraction of

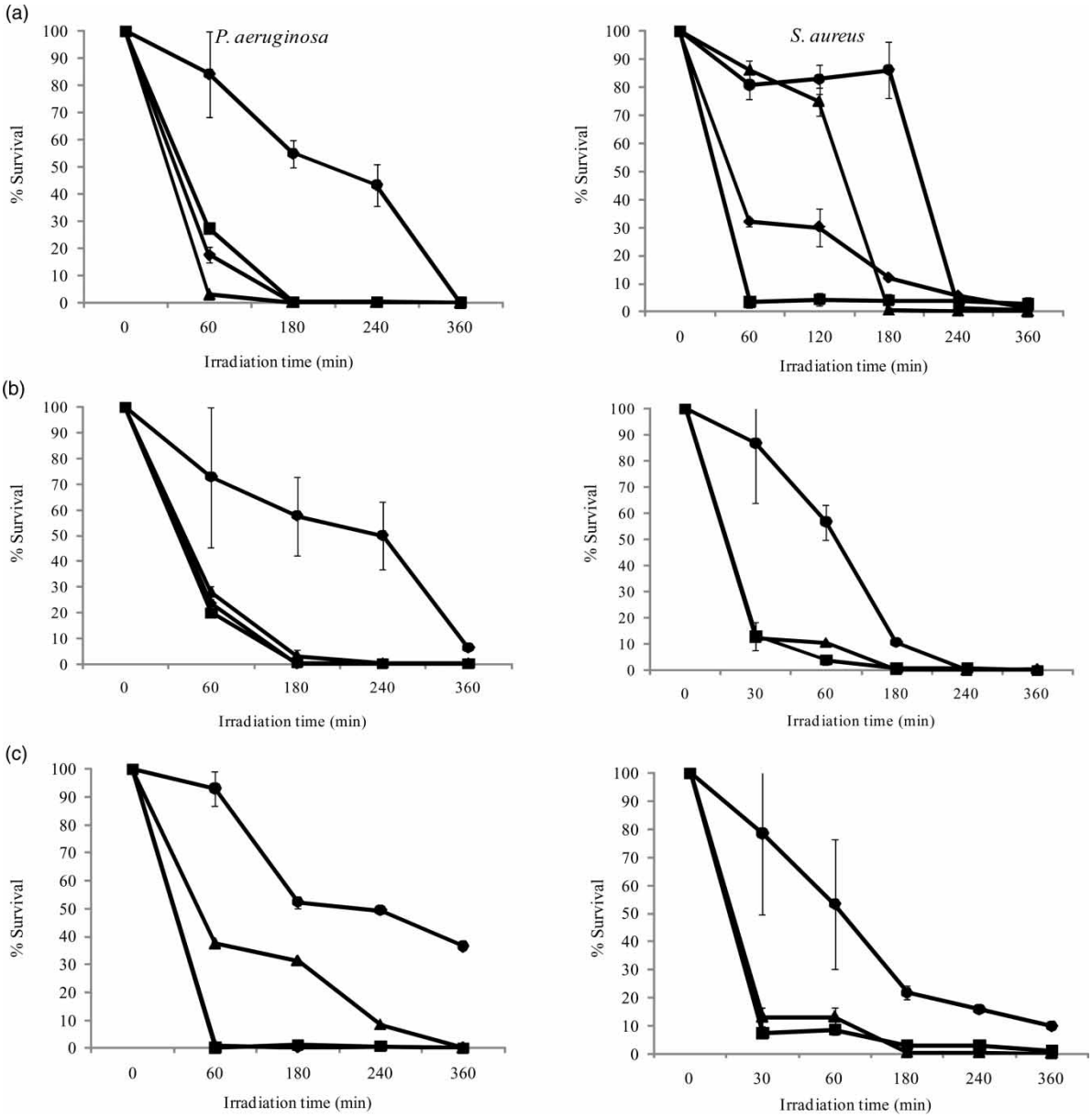

Figure 3 | Survival curves of *P. aeruginosa* and *S. aureus* suspended in NB exposed to different concentrations of photosensitizers: (♦) T_4MPyP; (■) RB; (▲) MB; (●) NR: (a) 20 μM; (b) 50 μM; and (c) 70 μM and different irradiations times. The cultures were left for 10 min in the dark with the indicated PSs and then irradiated at 50 mW cm^{-2} for 360 min. Data are the average of three independent experiments. Vertical bars represent the standard deviation.

27.35 ± 0.07% (a 0.56 log units decrease of viable cells). *Staphylococcus aureus* was found to be much more sensitive than *P. aeruginosa* to the xanthene PS. Besides, much higher concentrations of RB were not needed to kill these two strains and RB was not notably effective against these species after increasing concentrations and irradiation time.

The photodynamic action of MB as phenothiazine dye was determined when directed against *P. aeruginosa* and *S. aureus*, under irradiation conditions (Figure 3). For these two microorganisms tested, as a dye MB was found to be more photoactive than RB at a concentration of 20 μM. Under these conditions, MB induced a reduction in the numbers of CFUs per milliliter of *P. aeruginosa* and *S. aureus* in relation to the control group and to the RB. Under visible light (60 min) and when MB was used at 20 μM, there was a *P. aeruginosa* survival fraction of 3.1%.

Paradoxically, for the same irradiation time and in the presence of a higher concentration of MB (70 μM), the reduction of *P. aeruginosa* surviving was diminished, corresponding to a survival fraction of 37.5 ± 0.5%.

For phenazine (or azine) dye, photodynamic action with NR (20 μM) against *P. aeruginosa* resulted in slightly lower mean number CFU ml^{-1} when compared to the three PSs tested in this study (ANOVA, $p < 0.05$). *Pseudomonas aeruginosa* was found to be resistant to photoinactivation with NR. Indeed, 55% survivors were obtained for a contact time beyond 180 min with 20 and 50 μM NR. The contact of 60 min allowed the rescue of more than 70% of *P. aeruginosa* survivors, suggesting that this light exposure was too short to obtain significant cell lethality on this bacterium, independently from the compound concentrations used. Among the two microorganisms tested, *S. aureus* proved to be less sensitive to NR, causing only a 1 log units reduction survivors at a concentration of 50 μM and illuminated for 180 min. Under the same conditions, *S. aureus* showed a 7.5 log units reduction in viable count for a time longer than 180 min corresponding to a total removal of bacteria (>99.99%). *Pseudomonas aeruginosa* was not affected at NR concentrations higher than 20 μM. Overall, there was a significant difference in potency for these dyes tested in this study (ANOVA, $p < 0.05$).

Photodynamic inactivation in PBS

It should be noted that the photodynamic inactivation process of PSs used at a concentration of 20 μM was rather slow, with high bactericidal effects found after 180 min irradiation time for microorganisms tested. On the other hand, less bacteria reduction was observed after 30 min. So, to minimize the possibility of a supplementary protection from the culture medium, above-mentioned, sensitization procedure was performed in saline medium instead of the culture medium. In fact, proteins can protect bacteria from cytotoxic species generated during photosensitization (Nitzan *et al.* 1989; Wilson 1993; Wilson and Pratten 1995; Bhatti *et al.* 1997). Bacterial culture of *S. aureus* strain described above was suspended in PBS to obtain a cell density of approximately 10^7 CFU ml^{-1}.

A photosensitizer concentration of 20 μM was used in this experiment. After addition of each PS tested, samples of 2.5 mL were irradiated with visible light at a fluence rate of 50 mW cm^{-2}.

The T_4MPyP, RB and MB photoantimicrobial actions against *S. aureus*, suspended in PBS, are outlined in Tables 2–4, respectively. Light and dark controls were carried out during the experiments. Small effects were observed in samples after illumination in the absence of PSs (light control) and in samples that were incubated with the PSs tested in the dark (dark control).

At a concentration of 20 μM for T_4MPyP and under 50 mW cm^{-2} of light intensity, *S. aureus* showed survival fractions of 0.2 ± 0.001% and 0.01 ± 0.0% after 10 and 30 min of visible light, respectively, (Table 2) corresponding to a reduction in viable count higher than 2.5 and 4.5 log units after 10 and 30 min of illumination, respectively. A significant increase in the reduction percentage of colony forming units for *S. aureus* as Gram-positive bacteria for 10 min with T_4MPyP present was obtained. The reduction percentage reached more than 99% for *S. aureus* (Table 2) after 10 min of irradiation which corresponded to a light dose of 30 J cm^{-2}. A significant increase in the reduction percentages of colony forming units for *S. aureus* tested upon treatment

Table 2 | Survival table of *S. aureus* ATCC25923 (5.1×10^7 CFU ml^{-1}) suspended in PBS (7.3) exposed to 20 μM T_4MPyP and two different light doses at defined irradiation times. The cells were left for 10 min in the dark and then irradiated by visible light (50 mW cm^{-2}) for 10 and 30 min at light fluence doses of 30 and 90 J cm^{-2}, respectively. Each value is the mean of three experiments ± standard deviation

Medium	Viability (CFU ml^{-1})	Survival fraction (%)	Inactivated bacteria (%)
	After 10 min of phototreatment		
L − T_4MPyP −	4.9×10^7	96.1 ± 2.0	3.9 ± 2.0
L − T_4MPyP +	2.6×10^7	51.0 ± 10.9	49.0 ± 10.9
L + T_4MPyP −	4.2×10^7	82.3 ± 5.7	17.6 ± 5.7
L + T_4MPyP +	1.0×10^5	0.2 ± 0.01	99.8 ± 0.01
	After 30 min of phototreatment		
L − T_4MPyP −	4.8×10^7	94.1 ± 1.9	5.88 ± 1.9
L − T_4MPyP +	1.6×10^7	31.4 ± 5.1	68.6 ± 5.1
L + T_4MPyP −	2.6×10^7	51.0 ± 4.2	49.0 ± 4.2
L + T_4MPyP +	0.0	0.01 ± 0.0	>99 ± 0.00

Bacterial strain submitted to treatment with a visible light L and tetracationic porphyrin T_4MPyP (L + T_4MPyP+).

L + T_4MPyP +: porphyrin and visible light; **L + T_4MPyP −**: bacterial suspension in saline solution and visible light; **L − T_4MPyP −**: bacterial suspension in saline solution only as control; **L − T_4MPyP +**: bacterial suspension in saline solution and porphyrin alone.

Table 3 | Survival table of *S. aureus* ATCC25923 (5.1×10^7 CFU ml^{-1}) suspended in PBS (7.3) exposed to 20 μM RB and two different light doses at defined irradiation times. The cells were left for 10 min in the dark and then irradiated by visible light (50 mW cm^{-2}) for 10 and 30 min at light fluence doses of 30 and 90 J cm^{-2}, respectively. Each value is the mean of three experiments ± standard deviation

Medium	Viability (CFU ml^{-1})	Survival fraction (%)	Inactivated bacteria (%)
	After 10 min of phototreatment		
L − RB −	4.9×10^7	96.1 ± 2.0	3.9 ± 2.0
L − RB +	3.9×10^7	76.5 ± 1.4	23.5 ± 1.4
L + RB −	4.2×10^7	82.3 ± 5.7	17.6 ± 5.7
L + RB +	1.05×10^7	20.6 ± 1.4	79.4 ± 1.4
	After 30 min of phototreatment		
L − RB −	4.8×10^7	94.1 ± 1.9	5.88 ± 1.9
L − RB +	2.8×10^7	54.9 ± 1.0	45.1 ± 1.0
L + RB −	2.6×10^7	51.0 ± 4.2	49.0 ± 4.2
L + RB +	4.1×10^6	08.0 ± 0.4	91.9 ± 0.4

Bacterial strain submitted to treatment with a visible light L and rose Bengal RB (L + RB+). **L + RB +** : rose Bengal and visible light; **L + RB −** : bacterial suspension in saline solution and visible light; **L − RB −** : bacterial suspension in saline solution only as control; **L − RB +** : bacterial suspension in saline solution and rose Bengal alone.

Table 4 | Survival table of *S. aureus* ATCC25923 (5.1×10^7) suspended in PBS (7.3) exposed to 20 μM MB and two different light doses a defined irradiation times. The cells were left for 10 min in the dark and then irradiated by visible light (50 mW cm^{-2}) for 10 and 30 min at light fluence dose 30 and 90 J cm^{-2}, respectively. Each value is the mean of three experiments ± standard deviation

Medium	Viability (CFU ml^{-1})	Survival fraction (%)	Inactivated bacteria (%)
	After 10 min of phototreatment		
L − MB −	4.9×10^7	96.1 ± 2.0	3.9 ± 2.0
L − MB +	4.4×10^7	86.3 ± 4.1	13.7 ± 4.1
L + MB −	4.2×10^7	82.3 ± 5.7	17.6 ± 5.7
L + MB +	1.6×10^7	31.4 ± 5.6	68.6 ± 5.6
	After 30 min of phototreatment		
L − MB −	4.8×10^7	94.1 ± 1.9	5.88 ± 1.9
L − MB +	4.0×10^7	78.4 ± 4.1	21.6 ± 4.1
L + MB −	2.6×10^7	51.0 ± 4.2	49.0 ± 4.2
L + MB +	1.0×10^5	0.2 ± 0.1	99.8 ± 0.1

Bacterial strain submitted to treatment with a visible light L and methylene blue MB (L + MB+). **L + MB +** : methylene blue and visible light; **L + MB −** : bacterial suspension in saline solution and visible light; **L − MB −** : bacterial suspension in saline solution only as control; **L − MB +** : bacterial suspension in saline solution and methylene blue alone.

with 20 μM of RB and MB for 30 min of visible irradiation time with a light dose of 90 J cm^{-2}. The photoinactivation percentage reached 91.9 for RB (Table 3) and 99.8% for MB (Table 4). In this case, we verified that the dyes decreased the viability of *S. aureus* under visible light, ranking in terms of the bactericidal activity in this order: mesotetracationic porphyrin > methylene blue > rose Bengal.

In the presence of culture medium (nutrient broth), photoinactivation of the tested microorganism using the light fluency of 30 and 90 J cm^{-2} for 10 and 30 min, respectively, of irradiation time was significantly inhibited (Figure 3(a)). The influence of culture medium (NB) on the T_4MPyP, RB and MB PDI of the microorganism is shown in Figure 3(a) in comparison to that obtained in PBS (Tables 2–4). Culture medium exerted a protective effect against the PDI of the microorganism. *Staphylococcus aureus* could be successfully photoinactivated by T_4MPyP at 20 μM when suspended in PBS at the least time exposure, followed by MB and RB.

DISCUSSION

The present study demonstrated the efficiency comparison of four PSs differing in dye groups with different structures of parent molecule, substituent groups, ionic number, physicochemical properties, concentrations and periods of irradiation on the PDI on two pathogenic bacteria (*S. aureus* and *P. aeruginosa*). Upon illumination, the PSs showed obvious photodynamic action against either bacterium under the conditions. At the highest concentrations tested (50 and 70 μM), a little reduction of survivors was observed. Under 180 min of irradiation, a better photodynamic efficiency (0.9–~3.5 log units reduction) was obtained with the PSs used at a concentration of 20 μM and increasing concentrations did not really improve the efficiency of the treatment. Porphyrin (20 μM) was the most active, causing about 3.5 log units of *P. aeruginosa* reduction. Thus, it is unnecessary to increase the concentration of porphyrin because it really does not improve its photobactericidal effectiveness. From these results, it seems sensible to work with the lowest concentrations. However, increasing the irradiation time allowed a possible improvement in yields. Cationic photosensitizers, such as positively charged porphyrins are known to be more active than the corresponding anionic or non-ionic compounds against both Gram-positive and Gram-negative bacteria (Merchat *et al.* 1996; Jori & Brown 2004). In this work, we have designed these PSs and tested their ability to kill bacteria

suspended in liquid medium after exposure to visible light. Then, a comparison of differently structured molecules was performed. The positive charge and groups of dyes seem to have different effects in the photoinactivation of both bacteria. As cationic PSs, T_4MPyP and MB proved to have the highest survival reduction of *P. aeruginosa* after the first 60 min of exposure time and smaller concentration used in this study (20 μM), probably due to electrostatic interactions between the positively charged of PSs such as porphyrins which has four cationic moieties (in mesosubstituted porphyrins) and negatively charged sites at the outer membrane of Gram-negative bacteria (Costa *et al.* 2008). For all the dyes tested, there was no observed resistance of the two microorganisms suspended in liquid culture (NB). As mentioned above, Thiazine dye as MB with alkylamino functional groups at either or both the 3 and 7 positions had good antibacterial activity (Adamcikova *et al.* 2000). The RB, as an anionic compound was less effective than T_4MPyP and MB against *P. aeruginosa* and *S. aureus* for the first 60 min of visible light. For the given PSs, the presence of heavy atoms usually increases the maximum wavelength of absorption considerably on one side (Wainwright *et al.* 2007). On the other side, with the increasing number of halogen substituents, the singlet oxygen yields increased too (Gollnick & Schenck 1964; Gandin & Van De Vorst 1983; Neckers 1989). Some research had shown that the presence of heavy bromine or iodine atoms enhanced the intersystem crossing yield to the reactive triplet state of the xanthene dyes (Jori & Reddi 1991; Wang *et al.* 2006), with excited triplet yield of 0.76 for RB and of 0.6 and 0.3 for erythrosine and eosin Y, respectively (Neckers 1989). Indeed, there was an obvious correlation between increasing number of xanthene dyes halogen substituents and activity (Sabbahi 2010). Generally, effective PS molecules are able to populate the excited triplet state ($^*T_1$) significantly (Wainwright *et al.* 2007). The $^*T_1$ state can itself be stabilized by the inclusion of atoms of a high atomic number due to their large spin-orbital coupling constants (the 'heavy atom' effect) (Cincotta *et al.* 1988; Wainwright *et al.* 2007). The closely related phenazine photosensitizer NR has been used as an effective photosensitizing antiviral, especially against the *herpes simplex* virus (Bockstahler *et al.* 1979) by exerting its photodynamic action against the viral envelope. At 20 μM concentration, neutral red or NRH^+ Cl^- as a phenazine-based dye was notably less efficient than the other PSs against the bacterial strains tested. In most of the organic solvents, both the neutral (NRH^+ Cl^-) and the cationic (NRH+) forms of the dye coexist in these solutions. The contribution of each form is seen to depend largely on the concentration of the dye as well as on the nature of the solvent. In fact, Singh *et al.* (1999) demonstrated by using NR spectra study, that at sufficiently low concentrations (≤20 μM), the spectra closely resemble those of the neutral form (NR) of the dye with an absorption peak at around 450–460 nm. As the concentration is increased, a second absorption band slowly develops at around 530–540 nm. This latter band, which develops at higher concentrations of NRH^+Cl^-, is attributed to the cation form (NRH^+). *Staphylococcus aureus* could be successfully photoinactivated by NR at higher concentrations and long time exposure to visible light. In contrast, no influence of elevated concentrations on the PDI of *P. aeruginosa* was observed when NR was used as PS. Most likely this is due to the unavailability of free electrons with the dimethylamino group due to protonation (Singh *et al.* 1999) at high concentrations, indicating that NR has a different mode of photodynamic action with its concentration used according to the two bacterial Gram types. Porphyrin dissolved in PBS photosensitizer solvent enhanced mainly disinfection potential of photodynamic treatment of *S. aureus* strain, followed by MB and RB. From the experiments done in the absence of culture medium (in saline), it seems that the photodynamic effect is greater in such conditions. This fact alone indicates that the culture medium has a quenching character, probably by catching and binding a portion of the PS before reaching the bacteria cell (Nitzan *et al.* 1989). Further work will thus be focused on the comprehension of the relationships among PSs tested here, bacterial superficial composition, changing the suspending medium, PSs molecular structures and physico-chemical properties on the observed photodynamic activity.

CONCLUSIONS

Our experiments have demonstrated that *P. aeruginosa* and *S. aureus* can be photoinactivated using T_4MPyP, RB, MB and NR as PSs used in micromolar concentrations. However, the degree of microbial PDI strongly depend on the type of PS used, its concentrations and their spectral and

physico-chemical properties, irradiation time, type of bacteria and suspending medium. On the whole, our data confirm that the PSs tested demonstrated a photodynamic effect against the two microorganisms tested and that the presence of positive charges on the PSs such as T_4MPyP and MB can be envisaged as a good candidate for the photodynamic treatment of the pathogens tested. Furthermore, activity data suggest that the presence of heavy atoms increase the photodynamic action of xanthene dyes such as RB. There exists a sound understanding concerning important molecular structure and substituents of PSs relationships with increasing antimicrobial efficiencies.

Interestingly, the use of PSs with positive charges, heavy atoms and alkylamino functional groups at either or both the 3 and 7 positions provided a number of highly antibacterial Gram (+) and Gram (–) targets, having equal or greater activity against *S. aureus* and *P. aeruginosa*. So, the photodynamic inactivation method could be effectively used against different pathogenic and harmful microorganisms.

REFERENCES

Adamcikova, L., Paylikova, K. & Sevcik, P. 2000 The decay of methylene blue in alkaline solution. *Reaction Kinetics and Catalysis Letters* **69**, 91–94.

Banfi, S., Caruso, E., Buccafurni, L., Battini, V., Zazzaron, S., Barbieri, P. & Orlandi, V. 2006 Antibacterial activity of tetraaryl-prophyrin photosensitizers: An in vitro study on Gram negative and Gram positive bacteria. *Journal of Photochemistry and Photobiology B: Biology* **85**, 28–38.

Baptista, M. S. & Wainwright, M. 2011 Photodynamic antimicrobial chemotherapy (PACT) for the treatment of malaria, leishmaniasis and trypanosomiasis. *Brazilian Journal of Medical and Biological Research* **44**, 1–10.

Bhatti, M., MacRobert, A., Meghji, S., Henderson, B. & Wilson, M. 1997 Effect of dosimetric and physiological factors on the lethal photosensitization of *Porphyromonas gingivalis* in vitro. *Photochemistry and Photobiology* **65**, 1026–1031.

Bockstahler, L. E., Coohill, T. P., Hellman, K. B., David Lytle, C. & Roberts, J. E. 1979 Photodynamic therapy of Herpes simplex: A critical Review. *Pharmacology and Therapeutics* **42**, 473–499.

Calin, M. A. & Ion, R. M. 2010 The efficacy of photodynamic inactivation of the microorganisms using laser sources and methylene blue as sensitizer. *Journal of Optoelectronics and Advanced Materials* **12**, 141–145.

Calin, M. A. & Parasca, S. V. 2009 Light sources for photodynamic inactivation of bacteria. *Lasers in Medical Science* **24**, 453–460.

Carvalho, C. M. B., Gomes, A. T. P. C., Fernandes, S. C. D., Prata, A. C. B., Almeida, M. A., Cunha, M. A., Tomé, J. P. C., Faustino, M. A. F., Neves, M. G. P. M. S., Tomé, A. C., Cavaleiro, J. A. S., Lin, Z., Rainho, J. P. & Rocha, J. 2007 Photoinactivation of bacteria in wastewater by porphyrins: Bacterial β-galactosidase activity and leucine-uptake as methods to monitor the process. *The Journal of Photochemistry and Photobiology B* **88**, 112–118.

Cincotta, L., Foley, J. W. & Cincotta, A. H. 1988 Novel phenothiazinium for photodynamic therapy. *SPIE Advanced Photochemotherapy* **997**, 145–153.

Costa, L., Alves, E., Carvalho, C., Tomé, J., Faustino, M., Neves, M., Tomé, A., Cavaleiro, J., Cunta, Â. & Almeida, A. 2008 Sewage bacteriophage photoinactivation by cationic porphyrins: a study of charge effect. *Photochemical and Photobiological Sciences* **7**, 415–422.

Dahl, T. A., Midden, W. R. & Hartman, P. E. 1989 Comparison of killing of Gram-negative and Gram-positive bacteria by pure singlet oxygen. *Journal of Bacteriology* **171**, 2188–2194.

Donnelly, R. F., McCarnon, P. A. & Tunney, M. M. 2008 Antifungal photodynamic therapy: A review. *Microbiological Research* **163**, 1–12.

Gandin, Y. L. & Van De Vorst, A. 1983 Quantum yield of singlet oxygen production by xanthenes derivatives. *Photochemistry and Photobiology* **37**, 271–278.

Gollnick, K. & Schenck, G. O. 1964 Mechanism and stereoselectivity of photosensitized oxygen transfer reactions. *Pure and Applied Chemistry* **9**, 507–579.

Gonzales, F. P., Da Silva, S. H., Roberts, D. W. & Braqa, G. U. 2010 Photodynamic inactivation of Conidia of the fungi *Metharhizium anisopliae* and *Aspergillus nidulans* with methylene blue and toluidine blue. *Photochemistry and Photobiology* **86**, 653–661.

Guo, Y., Rogeli, Z. & Zhang, P. 2010 Rose Bengal-decorated silica nanoparticules as photosensitizers or inactivation of Gram-positive bacteria. *Nanotechnology* **21**, 065–102.

Huang, L., Dai, T. & Hamblin, M. R. 2010 Antimicrobial inactivation and photodynamic therapy for infections. *Methods in Molecular Medicine* **635**, 155–173.

Jemli, M., Alouini, Z., Sabbahi, S. & Gueddari, M. 2002 Destruction of faecal bacteria in wastewater by three photosensitizers. *Journal of Environmental Monitoring* **4**, 511–516.

Jemli, M., Alouini, Z., Sabbahi, S. & Gueddari, M. 2003 Désinfection photochimique des eaux usées par la porphyrine tétra-méso-cationique (T_4MPyP). *Vecteur Environnement* **36**, 68–75.

Jori, G. & Brown, S. B. 2004 Photosensitized inactivation of microorganisms. *Photochemical and Photobiological Sciences* **3**, 403–405.

Jori, G. & Reddi, E. 1991 Second generation photosensitizer for the photodynamic therapy of tumors. In: *Light in Biology and medicine* (R. H. Douglas, J. Moan & G. Ronto, eds). Plenum Press, London, pp. 253–266.

Lambrechts, S. A. G., Aalders, M. C. G. & Van Marle, J. 2005 Mechanistic study of the photodynamic inactivation of

Candida albicans by a cationic porphyrin. *Antimicrobial Agents and Chemotherapy* **49**, 2026–2034.

Lambrechts, S. A. G., Aalders, M. C. G., Langeveld-Klerks, D. H., Khayali, Y. & Lagerberg, J. W. M. 2003 Effect of monovalent and divalent cations on the photoinactivation of bacteria with meso-substituted cationic porphyrins. *Journal of Photochemistry and Photobiology* **79**, 297–302.

Maischt, T. 2006 Anti-microbial photodynamic therapy: useful in the future? *Lasers in Medical Science* **22**, 83–91.

Maragakis, L. L., Perencevich, E. N. & Cosgrove, S. E. 2008 Clinical and economic burden of antimicrobial resistance. *Expert Review of Anti-infective Therapy* **6**, 751–763.

Merchat, M. 1995 Mise en évidence des propriétés photoinactivatrices des porphyrines. Thèse de Doctorat en Science, Université Paris XI Orsay, Paris, p. 158.

Merchat, M., Bertoloni, G., Giacomini, G., Villanueva, A. & Jori, G. 1996 Meso-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. *Journal of Photochemistry and Photobiology B: Biology* **32**, 153–157.

Nakonechny, F., Firer, M. A., Nitzan, Y. & Nisnevitch, M. 2010 Intracellular antimicrobial photodynamic therapy: A novel technique for efficient eradication of pathogenic bacteria. *Photochemistry and Photobiology* **86**, 1350–1355.

Neckers, D. C. 1989 Rose Bengal. *Journal of Photochemistry and Photobiology A: Chemistry* **47**, 1–29.

Nitzan, Y., Balzam-Sudakevitz, A. & Ashkenazi, H. 1998 Eradication of Acinetobacter baumannii by photosensitized agents in vitro. *Journal of Photochemistry and Photobiology* **42**, 211–218.

Nitzan, Y., Shainberg, B. & Malik, Z. 1989 The mechanism of photodynamic inactivation of *Staphylococcus aureus* by deuteroporphyrin. *Current Microbiology* **19**, 265–269.

Oriel, S. & Nitzan, Y. 2012 Mechanistic aspects of photoinactivation of Candida albicans by exogenous porphyrins. *Photochemistry and Photobiology* **88**, 604–612.

Raab, O. Z. 1900 Ueber die Wirkung fluoreszierender stoffe auf infusorien. *Zeitschrift fuer Biologie* **39**, 524–546.

Redmond, R. W. & Gamlin, J. N. 1999 A compilation of singlet oxygen yields from biologically relevant molecules. *Photochemistry and Photobiology* **70**, 391–475.

Romanova, N. A., Brovko, L. Y., Moore, L., Pomehen, E., Savitsky, A. P., Ugarova, N. M. & Griffiths, M. W. 2003 Assessment of photodynamic destruction of *Escherichia coli* O157: H7 and *Listeria monocytogenes* by using ATP bioluminescence. *Applied and Environmental Microbiology* **69**, 6393–6398.

Sabbahi, S. 2010 Désinfection photodynamique des eaux usées. Thèse de Doctorat en Biologie, Faculté des Sciences de Tunis, Tunis, p. 163.

Sabbahi, S., Alouini, Z., Ben Ayed, L., Jemli, M. & Boudabbous, A. 2010 Inactivation of faecal bacteria in wastewater by methylene blue and visible light. *Desalination and Water Treatment* **20**, 209–219.

Sabbahi, S., Alouini, Z., Jemli, M., Ben Ayed, L., Boudabbous, A. & Lahmar, S. 2008a Destruction photodynamique des œufs de Taeniidés et de Toxocara sp. dans l'eau. *Cahier de l'Association Scientifique Européenne pour l'Eau et la Santé* **13**, 31–42.

Sabbahi, S., Alouini, Z., Jemli, M. & Boudabbous, A. 2008b The role of reactive oxygen species in *S. aureus* photoinactivation by methylene blue. *Water Science and Technology* **58** (5), 1047–1055.

Singh, M. K., Pal, H., Bhasikuttan, A. C. & Sapre, A. V. 1999 Photophysical properties of the cationic form of neutral red. *Photochemistry and Photobiology* **69**, 529–535.

Souza, R. C., Junqueira, J. C., Rossoni, R. D., Pereira, C. A., Munin, E. & Jorce, A. O. C. 2010 Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low-power laser irradiation alone against *Candida albicans*. *Lasers in Medical Science* **25**, 385–389.

Tavares, A., Carvalho, C. M. B., Faustino, M. A., Neves, M. G. P. M. S., Tomé, J. P. C., Tomé, A. C., Cavaleiro, J. A. S., Cunha, Â., Gomes, N. C. M., Alves, E. & Almeida, A. 2010 Antimicrobial photodynamic therapy: study of bacterial recovery and potential development of resistance after treatment. *Marine Drugs* **8**, 91–105.

Tome, J. P. C., Neves, M. G. P. M. S., Tome, A. C., Cavaleiro, J. A. S., Soncin, M., Magaraggia, M., Ferros, M. & Jori, G. 2004 Synthesis and antimicrobial activity of new poly-S-lysine-porphyrin conjugates. *Journal of Medicinal Chemistry* **47**, 6649–6652.

Wagner, S. J., Skripchenko, A., Robinette, D., Foley, J. W. & Cincotta, L. 1998 Factors affecting photoinactivation by a series of phenothiazine dyes. *Photochemistry and Photobiology* **67**, 343–349.

Wainwright, M., Mohr, H. & Walker, W. H. 2007 Phenothiazinium derivatives for pathogen inactivation in blood products. *Journal of Photochemistry and Photobiology B: Biology* **86**, 45–58.

Wainwright, M., Phoenix, D. A., Laycock, S. L., Wareing, D. R. A. & Wright, P. A. 1998 Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **160**, 177–181.

Wainwright, M., Smalley, H., Scully, O. & Lotfipour, E. 2012 Comparative photodynamic evaluation of new phenothiazinium derivatives against propionibacterium acnes. *Photochemistry and Photobiology* **88**, 523–526.

Wang, H., Lu, L., Zu, S., Li, Y. & Cai, W. 2006 The phototoxicity of xanthene derivatives against *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*. *Current Microbiology* **52**, 1–5.

Wilson, M. 1993 Photolysis of oral bacteria and its potential use treatment of caries and periodontal disease. *Journal of Applied Microbiology* **75**, 299–306.

Wilson, M. & Pratten, J. 1995 Lethal photosensitization of *Staphylococcus aureus* in vitro. Effect of growth phase, serum, and pre-irradiation time. *Lasers in Surgery and Medicine* **16**, 272–276.

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