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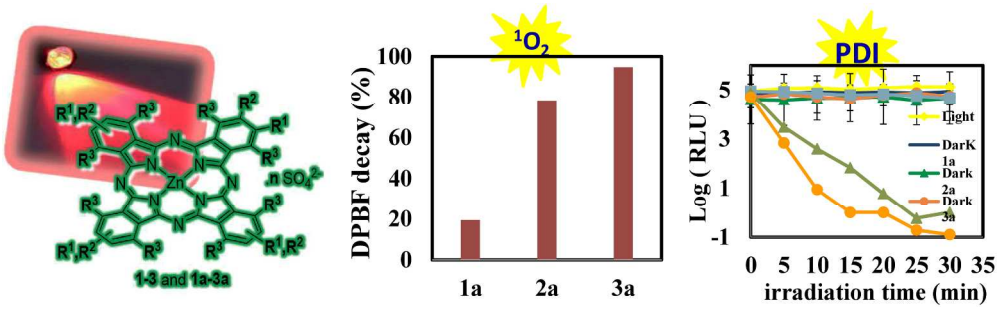

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ARTICLE

Photodynamic inactivation of *Escherichia coli* with cationic ammonium Zn(II)phthalocyanines

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The aim of this work was the development of a family of novel water soluble Zinc(II) phthalocyanines (Pc) for the photodynamic inactivation of Gram-negative bacteria. Pc derivatives **1a**, **2a** and **3a** containing trimethylammonium groups with varied number and nature of the groups at peripheral positions were synthesized by cyclotetramerization of dimethyl amino substituted phthalonitriles in the presence of zinc powder, using 1-chloronaphthalene as solvent, followed by cationization using dimethyl sulfate. The solubility, singlet oxygen generation ($^1O_2$) and stability/photostability of each Pc were evaluated as well as the affinity to bacterial cells and their photosensitizing potential against recombinant bioluminescent *Escherichia coli* strain, used as biological model of Gram negative bacteria. The efficiency of photodynamic inactivation was assessed under white and red light at an irradiance of 150 mW cm^{-2}. All Pc were soluble in phosphate buffer saline and in dimethyl sulfoxide and demonstrated good stability/photostability. The photochemical parameters reveal that Pc **2a** and **3a** are more efficient singlet oxygen producers than Pc **1a**, for which singlet oxygen generation could not be demonstrated. Pc **2a** and **3a** caused photosensitization in *E. coli*. The inactivation factors attained with red light were, however, generally higher than with white light. Under red light Pc **3a** and **2a** caused, respectively, 5.6 and 4.9 log reduction in the bioluminescence of the *E. coli* while, with white light the corresponding inactivation factors were 2.5 and 0.5 log. The order of the PDI efficiency (**3a**> **2a** >>> **1a**) was determined by the combined effect of solubility, singlet oxygen generation ability and affinity to bacterial cells. Ammonium phthalocyanines with eight charges or containing halogen atoms such as chlorine, when irradiated with red light can, therefore, be regarded as promising photosensitizers for the inactivation of Gram-negative bacteria.

1. Introduction

Resistance of common pathogenic microorganisms to different classes of antibiotics is a global concern issue. Significant research efforts have been directed to control the spreading of resistance among common pathogens. Tight prescription policies and the search for alternative antimicrobial approaches are currently considered imperative for the sustainability of antimicrobial chemotherapy. Photodynamic inactivation (PDI) of microorganisms has been recurrently pointed as a promising perspective [1,2] which is reinforced by the demonstration that multi-resistant bacteria and fungi can be as efficiently inactivated as their non-resistant counterparts.[3,4] PDI involves three non-toxic elements: a photosensitizer (PS), light of appropriate wavelength and molecular oxygen (O_2). When these elements are combined, the production of reactive oxygen species, namely singlet oxygen ($^1O_2$), is started and will ultimately lead to cell death. The efficiency of the process depends on the parameters related to these elements, such as the PS structure, concentration, singlet oxygen generation yield, solubility and affinity to cellular material, availability of molecular oxygen and wavelength, type, dose and irradiance of the light source.[1,5,6] PDI has proved to be efficient against viruses,[7]

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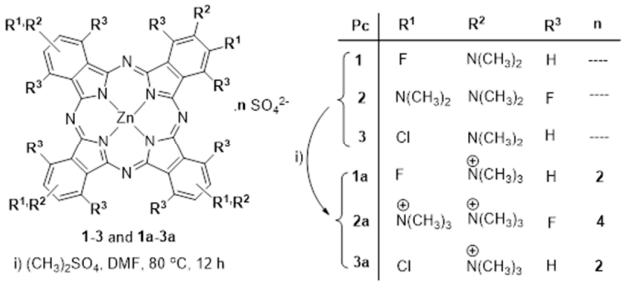
bacteria,[3,7] fungi[8,9] and parasites[10], although with different efficiency and, therefore, requiring suitable PS and PDI protocols.[11] The structure and composition of the cell coating is a major determinant of the susceptibility to PDI. In fact, bacterial endospores are less susceptible to PDI than vegetative cells[12] and Gram-positive bacteria, presenting a less structured cell wall are more easily photosensitized by neutral or anionic PS.[13] Gram-negative bacteria, by having an outer membrane, are less prone to photodynamic damage.[14] PDI of Gram-negative bacteria requires either cationic PS, or the combined use of membrane disruptors, such as polylysines, nona-peptide polymyxin B or ethylenediamine tetra-acetic acid (EDTA).[15,16] Phthalocyanines (Pc) have been gaining interest for PDI application and recognized as efficient PS for inactivation of microbial pathogens, namely Gram-positive, Gram-negative bacteria, fungus and virus.[13,17–19] The advantage of Pc for PDI applications are related with some interesting features of this class of molecules: they produce singlet oxygen in high yields and strongly absorb light in the red region, better matching the wavelength range of maximum penetration in living tissues.[20] Solubility is, however, an important limitation of Pc, because they tend to aggregate in aqueous media, which significantly decreases singlet oxygen production and ultimately reduces PDI efficiency. To overcome the solubility problem, cationic Pc which are more water-soluble, have been synthesized. Cationic Pcs are also more likely to target Gram-negative bacteria because of the electrostatic interactions between the positive charges of the Pc and the negatively charged outer membrane of the bacteria.[12,17] Cationic Pc have been successfully used against Gram-positive and Gram-negative bacteria, fungi and parasites.[16,18,21–23] Previous experiments conducted with the bioluminescent strain of *Escherichia coli* demonstrated that an inactivation factor corresponding to a 5 log reduction in light emission could be attained by photosensitization with a tetrathiopyridinium Pc.[17] Other studies have reported interesting PDI efficiencies with two tetra-cationic Pc (*N,N,N*-trimethylaminoethoxy and *N*-trimethylpyridoxy derivatives) against the Gram-positive *Streptococus mitis*, and the yeast *Candida albicans*, with reductions of ~4 log in the concentration of viable cells upon irradiation with white light.[21,24]

The objective of this wok was to evaluate the efficiency of a series of novel ammonium derived ZnPc, differing in the nature and number of the substituent groups, aiming the development of new PS with the photophysical and photochemical attributes required for the efficient photodynamic inactivation of Gram negative bacteria.

2. Experimental section

All the reagents were purchased from Sigma-Aldrich and used as received. NMR spectra (^{1}H and ^{19}F) were recorded on Bruker Avance 300 spectrometer at 300.13 and 282.38 MHz NMR, using tetramethylsilane (TMS) as internal reference.



Scheme 1 Synthetic route for the preparation of water soluble ZnPc **1a**, **2a** and **3a**.

The chemical shifts are reported in δ (ppm) and the coupling constants (J) in hertz (Hz). The mass of the compounds was confirmed using MALDI-Micromass Q-TOF2 equipment. UV–Vis spectra were recorded on a Shimadzu UV-2501PC spectrophotometer. Steady-state fluorescence emission analysis was carried out on a Jobin-Yvon FluoroMax 3 spectrofluorometer. Elemental analyses for C, H, N and S were performed with a LECO 630-200-200 Truspec CHNS Micro analyser.

2.1. Synthesis

Dimethyl amine substituted phthalonitriles and corresponding Zn(II)phthalocyanines (Pc **1, 2** and **3**) were synthesized in 1-chloronaphthalene at 200 °C, as previously reported.[25] Methylated Zn(II)phthalocyanines **1a**, **2a** and **3a** were obtained by direct methylation of the corresponding Pcs **1**, **2** and **3**, using dimethyl sulfate as methylation source in dimethyl formamide (DMF) at 80 °C (Scheme 1).

2.2. Methylation of Pcs 1, 2 and 3

For the preparation of Pc **1a**, an excess of dimethyl sulphate (1 mL, 100 mmol) was added to a suspension of Pc **1** (100 mg, 0.12 mmol) in dry DMF (2 mL). The reaction mixture was maintained under magnetic stirring for 12 h at 80 °C in a closed flask. After this period, the mixture was cooled and the product was washed with diethyl ether. The obtained viscous oil was dissolved in acetone/methanol and precipitated with and excess of acetone. The solid was dissolved in acetone/water (1:1) and was re-precipitated. The methylated product **1a** was dried under vacuum yields a green solid powder (72 mg, 58%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.19-8.81 (m, 8H, Pc-α-H), 4.33 (s, 36H, -N(CH$_3$)$_3$,). ^{19}F NMR (282.38 MHz, DMSO-d_6): δ -146.03 (d, J = 8.5 Hz, β-F). UV-vis (DMSO) λ_{max} nm (log ε): 333 (4.47), 680 (4.42) MALDI-TOF MS: m/z calculated for $C_{44}H_{44}F_4N_{12}Zn$, 880.3; found; 881.3 $[M+H]^+$. Elemental analysis ($C_{44}H_{44}F_4N_{12}O_8S_2Zn$): Calcd (%): C, 49.19; H, 4.13; F, 7.07; N, 15.64; O, 11.91; S, 5.97; Zn, 6.09. Found (%): C, 49.17; H, 5.15; N, 15.86, S, 6.02.

Compound **2a** was obtained from Pc **2** (100 mg, 0.10 mmol) and dimethyl sulfate (1 mL, 100 mmol) in DMF at 80 °C as described for **1a**. Yield: 88 mg, 57%. ^{1}H NMR (300 MHz, DMSO-d_6): 4.47 (s,72H, -N(CH$_3$)$_3$). ^{19}F NMR (282.38 MHz, DMSO-d_6): δ

-132.49 (d, *J* = 11.3 Hz, α-F). UV-vis (DMSO) λ_{max} nm (log ε): 383 (4.55), 698 (4.79) MALDI-TOF MS: m/z calculated for $C_{56}H_{72}F_8N_{16}Zn$, 1184.5 found; 1185.5 $[M+H]^+$. Elemental analysis ($C_{52}H_{60}F_8N_{16}O_{16}S_4Zn$): Calcd (%):C, 41.34; H, 4.00; F, 10.06; N, 14.83; O, 16.94; S, 8.49; Zn, 4.33. Found (%): C, 41.42; H, 5.22; N, 14.87, S, 8.59.

Compound **3a** was obtained from Pc **3** (100 mg, 0.11 mmol) and dimethyl sulfate (1 mL, 100 mmol) in DMF at 80 °C as described for **1a**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.23-8.76 (m, 8H, Pc-α-H),4.36 (s, 36H, -N(CH_3)$_3$). UV-vis (DMSO) λ_{max} nm (log ε):368 (4.67), 684 (5.10) MALDI-TOF MS: m/z calculated for $C_{44}H_{44}Cl_4N_{12}Zn$, 944.18; found; 945.14 $[M+H]^+$. Elemental analysis ($C_{44}H_{44}Cl_4N_{12}O_8S_2Zn$): Calcd (%):C, 46.35; H, 3.89; Cl, 12.44; N, 14.74; O, 11.23; S, 5.62; Zn, 5.73. Found (%): C, 46.44; H, 4.98; N, 14.86, S, 5.66.

2.3 Preparation of photosensitizer stock solutions

The stock solutions used in all the photophysical and biological experiments were prepared in dimethyl sulfoxide (DMSO) at a final concentration of 500 μM.

2.4 Solubility

The solubility of the cationic Pcs **1a**, **2a** and **3a** in PBS and in DMSO was assessed by UV-Visible spectroscopy. Analysis was conducted in a range of concentrations between 2.0 and 20 μM achieved by the sequential addition aliquots of stock solutions of each Pc. For the verification of Beer-Lambert law, an analysis of linear regression between the intensity of Q-band and the concentration of the Pc was conducted.

2.5. Singlet oxygen generation ability

The ability of the Pc to generate $^1O_2$ was assessed by the qualitative method of the 1,3-diphenylisobenzofuran (DPBF), in which DPBF is degraded by $^1O_2$. In fact, the irradiation of the PS in the presence of dissolved oxygen will results in the formation of $^1O_2$, which is trapped by DPBF resulting into colorless *o*-dibenzoylbenzene, after the Diels-Alder like reaction with $^1O_2$.[26] A mixture of 50 μM of DPBF and 0.5 μM of each Pc in DMF/water (9:1) was irradiated at ambient temperature during 180 seconds, with white light filtered through a cut-off filter for wavelengths <540 nm in order to prevent the DPBF photodegradation, at an irradiance of 9.0 mW cm^{-2}. The absorption of DPBF at *ca.* 414 nm was measured at different irradiation time intervals during irradiation.

2.6. Stability and photostability

Photostability of Pc **1a**, **2a** and **3a** was evaluated by irradiating 3 mL of a diluted solution of each Pc in PBS (Abs ~1) at ambient temperature, under the same condition as used in the PDI assay (150 mW cm^{-2} during 30 min). The UV-Vis spectra were collected at different time intervals. The photostability factor was calculated as I_t/I_0 (I_t is the intensity of the band at a given irradiation time, I_0 is the intensity of the band before irradiation) and expressed as percentage. Stability was evaluated in a similar way in dark conditions, to account for the effect of aggregation as a source of light-independent decay.

2.7. Bacterial strain

For the photodynamic inactivation assays, a recombinant bioluminescent strain of *Escherichia coli* was used.[27] A fresh culture was prepared in Tryptic Soy Broth (TSB), amended with the antibiotics ampicillin (100 mg mL^{-1}) and chloramphenicol (25.0 mg mL^{-1}), and allowed to growth during 20 h at 25 °C with gentle stirring (120 rpm).

2.8. Photodynamic inactivation of *E. coli*

Fresh cultures were 10-fold diluted in phosphate saline buffer (PBS, pH=7.4), to a final concentration of 10^7 colony forming units per milliliter (CFU mL^{-1}). The bacterial suspension was allowed to stabilize at ambient temperature under gentle stirring for 10 min. The suspension was distributed in 12-well plates and appropriate quantities of 500 μM stock solutions of the Pc derivatives were added to achieve a final concentration of 20 μM, in a total volume of 4 mL per well. The suspensions were protected from light with aluminium foil and incubated for 15 min under gentle stirring, at ambient temperature, to allow for the adsorption of the PS to cellular material.

For the irradiation of the samples with red light (620-750 nm) or white light (400-800 nm), an illumination system (LC-122-LumaCare, London) containing a halogen/quartz 250 W lamp coupled to different interchangeable optic fiber probes (620-750 nm and 400-800 nm) was used. The light was delivered at an irradiance of 150 mW cm^{-2}, measured with a Power Meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor. Samples were irradiated during 30 min, under gentle stirring, at ambient temperature. Light and dark controls were included in all experiments. Light controls were irradiated as the samples but without added Pc and the dark control, containing 20 μM of the tested Pc, were protected from light with aluminum foil throughout the experiments.

The inactivation kinetics was followed by periodically measuring the bioluminescence of the cell suspension during the irradiation time. Triplicate aliquots of treatments and controls were collected at times 0, 5, 10, 15, 20, 25 and 30 min bioluminescence, expressed in Relative Light Unis (RLU) was read in a TD-20/20 Luminometer (Turner Designs, Inc., USA) with a wavelength detection peak at 420 nm. Three independent assays were conducted with each compound.

To confirm the correlation between light emission expressed in relative light units (RLU) and the concentration of colony forming units (CFU), a culture of the recombinant bioluminescent *E. coli* strain was grown overnight and serially diluted (10^{-1} to 10^{-7}) in PBS. The luminescence was measured in each dilution and a parallel aliquot was serially diluted and pour-plated in triplicate in Tryptic Soy Agar (TSA). Cultures

were incubated at 37 °C for 24 h. Colonies counted in the most suitable dilutions and the concentration of viable cells was calculated as the average number of colonies in the most suitable dilution corrected for the dilution factor (CFU mL^{-1}).

2.9. Uptake

A bacterial suspension (10^7 CFU mL^{-1}) was incubated for 15 min in the dark, with gentle magnetic stirring, at ambient temperature, in the presence of the Pc derivatives to allow the binding of PS molecules to the cells. After incubation, 1 mL of suspension was centrifuged during 15 min at 13,000 rpm (Thermo Scientific-Heraeus Pico 17 Centrifuge). In order to remove the unbounded PS, the supernatant was rejected and the pellet was washed twice with 1 mL of PBS. Cells were finally re-suspended in 1 mL of a digesting solution containing 2% of dodecyl sodium sulfate (SDS) and 0.1 g L^{-1} of NaOH. The mixture was kept for at least 24 h in the dark, at ambient temperature, until the clearance of the solution. The concentration of PS in the digested cells was determined by fluorescence analysis. Samples were excited at *ca.* 610 nm and the emission spectra were recorded in 620-850 nm range. The fluorescence intensity of the samples allowed the determination of PS concentration bounded to the cells by interpolation with a calibration plot, build with solutions of known PS concentration using the digestion solution as solvent. The uptake values were determined as the ratio between the number of PS molecules, calculated from the concentration by using the Avogadro number, and the number of viable cells expressed as CFU.[28]

3. Results

3.1. Synthesis

Dimethylamine substituted phthalonitriles were obtained in good yields by nucleophilic substitution of fluoro and chloro phthalonitrile derivatives by dimethylamine obtained by decomposition of DMF at 160 °C, in the presence of catalytic amounts of triethylphosphite. Zn(II) phthalocyanines **1**, **2** and **3** were prepared via cyclotetramerization reaction of respective phthalonitriles and metallic zinc in α-chloronaphthalene at 200 °C. The quaternized derivatives of Zn(II) phthalocyanines **1a**, **2a** and **3a** were obtained by using dimethyl sulfate as methylating source in DMF at 80 °C for 12 h. The structures of the final products were confirmed by NMR (Figs. S1-S5, ESI†) and MALDI-TOF spectrometry techniques (Figs. S6-S8, ESI†).

3.2. Absorption

The absorption spectra of Pc **1a**, **2a** and **3a** in PBS and in DMSO are shown in Figure 1. In PBS, the soret bands (B-band) appear at *ca.* 342 nm, 375 nm and 345 nm respectively. In DMSO, the corresponding bands are observed at *ca.* 341 nm, 392 nm and 380 nm. The Q-bands were found to be broad in both solvents with maxima peaking at *ca.* 695 nm, 697 nm and 695 nm (in PBS) and *ca.* 691 nm, 700 nm and 686 nm (in DMSO) for Pc **1a**, **2a** and **3a**, respectively.

3.3. Solubility

The solubility of the three cationic Pc was tested in DMSO and in PBS. Different concentrations of each Pc, ranging from 2 to 20 µM, were prepared and the UV-Visible spectra were collected. Results show modifications in the absorbance of Q-band of each phthalocyanine at different concentrations. The intensity of Q-bands of the three Pc increased with increasing of concentrations. However, Q-bands were found to be much intense in DMSO than in PBS. There was a significant linear correlation between the intensity of the Q-bands and the concentration, in either DMSO or in PBS suggesting compliance with the Beer-Lambert law is both solvents (Figs. S9-S10, ESI†).

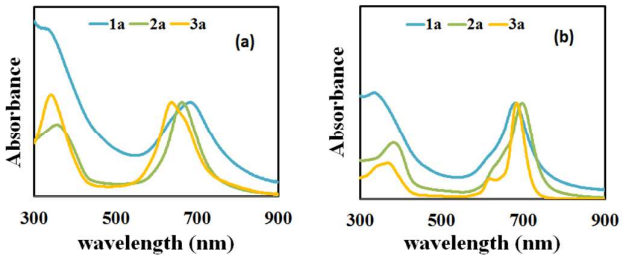


Fig. 1 Normalized UV-visible spectra of phthalocyanines **1a**, **2a** and **3a** in PBS (a) and DMSO (b).

3.4. Singlet oxygen generation ability

The results of the photo-oxidation of DPBF upon irradiation in the presence of all Pc derivatives (neutral 1, 2 and 3 and 1a, 2a and 3a), used as an indicator of singlet oxygen generation, are represented in Figure 2. The results obtained showed that neutral Pc 1, 2 and 3 exhibit, in general, higher singlet oxygen generation ability than the cationic Pc 1a, 2a and 3a although all of them with lower ability than ZnPc, a well-known singlet oxygen generator (data not shown). However, due to the low solubility of the neutral Pc in aqueous media and inefficiency against Gram-negative bacteria, they were not used in *E. coli* PDI experiments.[13] Pc 1a failed to cause any significant decay in DPBF absorbance when compared with the control (DPBF without PS).

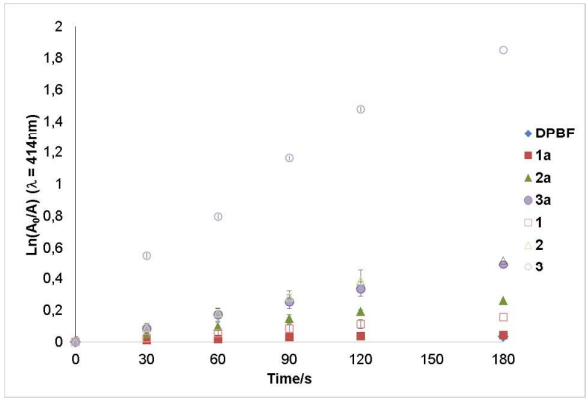


Fig. 2 Time-dependent decomposition of DPBF (50 µM) photosensitized by the neutral Pc **1**-**3** and the cationic Pc **1a**-**3a** in DMF/H_2O (9:1) upon irradiation with white light filtered through a cut-off filter for λ < 540 nm, at an irradiance of 9.0 mW cm^{-2} with (tests) or without (control) PS (0.5 µM). Values correspond to the average of three independent experiments

3.5. Stability and photostability

Table 1 summarizes the results of the assays of stability and photostability conducted under red light with Pcs **1a**, **2a** and **3a**. The photostability studies show that significant reductions in the absorbance of Pc **3a** did not occur and that the decrease in the absorbance of Pc **1a** and **2a** upon irradiation with red light was very small (6%). The later compounds were also stable in the absence of light, maintaining 97% and 90% of the initial absorbance, respectively.

Table 1 Photostability and stability of the cationic Pc **1a**, **2a** and **3a** in PBS, upon irradiation for 20 min with red light (620-750 nm) at an irradiance of 150 mW cm^{-2}. The values represent the percentage decay of intensity of the Q band and correspond to the average of three independent assays.

Compound	Photostability (%)	Stability (%)
1a	94	97
2a	94	90
3a	100	-

3.6. Photodynamic inactivation of *E. coli*

The results of the photodynamic inactivation assays conducted with the recombinant bioluminescent *E. coli* strain (Figure 3) show that three cationic Pc have distinct performance in terms of inactivation efficiency (P<0.05 ANOVA). In the assays with white light, significant inactivation occurred only with Pc **3a**, which caused a reduction of 2.3 log in light emission. Under these conditions, Pc 1a and 2a failed to significantly inactivate *E. coli*. With red light, the inactivation was more efficient than with white light. Within a period of 30 min, Pc **3a** caused the inhibition of light emission to the detection limit (~5.6 log reduction) and **2a** caused a 4.9 log reduction. The reductions, however, are not statistically different between these two Pc (P<0.05 ANOVA). Pc **1a** did not show any significant inhibition of *E. coli* bioluminescence, similarly to what was observed in the experiments with white light. According to the inactivation efficiency, the new Pc can be ordered as **1a**<<<**2a**<**3a**. Results of the controls show that the bacterial strain was not directly inactivated by light in absence of the PS, and that none of the Pc showed dark toxicity in the tested concentration (20 µM).

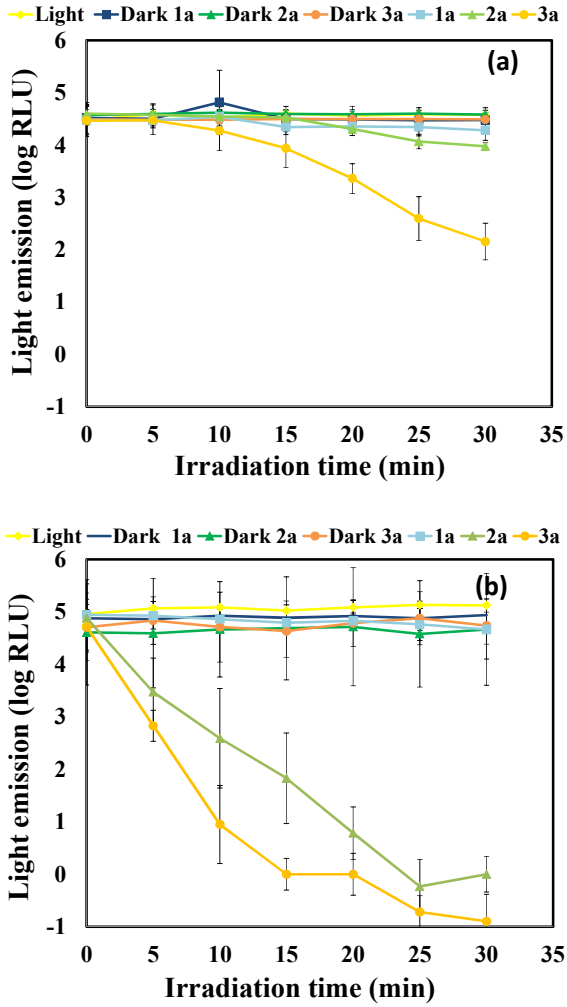


Fig. 3 Reduction of light emission by a recombinant bioluminescent strain of *E. coli* in presence of 20 µM of each phthalocyanine, upon irradiation with white (a) (400-800 nm) or (b) red (620-750 nm) light (150 mW cm^{-2}). Values correspond to the average of three independent experiments. Error bars represent the standard deviation.

3.7. Relation between bioluminescence and concentration of viable cells

The relation between the bioluminescence (Relative Light Units) and the concentration of viable cells (CFU mL^{-1}) of the recombinant *E. coli* strain is represented in Figure 4. The results confirm the

significant correlation (R^2 = 0.985) between light emission and the concentration of viable cells.

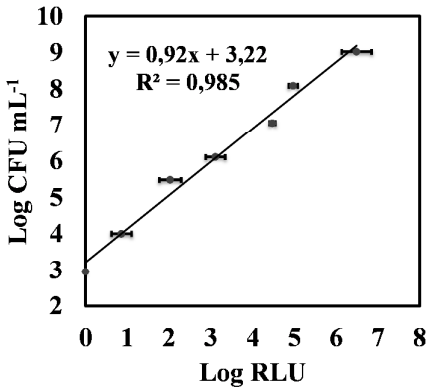


Fig. 4 Correlation between bioluminescence and colony counts of an overnight culture of recombinant bioluminescent *E. coli*. Bioluminescence is expressed in Relative Light Units (RLU) and colony counts are expressed in Colony Forming Units per millilitre (CFU mL^{-1}). Data points represent two independent assays and error bars represent the standard deviation.

3.8. Uptake

The results of the quantification of the Pc adsorbed to the cells of the bioluminescent *E. coli* indicate different affinity of the tested Pc towards cellular material (Figure 5) (P<0.05 ANOVA). Pc **2a** showed the highest uptake value (7.62 x 10^6 PS molecules CFU^{-1}), followed by Pc **3a** (1.90 x 10^6 PS molecules CFU^{-1}) and finally by Pc **1a**, which showed the lowest uptake value (8.78 x10^4 PS molecules CFU^{-1}).

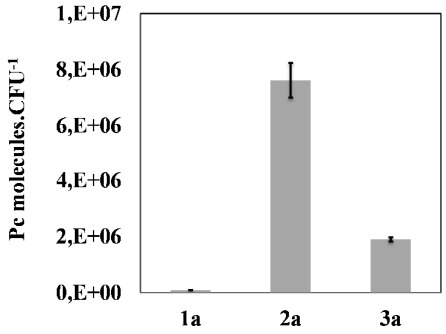


Fig. 5 Adsorption of phthalocyanines **1a**, **2a** and **3a** (20 µM) to the cells of the bioluminescent *E. coli* strain after 15 min of incubation in the dark and two washing steps. Bars represent the average of three replicates and error bars represent the standard deviation.

4. Discussion

The possibility to create new Pc, by the introduction of substituents in the macrocycle core, allows the improvement of photophysical and photochemical properties of the molecules leading to enhanced PDI efficiency. In this work, we reported the development of new cationic Pc with amphiphilic ammonium groups at different peripheral positions of the macrocycle, considering that this type of cationic Pc tends to be soluble in aqueous media and to exhibit better affinity towards Gram-negative bacteria, which is an additional advantage in PDI.

None of the tested Pc showed dark cytotoxicity against the recombinant bioluminescent *E. coli* strain. Pc **3a** and **2a** presented good PDI efficiencies when irradiated with red light but with white light, a moderate photosensitization could only be achieved with Pc **3a**. The amount of energy required to excite the PS is an important factor in the process of the photosensitization of bacteria.[5,29,30] The most appropriate light sources are those displaying emission peaks close to the absorption maxima of the PS.[31] All Pc tested in this work have Q-bands within the red region of the visible spectrum (Fig. S11, ESI†). Considering the emission maxima of the red (*ca*. 673 nm) and white (*ca*. 573 nm) light sources, when red light was used, the energy provided by the illuminated system could be very effectively absorbed whereas in the case of white light, some of the energy was dissipated, thus explaining the lower inhibition activity. However, taking into account that the Q-band of Pc **2a** corresponds to a higher wavelength than that of Pc **3a**, that is, further in the red region and closer to the peak of light emission, it would be expected that under red light, Pc **2a** would exhibit better performance than Pc **3a**. Experimental data, however, show that this was not the case. The inactivation outcome may be more related to the fact that, comparing with Pc **2a**, Pc **3a** needs a smaller amount of photons to generate the same amount of $^1O_2$ as inferred from the $^1O_2$ assays. In a similar fashion, if the absorption peak of the molecules were the major factor determining the photosensitization efficiency, Pc **1a** would have produced the best results under red light because the Q-band appears at higher wavelengths. However, this PS was ineffective because it showed a very poor ability to generate singlet oxygen. Singlet oxygen is the main ROS involved in type-II PDI mechanism, which is responsible by the irreversible damage caused to cells by oxidative stress.[32] In this work, we have observed that the PDI efficiency of cationic Pc followed the same trend as $^1O_2$ generation ability, **3a**>**2a**>>>**1a**. The ability to produce $^1O_2$ can be, in turn, associated to different PS properties, namely PS structure, stability and solubility.

The aggregation and stability studies indicate that all Pc follow the Beer-Lambert law, either in DMSO or PBS. However, Pc **1a** and **3a** presented a broader Q-band in PBS than in DMSO suggesting that in PBS they occur as non-monomer structures.[33] This phenomenon may be due to the presence of the fluorine and chlorine atoms in the macrocycles, which leads to the formation of planar aggregates that tend to stack in solution. This effect is less likely to have occurred in Pc **2a**, due to the extra four ammonium groups that increase the electrostatic repulsion between molecules and keep them more spaced.[34] The formation of non-monomeric structures of Pc **1a** explains the low $^1O_2$ generation capacity, because aggregates tend to dissipate the energy of the excited states.[34] In contrast, non-monomeric structures of Pc **3a** do not seem to affect the capacity to produce $^1O_2$. It is known that the presence of heavy atoms, such as halogens can increase the rates of triplet-state

formation in the PS, which favors the dissipation of energy by intersystem crossing and, consequently, increases $^1O_2$ generation.[34] It is likely that, for Pc **3a** the effect caused by chlorine atoms in the $^1O_2$ generation compensates the effect caused by non-monomeric structures.

Other studies have demonstrated that the uptake of PS by microorganisms can significantly determine the outcome of the photosensitization process. The effect of PS uptake was identified as a critical factor in the efficiency of photodynamic inactivation of bacteria and fungi, namely the Gram positive *Staphylococcus aureus*, the Gram-negative *Pseudomonas aeruginosa*, and conidia of filamentous fungus *Penicillium chrysogenum*.[8,35] In this study, however, PS uptake was less important that other factors in determining the photosensitization efficiency of the tested Pc. Pc **2a** displayed the highest uptake value but was not the most efficient PS. This may indicate that above the critical uptake threshold, which was overwhelmed by Pc **2a** and **3a**, singlet oxygen generation and other photophysical features such as solubility may be more important. The overall analysis of the results indicate that for the new Pc tested, the major factor influencing the potential for photosensitization of the model Gram-negative strain was the production of $^1O_2$. Even producing less $^1O_2$ than the previously reported $ZnPcH_{12}(SPyMe)_4$,[17] Pc **3a** caused higher inactivation of the bioluminescent *E. coli*, almost to the detection limit.

5. Conclusions

In conclusion, work we have demonstrate that PDI efficiency is strongly dependent on the combined effect of $^1O_2$ generation ability, spectrum of irradiating light and nature of the substituents introduced in the PS structures, which impact the stability and solubility of the Pc and the affinity to bacterial external structures. The results confirm that ammonium Pc **2a** and **3a**, with the convenient modifications, may become promising PS for the inactivation of Gram-negative bacteria. Tuning the number and nature of amphiphilic groups on the Pc molecule may improve photophysical properties for clinical applications.

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