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Synthesis, properties, and photodynamic inactivation of *Escherichia coli* using a cationic and a noncharged Zn(II) pyridyloxyphthalocyanine derivatives

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Abstract—The photodynamic effect of a cationic Zn(II) *N*-methylpyridyloxyphthalocyanine (ZnPc **2**) and a noncharged Zn(II) pyridyloxyphthalocyanine (ZnPc **1**) has been compared in both homogeneous media bearing photooxidizable substrates and in vitro using a typical Gram-negative bacterium *Escherichia coli*. Absorption and fluorescence spectroscopic studies were analyzed in different media. Fluorescence quantum yields (ϕ_F) of 0.23 for ZnPc **1** and 0.22 for ZnPc **2** were calculated in *N*,*N*-dimethylformamide (DMF). The singlet molecular oxygen, $O_2(^1\Delta_g)$, production was evaluated using 9,10-dimethylanthracene (DMA) in DMF yielding values of $\Phi_\Delta = 0.56$ for ZnPc **1** and 0.59 for ZnPc **2**. A faster decomposition of L-tryptophan (Trp), which was used as biological substrate model, was obtained using ZnPc **2** as a sensitizer with respect to ZnPc **1**. In biological medium, the *E. coli* cultures were treated with 10 μM of sensitizer for different times at 37 °C in the dark. Both ZnPcs **1** and **2** are rapidly bound to *E. coli* cells in 5 min and the amount of cell-bound sensitizer is not appreciably changed incubating the cultures for longer times. The recovered ZnPc **2** after one washing step is ~3 times higher than **1**, reaching a value of ~3 nmol/10^6 cells. After irradiation with visible light, a higher photoinactivation of cells was found for ZnPc **2**. Thus, a ~4.5 log (99.997%) decrease of cell survival was obtained after 30 min of irradiation. On the other hand, a very low photodamage was found for cells treated with ZnPc **1** (~0.5 log). Also, these results were established by stopping of growth curves for *E. coli*. In the structure of ZnPc **2**, the cationic centers are isolated from the phthalocyanine ring by an ether bridge, which also provides a higher mobility of the charges facilitating the interaction with the outer membrane of the Gram-negative bacteria. These studies show that cationic ZnPc **2** is an efficient phototherapeutic agent with potential applications in photodynamic inactivation of bacteria.

1. Introduction

Photodynamic therapy (PDT) is an innovative treatment for several types of cancer. This therapy is based on the administration of a photosensitizer, which is selectively incorporated in tumor cells. The subsequent exposure to visible light in the presence of oxygen specifically inactivates neoplastic cells.[1,2] Two photoreaction processes are principally implicated in the photodamage of cells, one involves the generation of free radicals (type I) and the other produces singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II), as the main species responsible for cell inactivation.[3,4] The incidence of these mechanisms depends mainly on the sensitizer, substrate, and the nature of the medium.[5]

Recently, a nononcological application of PDT has been established for photoinactivation of bacteria in an attempt to overcome the problem of bacterial strains resistant to current antibiotics.[6,7] Thus, pathogenic microorganisms growing in vivo as localized foci of infection, on skin or on accessible mucous membrane, would be candidates for photodynamic destruction. Previous investigation showed that phthalocyanine derivatives can photosensitize the inactivation of various microbial pathogens.[8–11] Gram-positive bacteria are efficiently photoinactivated by a variety of sensitizers, whereas Gram-negative bacteria are usually resistant to the action of negatively charged or neutral photosensitizers.[10,12] However, the presence of positively

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charged functional groups in the photosensitizer structure allows an extensive photoinduced inactivation of Gram-positive and also of Gram-negative bacterial cells. The positive charge on the photosensitizer molecule appears to promote a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cells. This association increases the efficiency of the photoinactivation processes.[8,10]

In previous studies, we have investigated the photodynamic activity of porphyrin derivatives in different biomimetic media and in vitro on the Hep-2 human larynx carcinoma cell line.[13–15] In particular, the presence of a cationic charge in the sensitizer structure produces an increase in the uptake of this amphiphilic porphyrin into Hep-2 cells with respect to a nonionic porphyrin model.[16] In this case, a higher cellular incorporation is also accompanied by a higher photocytotoxic activity on Hep-2 cells. Recently, *meso*-substituted cationic porphyrin derivatives with asymmetric charge distribution were synthesized as efficient sensitizers to inactivate *Escherichia coli* cells.[17]

In this paper, the photodynamic activity of a cationic Zn(II) *N*-methylpyridyloxy phthalocyanine (ZnPc **2**) was compared with that of a noncharged Zn(II) pyridyloxyphthalocyanine (ZnPc **1**), in homogeneous medium bearing a photooxidizable substrate and in vitro using a typical Gram-negative bacterium *E. coli*. Phthalocyanines derivatives exhibit a high absorption coefficient in the visible region of the spectrum, mainly in the phototherapeutic window (600–800 nm) and a long lifetime of triplet excited state to produce efficiently $O_2(^1\Delta_g)$.[3] The use of phthalocyanines for sterilization of infection contaminants in red blood cells has been of major interest because of the intense absorption of red light where hemoglobin absorption is minimal.[7,18] The behaviors of ZnPcs **1** and **2** in solution were compared with that of Zn(II) phthalocyanine (ZnPc). These neutral and cationic phthalocyanines derivatives have appropriate photophysical properties to be used as photosensitizer in PDT.[3] In biological medium, ZnPc **1** was used as a noncationic model. Thus, the main purpose of this study was to evaluate the photoinactivation efficiencies of these photosensitizers in a Gram-negative representative bacterium *E. coli*.

2. Results and discussion

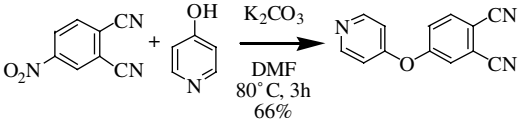
2.1. Synthesis

ZnPc **1** was synthesized by a two-step procedure.[19,20] First, 4-(4-pyridyloxy)phthalonitrile was prepared by a nucleophilic *ipso*-nitro substitution reaction of 4-nitrophthalonitrile with 4-hydroxypyridine in the presence of K_2CO_3 (Scheme 1). Dinitrile derivative was isolated by flash chromatography with 66% yield. The cyclotetramerization of dinitrile with zinc(II) acetate in the presence of organic base 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) was performed in *n*-pentanol (Scheme 2). After reflux for 18 h, the reaction results in the formation of the corresponding ZnPc **1** as mixtures of constitutional isomers with 68% yield.

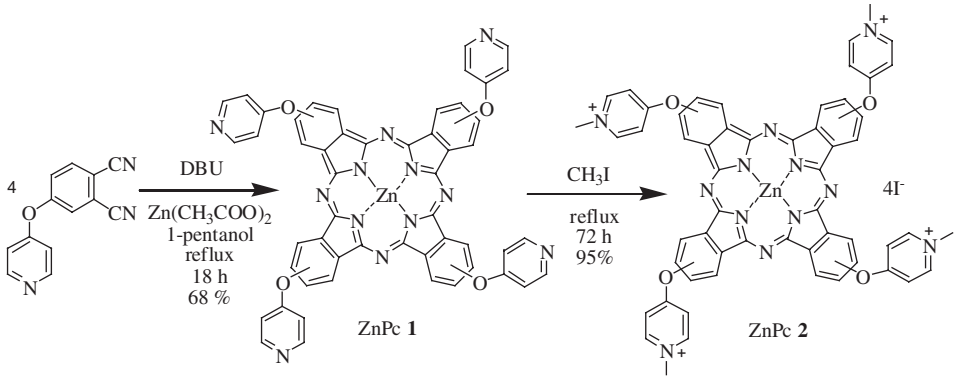
Cationic sensitizer **2** was obtained treating the ZnPc **1** with methyl iodide for 48 h at reflux in acetone (Scheme 2). The exhaustive methylation produces ZnPc **2** with 95% yield. In the structure of ZnPc **2**, the cationic centers are isolated from the phthalocyanine macrocycle ring by ether bonds. This spacer provides a higher mobility of the charges, which could facilitate the interaction with the outer membrane of the Gram-negative bacteria.

2.2. Spectroscopic studies and phthalocyanine properties

The absorption spectra of ZnPcs **1** and **2** in *N*,*N*-dimethylformamide (DMF) are gathered in Figure 1. The spectra show the typical Soret and Q-bands, characteristic of zinc(II) phthalocyanines (ZnPc).[3,21] The values of molar coefficient (ε) in DMF are reported in Table 1. The Q-band presents a bathochromic shift by ~10 nm when compared with that of ZnPc (670 nm). In addition, the wavelength absorption maxima of these ZnPcs were



Scheme 1. Synthesis of 4-(4-pyridyloxy)phthalonitrile.



Scheme 2. Synthesis of ZnPcs **1** and **2**.

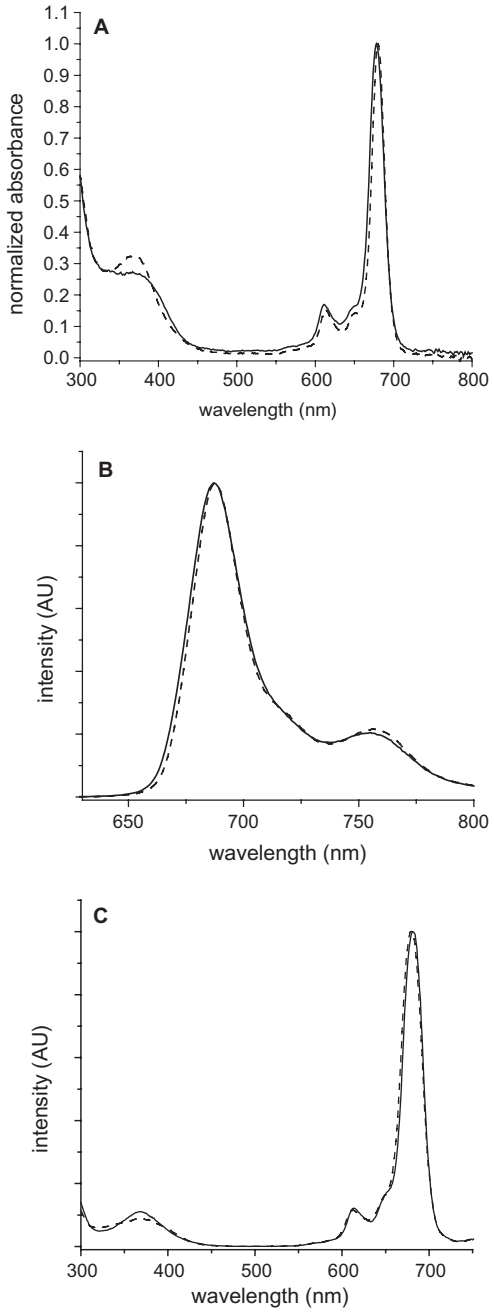


Figure 1. (A) Absorption spectra; (B) fluorescence emission spectra, λ_{exc} = 612 nm; and (C) fluorescence excitation spectra, λ_{em} = 760 nm, of ZnPc **1** (- - -) and ZnPc **2** (—) in DMF.

Table 1. Spectroscopic data in DMF and partition coefficients (*P*) of ZnPcs **1** and **2**

Phthalocyanine	ε (M^{-1} cm^{-1})	ϕ_F[c]	E_s (eV)	*P*
ZnPc **1**	1.7×10^5[a]	0.23 ± 0.01	1.81	1.6 ± 0.5
ZnPc **2**	1.1×10^5[b]	0.22 ± 0.01	1.81	0.88 ± 0.1

[a] 680 nm.
[b] 678 nm.
[c] ϕ_F(ZnPc) = 0.28.

studied in different media (Table 2). A sharp absorption band was obtained in organic solvents indicating that

Table 2. Absorption and fluorescence emission data for ZnPcs **1** and **2** in different solvents

Medium	Absorption λ_{max} (nm)			Emission λ_{max} (nm)[a]	
ZnPc 1					
DMF	369	613	680	688	758
Methanol	353	607	671	677	746
PBS	347[c]	613[d]	—	—	—
2% w/v SDS[b]	367	611	676	683	755
ZnPc 2					
DMF	374	612	678	687	756
Methanol	351	605	669	678	744
PBS	340	629[d]	—	—	—
2% w/v SDS[b]	368	610	675	683	753

[a] λ_{exc} = 608 nm.
[b] 2% w/v in water.
[c] Shoulder.
[d] Broadening.

there is not aggregation of these ZnPcs in the systems. A solvatochromic effect in pure solvents is observed on the location of the absorption band showing a blue-shift (~10 nm) upon solubilization in methanol. On the other hand, these ZnPcs are aggregated in PBS, as it is typical for many phthalocyanine derivatives.[3,22] The cationic ZnPc **2** shows a broadening band at 629 nm. However, they are solubilized as monomers in 2% w/v SDS, which was used as a medium to evaluate the amount of phthalocyanine bound to cells.

The steady-state fluorescence emission spectra of ZnPcs **1** and **2** were performed in DMF (Fig. 1B). The spectra show two bands in the red spectral region, which are characteristic for similar Zn(II) phthalocyanines.[21] By comparison with ZnPc as a reference, the values of fluorescence quantum yields (ϕ_F) were obtained in DMF. Values of ϕ_F are shown in Table 2. Also, emission spectra were obtained in different media (Table 2). As expected, no emission was observed in PBS where these phthalocyanines are mainly aggregated. A small Stokes shift (~7 nm) was observed, indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. Taking into account the energy of the 0–0 electronic transitions, the energy levels of the singlet excited states (E_s) were calculated (Table 1). These results are in agreement with those previously reported for similar phthalocyanines in different media.[22]

The corrected fluorescence excitation spectra of the ZnPcs **1** and **2** were measured in DMF (Fig. 1C), monitoring the emission at 760 nm. In both cases, the spectra resemble the absorption spectra (Fig. 1A), indicating that these sensitizers are essentially unaggregated in this medium.

The fluorescence spectrum of ZnPc **2** was also analyzed in biological medium. The *E. coli* cells were treated with 10 μM of sensitizer **2** for 30 min at 37 °C in the dark. After one washing step, a cell suspension in PBS showed a shape close to that of the fluorescence emission in homogeneous media (Fig. 1B) with maxima at 686 and 755 nm. This result indicates that the binding

of *s* ZnPc **2** to *E. coli* cells leads to photosensitizer disaggregation.[16]

On the other hand, the *n*-octanol/water partition coefficients ($P = [\text{phthalocyanine}]_o/[\text{phthalocyanine}]_w$) of these phthalocyanines were evaluated at 25 °C.[3,15,23] The values of *P* obtained are reported in Table 1. As expected, the hydrophilic character increases in cationic ZnPc **2** with respect to **1**.[19]

2.3. Photosensitized decomposition of substrates

2.3.1. Photooxidation of 9,10-dimethylanthracene (DMA). The aerobic irradiation of photosensitizers **1** and **2** in DMF was performed in the presence of DMA. This substrate quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction.[14] Typical semilogarithmic plots, describing the progress of the reaction for DMA are shown in Figure 2A. From these plots the values of the observed rate constant (k_{obs}^{DMA}) were obtained (Table 3). The quantum yields of $O_2(^1\Delta_g)$ production (Φ_Δ) were calculated comparing the slope for the phthalocyanines with the corresponding slope obtained for the reference, ZnPc ($\Phi = 0.56$).[24,25] As can be observed in Table 3, a similar efficiency in the $O_2(^1\Delta_g)$ production was found for ZnPc **1** and ZnPc in DMF, while ZnPc **2** presents a slightly higher Φ_Δ value. These are quite reasonable values for Zn(II) phthalocyanines in this solvent.[21,24] However, the values of Φ_Δ can significantly change in a different medium, diminishing when the sensitizer is partially aggregated.

2.3.2. Decomposition of tryptopha (Trp). The amino acid Trp represents a suitable substrate for testing the efficiency of photosensitizing agents, since it is efficiently photooxidized by both type I and type II reaction mechanisms.[26] The photoprocess follows first-order kinetics with respect to Trp concentration, as shown in Figure 2B for [Trp] = 25 μM. From the plots in Figure 2B, the values of the k_{obs}^{Trp} were calculated for ZnPcs **1** and **2**. As can be observed in Table 2, the photooxidation rate is about five times higher for cationic ZnPc **2** than that for **1** and ZnPc. Thus, these results are not in agreement with those obtained for the DMA photooxidation, indicating that the other photoreaction process, probably an electron transfer process, is also involved in the decomposition of Trp sensitized by ZnPc **2**. In addition, the photosensitized oxidation of Trp was found to be more than two orders of magnitude more efficient via electron transfer than via $O_2(^1\Delta_g)$.[27] Also, electron transfer quenching of the sensitizer singlet excited state by Trp has been postulated.[28] Since cationic sensitizers can bind to these substrates, probably by electrostatic attraction, an electron transfer pathway may also be contributing together with Type II photoprocess to Trp decomposition in DMF.[5,16]

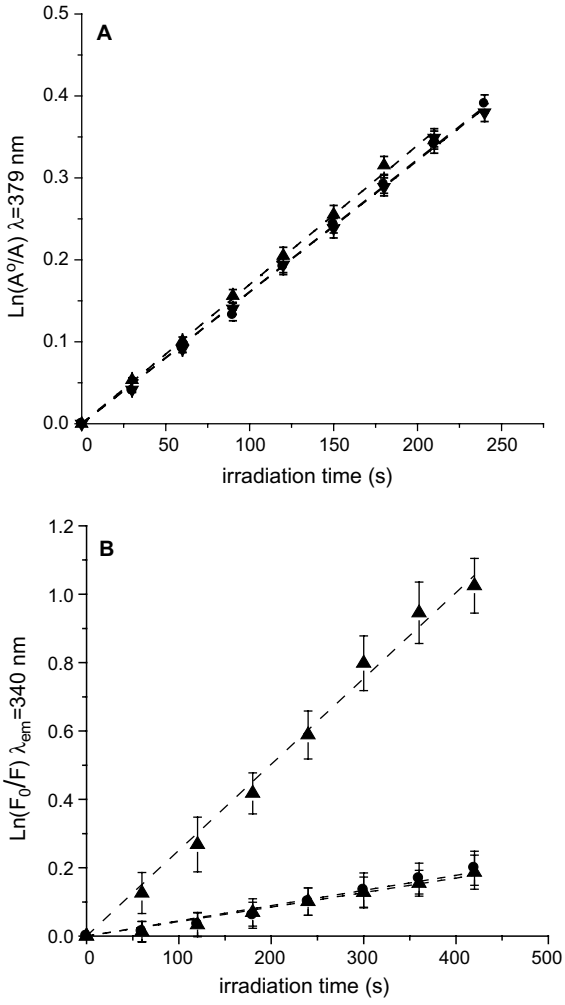


Figure 2. First-order plots for the photooxidation of (A) DMA (35 μM) and (B) Try (25 μM) photosensitized by ZnPc **1** (▼), ZnPc **2** (▲), and ZnPc (●) in DMF; λ_{irr} = 590–800 nm. Values represent means ± standard deviation of three separate experiments.

2.4. Photobleaching of sensitizers

Photobleaching of ZnPcs was carried out under the same irradiation conditions used for photoinactivation of *E. coli* cultures (see Experimental). The decompositions were analyzed following the decrease in absorption of the phthalocyanine Q-band. Figure 3A shows the

Table 3. Kinetic parameters of ZnPcs **1** and **2** in DMF

Parameter	ZnPc **1**	ZnPc **2**	ZnPc
k_{obs}^{DMA} (s^{-1})	$(1.6 \pm 0.1) \times 10^{-2}$	$(1.7 \pm 0.1) \times 10^{-2}$	$(1.6 \pm 0.1) \times 10^{-2}$
Φ_Δ[a]	0.56	0.59	0.56
k_{obs}^{Try} (s^{-1})	$(4.5 \pm 0.1) \times 10^{-4}$	$(2.5 \pm 0.1) \times 10^{-3}$	$(4.2 \pm 0.1) \times 10^{-4}$
k_{obs}^{P} (s^{-1})	$(2.9 \pm 0.2) \times 10^{-4}$	$(3.6 \pm 0.2) \times 10^{-4}$	$(2.6 \pm 0.2) \times 10^{-4}$
Φ_P	$(2.2 \pm 0.1) \times 10^{-5}$	$(5.6 \pm 0.3) \times 10^{-5}$	$(1.6 \pm 0.1) \times 10^{-5}$

[a] Ref. 25 Φ_Δ(ZnPc) = 0.56.

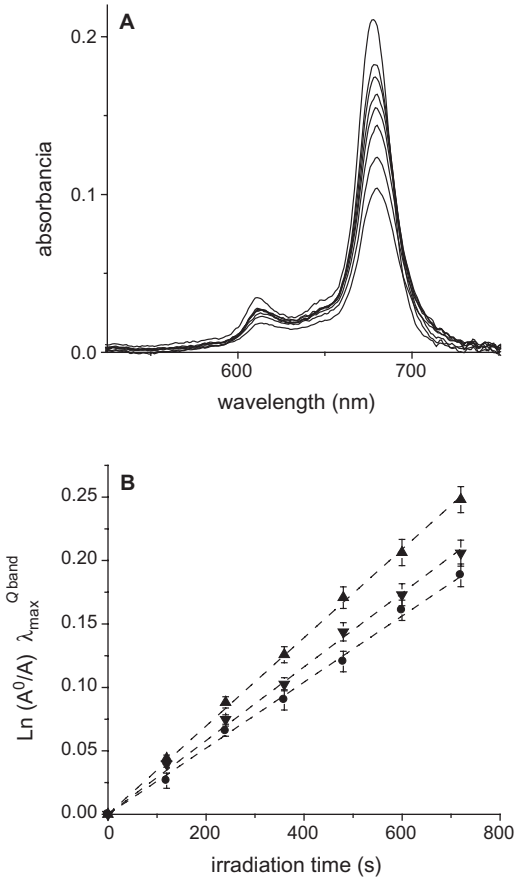


Figure 3. (A) Absorption spectra changes of ZnPc **2** irradiated with visible light in DMF ($\Delta t = 10$ min). (B) First-order plots for the photobleaching of ZnPc **1** (▼), ZnPc **2** (▲), and ZnPc (●) in DMF irradiated with visible light. Values represent means ± standard deviation of three separate experiments.

absorption spectra changes observed on photolysis of ZnPc **2** in DMF. As can be observed, the diminishments in the Q-bands are not accompanied by the formation of a new band in this region. The photobleaching reactions follow a first-order kinetic (Fig. 3B). The values of k_{obs}^{P} and the initial quantum yield of photobleaching (Φ_P) are shown in Table 3. The results of Φ_P for ZnPcs **1** and **2** are similar to those obtained for other structurally comparable Zn(II) phthalocyanine derivatives.[3,21] Under these conditions, ZnPc **2** presents a higher efficiency of photobleaching in DMF. However, the mechanism of phthalocyanine photodegradation can be complex and differs in solution and in biological media.[5]

2.5. Studies in vitro on *E. coli* cells

2.5.1. Binding of phthalocyanines to cells. The ability of cationic ZnPcs **2** to bind to bacterial cells was compared with that obtained for a noncationic ZnPc **1**. The *E. coli* cultures were incubated with phthalocyanine for different times at 37 °C in the dark. In each case, the sensitizer associated with cells was determined by fluorescence analysis (see Experimental). The results after one washing step are summarized in Figure 4A. Under these conditions, these phthalocyanines are rapidly bound to *E. coli* cells in 5 min and the binding is not appreciably changed incubating the cultures for 30 min. However, the amount of cationic ZnPc **2** recovered after one washing step is considerably higher than noncationic ZnPc **1** and it reaches a value of ~3 nmol/10^6 cells. Thus, the presence of cationic *N*-methylpyridyl groups, on the periphery of phthalocyanine macrocycle, considerably increases the binding affinity of sensitizer **2** for *E. coli* cells.

2.5.2. Photosensitized inactivation of *E. coli*. After the treatment of *E. coli* cells with 10 μM of sensitizers **1** and **2** for 30 min at 37 °C in the dark, the cultures were irradiated with visible light after one washing step. The survival curves at different light exposure levels are shown in Figure 4B. As can be observed, the photoinactivation activities of cationic ZnPc **2** are considerably

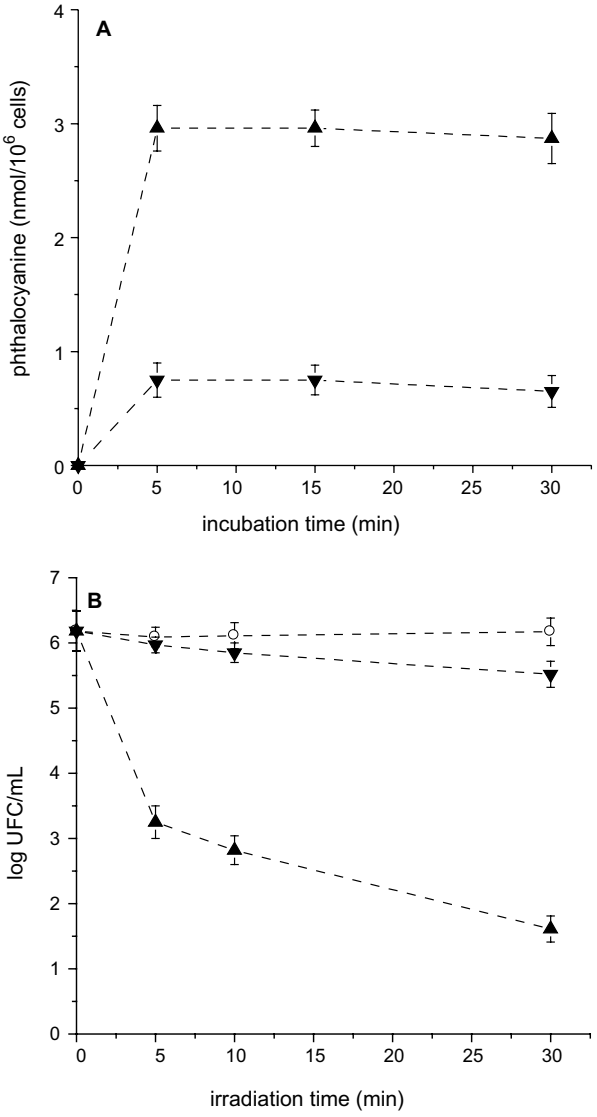


Figure 4. (A) Amount of ZnPcs **1** (▼) and **2** (▼) recovered after one washing step from *E. coli* cells treated with 10 μM of sensitizer for different incubation times, at 37 °C in the dark. (B) Survival curves of *E. coli* incubated with 10 μM of ZnPcs **1** (▼) and **2** (▲) for 30 min at 37 °C in the dark, washing once before illumination and exposed to visible light for different irradiation times. Values represent means ± standard deviation of three separate experiments.

higher than that found for ZnPc **1**. Thus, the photodynamic activity of ZnPc **2** produces a ~4.5 log decrease of *E. coli* cell survival, when the cultures are irradiated for 30 min. This diminishing represents ~99.997% of cell inactivation. While, under these conditions a very low diminishing in the cellular viability was found using ZnPc **1** as a sensitizer. On the other hand, control experiments showed that the viability of *E. coli* was unaffected by illumination alone or by dark incubation with 10 μM of the photosensitizer for 30 min, indicating that the cell mortality obtained after irradiation of the cultures is produced by the photosensitization effect of phthalocyanines.

Also, growth delay of *E. coli* cultures was performed in the medium (10% w/v TS broth) to ensure that photosensitization was still possible when the cells were not under starvation conditions or the potential damaging effects of phosphate buffer washing.[8] As can be observed in Figure 5, under these conditions, growth was arrested when *E. coli* was exposed to 10 μM of ZnPc **2**. After irradiation in the presence of sensitizer **2**, the cells no longer appeared to be growing as measured by turbidity at 550 nm. As observed above, the effect of ZnPc **1** is slower than the sensitizer **2**. On the other hand, *E. coli* cells exposed to 10 μM of sensitizers in the dark or not treated with sensitizer and illuminated showed no growth delay compared with controls. Therefore, the data illustrate that the observed growth delay is due to the photoinactivation effect of the sensitizer **2** on the cells.[8,10]

In conclusion, the spectroscopic and photodynamic properties of both sensitizers **1** and **2** in homogeneous medium are quite similar except for Trp, which presents a higher photodecomposition rate when the cationic phthalocyanine is used as a sensitizer. However, the studies in vitro show that the efficiency of *E. coli* inactivation is appreciably higher for cationic ZnPc **2** than noncharged ZnPc **1**. An analogous behavior was previously found using a cationic zinc pyridinium phthalocyanine derivative in comparison with a neutral tetra-diethanolamine phthalocyanine and a negatively charged tetrasulfonated phthalocyanine.[8] The insensitivity of Gram-negative bacteria to photoinactivation was attributed to the presence of the outer membrane, which endows the surface of Gram-negative bacteria with a negative charge.[10,29,30] In these cases, the binding of the sensitizer to *E. coli* cells appears to be necessary to exert its photocytotoxic effect. Consequently, $O_2(^1\Delta_g)$ is produced in the membrane microenvironment, where the agent is localized. Thus, the photodynamic activity is mainly associated with ZnPc **2**, which are tighter bound to cells.[8,10] On the other hand, when the sensitizer remains in the PBS, such as neutral or anionic agents, the photodynamic effect is almost not observed. Therefore, noncationic phthalocyanines, such as ZnPc **1**, are unsuccessful sensitizers for Gram-negative bacteria, unless that the outer membrane permeability is altered.[8,12,10] The results indicate that the failure of ZnPc **1** to show any photosensitizing activity on the bacteria is most probably due to its failure to bind to them. These data confirm that cationic ZnPc **2** is an efficient photosensitizer with potential applications in bacteria inactivation by photodynamic treatment.

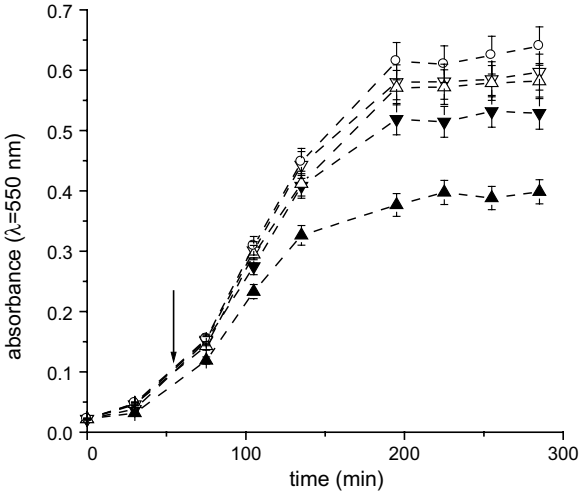


Figure 5. Growth delay curves of *E. coli* incubated with 10 μM of ZnPc **1** (▼) and ZnPc **2** (▲) and exposed to different irradiation times with visible light in 10% w/v TS broth at 37 °C. The arrow marks the time point where the appropriate concentration of phthalocyanine was added. Control culture untreated irradiated (○), treated with 10 μM of ZnPc **1** (▽) and ZnPc **2** (△) and not irradiated. Values represent means ± standard deviation of three separate experiments.

3. Experimental

3.1. General

UV–visible and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker ARX 300 multinuclear spectrometer at 300 MHz. Mass Spectra were taken with a Varian Matt 312 operating in EI mode at 70 eV and with a ZAB-SEQ Micromass equipment. All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Sodium dodecyl sulfate (SDS) from Merck (Darmstadt, Germany) and zinc phthalocyanine (ZnPc) from Aldrich were used as received. Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labonco equipment model 90901-01. The cultures were exposed for different time intervals to visible light. The light source used was a Novamat 130 AF slide projector equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. Wavelength ranges between 350–800 and 590–800 nm were selected by optical filters (OG590 cut-off filter). The light intensity at the treatment site was 30 mW/cm^2 (λ = 670 nm) (Radiometer Laser Mate-Q, Coherent).

3.2. Photosensitizers

According to the synthetic procedure, phthalocyanine macrocycle is obtained as a mixture of the corresponding regioisomers, which only one is named below.

3.2.1. 4-(4-Pyridyloxy)phthalonitrile. A solution of 4-nitrophthalonitrile (500 mg, 2.89 mmol) and 4-hydroxypyridine (302 mg, 3.18 mmol) in 15 mL of DMF was stirred for 10 min under argon atmosphere. Then, dry potassium carbonate (2 g, 14.5 mmol) was added and the mixture was heated at 80 °C for 3 h. The mixture was treated with water (50 mL) and extracted with dichloromethane/methanol (5%) (two portions of 50 mL each). The solvent was removed under reduced pressure. The product was purified by flash chromatography (silica gel, dichloromethane/methanol 10%) and yielded 422 mg (66%) of the pure 4-(4-pyridyloxy)phthalonitrile. TLC (silica gel) R_f (dichloromethane/methanol 10%) = 0.45. Melting point 126 °C. ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 6.15 (d, 2H, J = 7.8 Hz), 8.00 (d, 2H, J = 7.8 Hz), 8.01 (dd, 1H, J = 2.2 Hz, 8.6 Hz), 8.18 (d, 1H, J = 8.6 Hz), 8.34 (d, 1H, J = 2.2). MS [m/z] 221 (M^+) (221.0589 calculated for $C_{13}H_7N_3O$). Anal. Calcd C 70.58, H 3.19, N 19.00; found C 70.49, H 3.22, N 19.05.

3.2.2. Zinc(II) 2,9,16,23-tetrakis(4-pyridyloxy)phthalocyanine 1 (ZnPc 1). A solution of 4-(4-pyridyloxy)phthalonitrile (400 mg, 1.81 mmol) and zinc(II) acetate dihydrate (110 mg, 0.50 mmol) in 15 mL of *n*-pentanol was stirred for 10 min under argon atmosphere. Then, 300 μL of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 2.01 mmol) was added and the mixture was refluxed for 18 h. The reaction mixture was cooled to room temperature and precipitated with 50 mL of cyclohexane. The solid was filtered and washed with water and hexanes. The solid was recrystallized from methanol/water to yield 297 mg (68%) of ZnPc **1**. Melting point >300 °C. ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 6.2–6.8 (m, 8H), 8.0–8.3 (m, 8H), 8.1–9.4 (br, 12H). MS [m/z] 948 (M^+) (948.1648 calculated for $C_{52}H_{28}N_{12}O_4Zn$). Anal. Calcd C 65.73, H 2.97, N 17.69; found C 65.64, H 3.02, N 17.60.

3.2.3. Zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]-phthalocyanine iodide (ZnPc 2). A mixture of ZnPc **1** (240 mg, 0.25 mmol) and 20 mL of methyl iodide in 10 mL of acetone was stirred for 48 h at reflux. The solvents were removed under vacuum. The solid was re-suspended in cyclohexane and filtered. The solid was washed with hexanes, diethylether, and acetone, to yield 360 mg (95%) of ZnPc **2**. Melting point >300 °C. ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 4.2–4.3 (m, 12H, $-CH_3$), 8.0–9.5 (m, 28H). MS [m/z] 1516 (M^+) (1515.8766 calculated for $C_{56}H_{40}I_4N_{12}O_4Zn$). Anal. Calcd C 44.31, H 2.66, N 11.07; found C 44.38, H 2.75, N 11.15.

3.3. Spectroscopic studies

Absorption spectra were recorded at 25.0 ± 0.5 °C using 1 cm path length cells. The fluorescence quantum yields (ϕ_F) of phthalocyanines were calculated by comparison of the area below the corrected emission spectrum with that of ZnPc as a fluorescence standard, exciting at λ_{ex} = 608 nm.[31] A value of ϕ_F = 0.28 for ZnPc in DMF was calculated by comparison with the fluorescence spectrum in pyridine using ϕ_F = 0.30 and taking into account the refractive index of the solvents.[32]

3.4. Partition coefficient measurements

1-Octanol/water partition coefficients (P) were determined at 25 °C using equal volumes of water (2 mL) and 1-octanol (2 mL). Typically, a solution of each phthalocyanine (~10 μM) was stirred in the thermostat after the equilibrium was reached (~8 h). An aliquot (100 μL) of aqueous and organic phases was dissolved in 2 mL of DMF and the final phthalocyanine concentration determined by absorption spectroscopy.[15,23]

3.5. Steady state photolysis

Solutions of either 9,10-dimethylanthracene (35 μM in DMF, 2 mL) or L-tryptophan (Trp, 25 μM, 2 mL) and photosensitizer (λ = 680 nm, absorbance 0.1) in DMF were irradiated in quartz cuvettes with 590–800 nm light. The kinetics of photooxidation were studied by following the decrease of the absorbance (A) at λ_{max} = 378 nm for DMA and the fluorescence intensity (F) at λ = 340 nm for Trp. The Trp fluorescence was excited by 290 nm light. A control experiment showed that under these experimental conditions the fluorescence intensity is linearly correlated with Trp concentration. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of $\mathrm{Ln}\, A_0/A$ versus time or $\mathrm{Ln}\, F_0/F$ versus time. Photooxidation of DMA was used to determine singlet molecular oxygen, $O_2(^1\Delta_g)$, production by the photosensitizers.[14,24] ZnPc was used as the standard in DMF (Φ_Δ = 0.56).[24,25] Measurements of the sample and reference under the same conditions afforded Φ_Δ for ZnPc **1** and **2** by direct comparison of the slopes in the linear region of the plots. Sensitizer photobleaching was evaluated irradiating a phthalocyanine solution in DMF (λ = 680 nm, absorbance 0.2) with visible light in the same conditions described below for the treatment of *E. coli* cultures. Quantum yields of photobleaching (Φ_P) were calculated as [initial rate of disappearance of phthalocyanine]/[initial rate of absorption of photons by the reaction mixture].[33–35] All the experiments were performed at 25.0 ± 0.5 °C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%.

3.6. Bacterial strain and preparation of cultures

E. coli strain (EC7) recovered from clinical urogenital material was used. The strain was resistant to ampicillin and sulfamethoxazol–trimethoprim. *E. coli* strain was grown aerobically at 37 °C in 30% w/v tryptic soy (TS) broth overnight. Aliquots (~40 μL) of the culture were aseptically transferred to 4 mL of fresh medium (30% w/v TS broth) and incubated at 37 °C to mid logarithmic phase (absorbance ~0.6 at 660 nm). Cells in the logarithmic phase of growth were harvested by centrifugation of broth cultures (3000*g* for 15 min), washed once with 10 mM of PBS and re-suspended in 4 mL of PBS. Then, the cells were diluted 1/1000 in PBS, corresponding to ~10^6 colony forming units (CFU)/mL. In all the experiments, 2 mL of the cell suspensions in Pyrex brand

culture tubes (13 × 100 mm) was used and the sensitizer was added from a stock solution of phthalocyanine (3.0×10^{-3} M) in DMF. Viable bacteria were monitored and their number calculated by counting the number of colony forming units after appropriated dilution on agar plates.[8] Bacterial cultures grown under the same conditions and light exposures, but without addition of any photosensitizer, served as controls.

3.7. Phthalocyanine binding to bacteria cells

Suspensions of *E. coli* (2 mL, $\sim 10^6$ CFU/mL) in PBS were incubated in the dark at 37 °C with 10 μM of sensitizer for different times. The cultures were centrifuged (3000*g* for 15 min) and then washed with PBS. The cell pellets obtained by centrifugation (3000*g* for 15 min) were re-suspended in 2% aqueous SDS (2 mL), incubated overnight at 4 °C and sonicated for 30 min. The concentration of sensitizer in the supernatant was measured by spectrofluorimetry (λ_{exc} = 670 nm, λ_{em} = 684 nm) in solution of 2% SDS in PBS. The fluorescence values obtained from each sample were referred to the total number of bacteria contained in the suspension. The concentration of the phthalocyanine in this sample was estimated by comparison with a calibration curve obtained with standard solutions of the sensitizer in 2% w/v SDS ([sensitizer]∼0.05–0.5 μM).

3.8. Photosensitized inactivation of bacteria cells

Cell suspensions of *E. coli* (2 mL, $\sim 10^6$ CFU/mL) in PBS were incubated with 10 μM of phthalocyanine for 30 min in the dark at 37 °C. After that, the cultures were washed once with PBS and re-suspended in 2 mL of PBS. The cultures were exposed for different time intervals to visible light (350–800 nm). Control experiments were carried out without illumination in the absence and in the presence of sensitizer. Control and irradiated cell suspensions were serially diluted with PBS, each solution was plated in triplicate on TS agar and the number of colonies formed after 18–24 h incubation at 37 °C was counted. Each experiment was repeated separately three times.

3.9. Growth delay experiment

E. coli cells were grown overnight as described above. A portion (60 μL) of this culture was transferred to 20 mL of fresh TS broth (10%) medium. The suspension was homogenized and aliquots of 2 mL were incubated at 37 °C. The culture grown was measured by turbidity at 550 nm using a Tuner SP-830 spectrophotometer.[8] When the cultures reached the log phase (absorbance ∼0.1), 10 μM of sensitizer was added. Then, the flasks were irradiated with visible light as described above. Samples were also taken to determine the viability of the cells as described earlier.

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