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Porphyrins containing basic aliphatic amino groups as potential broad-spectrum antimicrobial agents

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ABSTRACT

New porphyrin derivatives bearing basic aliphatic amino groups were synthesized from the condensation of *meso*-4-[(3-*N*,*N*-dimethylaminopropoxy)phenyl]dipyrromethane, pentafluorobenzaldehyde and 4-(3-*N*,*N*-dimethylaminopropoxy)benzaldehyde. The reaction was catalyzed by trifluoroacetic acid in acetonitrile. This approach was used to obtain porphyrins with different patterns of substitution, of which three of them were isolated: 5,15-di(4-pentafluorophenyl)-10,20-di[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (F_{10}APP), 5-(4-pentafluorophenyl)-10,15,20-tris[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (F_5APP) and 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP). The UV–vis spectroscopic characterizations and the photodynamic effect of these compounds were compared in *N*,*N*-dimethylformamide. These porphyrins showed red fluorescence emission with quantum yields of 0.09-0.15. Moreover, they sensitized the production of singlet molecular oxygen, reaching quantum yields values of 0.33-0.53. Photodynamic inactivation was studied in two bacteria, *Staphylococcus aureus* and *Escherichia coli*, and a yeast *Candida albicans*. High amount of cell-bound porphyrin was obtained at short times (< 2 min) of incubation. After 15 min irradiation, a 7 log reduction of *S. aureus* was found for cells treated with 1 μM F_5APP. Similar photokilling was obtained in *E. coli*, but using 7.5 μM F_5APP and 30 min irradiation. Under these conditions, a decrease of 5 log was observed in *C. albicans* cells. An increase in cell survival was observed by addition of sodium azide, whereas a slight protective effect was found in the presence of D-mannitol. Moreover, the photoinactivation mediated by these porphyrins was higher in D_2O than in water. Thus, these porphyrins induced the photodynamic activity mainly through the intermediacy of $O_2(^1\Delta_g)$. In particular, F_5APP was a highly effective photosensitizer with application as a broad-spectrum antimicrobial. This porphyrin contains three basic aliphatic amino groups that may be protonated at physiological pH. In addition, it is substituted by a lipophilic pentafluorophenyl group, which confers an amphiphilic character to the tetrapyrrolic macrocycle. This effect can increase the interaction with the cell envelopment, improving the photocytotoxic activity against the microorganisms.

1. Introduction

Microbial resistance to antibiotics has increased considerably over the past few years [1]. As a result, infections caused by resistant bacteria are difficult to treat and even in many cases can not be healed with conventional medications [2]. Currently, there are few new antibiotics and effective alternative therapies for the treatment of resistant infections. In this sense, the opportunistic pathogen *Staphylococcus aureus* is the main cause of a wide variety of serious clinical diseases. In addition, *Escherichia coli* strains, which in an initial phase were susceptible to conventional antibiotics, started to acquire resistance to antimicrobial treatments. Although fungal infections are not as common as diseases caused by bacteria, they can produce higher mortality [3]. In particular, *Candida albicans* is the most frequent species associated with diseases and resistance detected to antifungal drugs in hospitals. Therefore, the search for new strategies that promote the elimination of resistant microbial cells is of vital importance [1]. For this purpose, photodynamic inactivation (PDI) of microorganisms has been planned as an alternative to kill microbial cells [4]. This approach is based in the addition of a photosensitizer that is rapidly bound to cells. Subsequent irradiation of the affected area with an appropriate wavelength generates reactive oxygen species (ROS), which react with a diversity of biological substrates. These reactions induce a decrease in biological functions that lead to the inactivation of the cells [5].

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Several compounds have been evaluated to photoinactivate different microorganisms. In this way, porphyrins have suitable properties as effective photosensitizers. Thus, the tetrapyrrolic macrocycle can be substituted to obtain strong candidates for better phototherapeutic agents [6,7]. In general, electrostatic interactions between porphyrins with intrinsic positive charges and the cell envelope of Gram-negative bacteria promote the destabilization of the cell wall. This effect allows the binding of the photosensitizer to the cells, which leads to a greater photoinactivation activity [8]. Also, cationic porphyrins showed a high photoactivity toward Gram-positive bacteria and yeasts [6,7]. However, a few PDI studies have been carried out using porphyrins that contain precursor groups of positive charges distributed in different substitution patterns.

In the present work, porphyrins containing amine groups were synthesized from the condensation of *meso*-4-[(3-*N*,*N*-dimethylaminopropoxy)phenyl]dipyrromethane with a binary mixture of pentafluorobenzaldehyde and 4-(3-*N*,*N*-dimethylaminopropoxy)benzaldehyde catalyzed by trifluoroacetic acid in acetonitrile. It was previously found that intrinsically non-charged porphyrins are interesting photosensitizer in aqueous media [9]. The basic aliphatic amino groups in these porphyrins can be protonated at physiological pH [10]. Moreover, fluorinated porphyrin derivatives represent an efficient platform for the development of new phototherapeutic agents [11]. These porphyrins can be used as ^{19}F magnetic resonance imaging agents in combination with fluorescence spectroscopy for *in vivo* assays. Furthermore, the pentafluorophenyl group can be covalently bond to different molecular structures by *ipso*-F nucleophilic aromatic substitution reactions [12,13]. Therefore, the spectroscopic properties and photodynamic activity of these porphyrins were compared in solution. Also, the capacity of these photosensitizers to inactivate microorganisms was investigated in *S. aureus*, *E. coli* and *C. albicans*. The results allow establishing the best conditions for the eradication of microorganisms mediated by these amino substituted porphyrins.

2. Materials and methods

2.1. General

Proton nuclear magnetic resonance spectra were achieved on a FT-NMR Bruker Avance DPX400 spectrometer (Bruker BioSpin, Rheinstetten, Deutschland). Mass spectra were attained on a Bruker micrO-TOF-QII (Bruker Daltonics, MA, USA) equipped with an ESI source (ESI-MS). Absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan), while fluorescence spectra were carried out on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc, Edison, NJ, USA). Fluence rates were measurement using a Radiometer Laser Mate-Q (Coherent, Santa Clara, CA, USA). The light source for photolysis studies was a Cole-Parmer illuminator 41720-series (Cole-Parmer, Vernon Hills, IL, USA) with a 150 W halogen lamp through a high intensity grating monochromator (Photon Technology Instrument, Birmingham, NJ, USA). A value of 0.45 mW/cm^2 was determined for the light fluence rate at 420 nm. Microorganisms were irradiated with a Novamat 130 A F projector containing a 150 W halogen lamp (Braun Photo Technik, Nürnberg, Germany). A wavelength range from 350 to 800 nm (fluence rate = 90 mW/cm^2) was established by optical filters. Compounds from Sigma-Aldrich (Milwaukee, WI, USA) were used as received. Silica gel thin-layer chromatography (TLC) plates 250 μm were purchased from Analtech (Newark, DE, USA) and silica gel 60 (0.040-0.063 mm, 230–400 mesh) from Merck (Darmstadt, Germany).

2.2. Synthesis of meso-substituted porphyrins

meso-[4-(3-*N*,*N*-Dimethylaminopropoxy)phenyl]dipyrromethane (ADPM) was obtained as previously described [14]. A solution of 4-(3-*N*,*N*-dimethylaminopropoxy)benzaldehyde (373 μL, 1.86 mmol), pentafluorobenzaldehyde (0.460 g, 2.35 mmol) and *meso*-[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]dipyrromethane (1.53 g, 4.73 mmol) in 464.1 mL of acetonitrile was purged with argon for 15 min. Then, trifluoroacetic acid (TFA, 2.60 mL, 33.8 mmol) was slowly added to the reaction mixture. The solution was stirred for 2 h at room temperature. After that, triethylamine (TEA, 14 mL, 9901 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 2.11 g, 9.22 mmol) were added and the solution was stirred for 3 h. The solvent was removed under vacuum and flash column chromatography (silica gel, dichloromethane/methanol 1–15 % gradient/ TEA 3%) afforded 93 mg (8%) of 5,15-di(pentafluorophenyl)-10,20-di[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (F_{10}APP) as first moving purple band, 188 mg (10%) of 5-(pentafluorophenyl)-10,15,20-tris[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (F_5APP) and 57 mg (6%) of 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP). F_{10}APP: TLC analysis (dichloromethane/methanol 10%/TEA 1%) R_f 0.66. ^{1}HNMR ($CDCl_3$, TMS) [ppm]: -2.87 (br, 2H, pyrrole NH), 1.98 (m, 4 H), 2.26 (s, 12H, -NCH_3), 2.45 (t, 4H, J = 7.0 Hz), 4.03 (t, 4H, J = 6.2 Hz), 7.14 (d, 4H, 10,20-Ar 2,6-H, J = 8.0 Hz), 7.92 (d, 4H, 10,20-Ar 3,5-H, J = 8.0 Hz), 8.70–8.85 (m, 8H, pyrrole). ESI-MS [*m/z*] 997.3288 $[M+H]^+$ (996.3210 calculated for $C_{54}H_{42}F_{10}N_6O_2$). F_5APP: TLC analysis (dichloromethane/methanol 15%/TEA 1%) R_f 0.50. ^{1}HNMR ($CDCl_3$, TMS) δ [ppm]: -2.86 (br, 2H, pyrrole NH), 1.99 (m, 6 H), 2.27 (s, 18H, -NCH_3), 2.48 (t, 6H, J = 7.0 Hz), 4.02 (t, 6H, J = 6.2 Hz), 7.12 (d, 6H, 10,20-Ar 2,6-H, J = 8.0 Hz), 7.90 (d, 6H, 10,20-Ar 3,5-H, J = 8.0 Hz), 8.70–8.85 (m, 8H, pyrrole). ESI-MS [*m/z*] 1008.4600 $[M+H]^+$ (1007.4521 calculated for $C_{59}H_{58}F_5N_7O_3$). TAPP: characterization data of this porphyrin agree with those previously reported [14].

2.3. Spectroscopic studies and partition coefficient measurements

A quartz cell (1 cm path length) was used to acquire UV–vis absorption and fluorescence spectra. Samples were excited at 420 nm and the emission spectra were integrated in the range 600–800 nm. The fluorescence quantum yield (Φ_F) of the porphyrins was calculated from the area below the corrected emission spectrum in DMF, using TAPP as a reference (Φ_F = 0.15) in DMF [10]. *n*-Octanol/water partition coefficients (P_{ow} = [porphyrin]$_o$/[porphyrin]$_w$) were determined at 25 °C as previously described [15].

2.4. Steady state photolysis

Solution of 9,10-dimethylanthracene (DMA, 35 μM) and porphyrin (A^{420} = 0.1) were prepared in DMF (2 mL). Samples were irradiated with monochromatic light at λ_{irr} = 420 nm in 1 cm path length quartz cells. The photooxidation rate of DMA were analyzed by determining the decrease in absorbance at λ_{max} = 378 nm. The observed rate constants (k_{obs}^{DMA}) were obtained by a linear least-squares fit of the pseudo-first order kinetic plots of ln A_0/A *vs*. time. Quantum yields of $O_2(^1\Delta_g)$ production (Φ_Δ) were calculated comparing the k_{obs}^{DMA} for the corresponding photosensitizer with that for TAPP, which was used as a reference (Φ_Δ = 0.53) [10].

2.5. Microbial cells strains and growth conditions

S. aureus ATCC 25923, *E. coli* (EC7) and *C. albicans* (PC31) were previously characterized and identified [16,17]. Microorganisms were grown under aerobiosis overnight at 37 °C in 4 mL tryptic soy for bacteria or Sabouraud broth for yeast. Then, 60 μL of the bacterial culture was dissolved in 4 mL of fresh tryptic soy broth. Cells were incubated at 37 °C to reach the exponential phase of growth (absorbance 0.7 at 660 nm). Cells were centrifuged at 3000 rpm for 15 min and the pellet resuspended in equal amount of 10 mM phosphate-buffered saline (PBS, pH = 7.4) solution, which correspond to ~10^8 colony forming units (CFU)/mL. After overnight cultures of *C. albicans*, cells were centrifuged

at 3000 rpm for 15 min and the pellet resuspended in PBS. Yeast cells suspension of A^{650} = 0.5 were diluted 1:4 in PBS to obtain ~10^6 CFU/mL.

2.6. Photosensitizer binding to microbial cells

Microbial cell suspensions (2 mL, ~10^8 CFU/mL bacteria and ~10^6 CFU/mL yeast) in PBS were treated with different photosensitizer concentrations (1.0–5.0 μM for *S. aureus* and 2.5–7.5 μM for *E. coli* and *C. albicans*) and incubation times (2–15 min) in dark at 37 °C in Pyrex culture tubes (13 x 100 mm). Porphyrin was added from a stock solution (~0.5 mM) in DMF. Cells were centrifuged at 3000 rpm for 10 min. Pellets were resuspended in 2 mL of aqueous 2% SDS. Cells were incubated overnight at 4 °C and sonicated for 30 min. The concentration of porphyrin in the supernatant was determined by spectrofluorimetry (λ_{exc} = 420 nm, λ_{em} = 653 nm). The fluorescence intensities of each sample were referred to the total number of cells. The concentration of the porphyrin in the solution was calculated by comparison with a calibration curve obtained with standard solutions (0.05-0.2 μM) of the photosensitizer in 2% SDS.

2.7. Photoinactivation of microorganisms

Cell suspensions were treated with different photosensitizer concentrations for 15 min in dark at 37 °C, as described above. Then, 200 μL of cell suspensions were placed in 96-well microtiter plates (Deltalab, Barcelona, Spain). Cells were irradiated for different periods (2–15 min for *S. aureus* and 5–30 min for *E. coli* and *C. albicans*) with visible light. For photoinactivation assays in D_2O, cells were centrifuged (3000 rpm for 15 min) and re-suspended in 2 mL PBS in D_2O. Then the cell suspensions were incubated with porphyrin as described above. Cell suspensions were incubated with 50 mM sodium azide or D-mannitol for 30 min at 37 °C in dark before the treatment with porphyrin. After each assay, cell suspensions were serially diluted 10-fold in PBS. Viable cells were determined by the spread plate counting method in triplicate. The amount of CFU was counted after incubation at 37 °C in the dark for ~24 h (bacteria) or ~48 h (*C. albicans*).

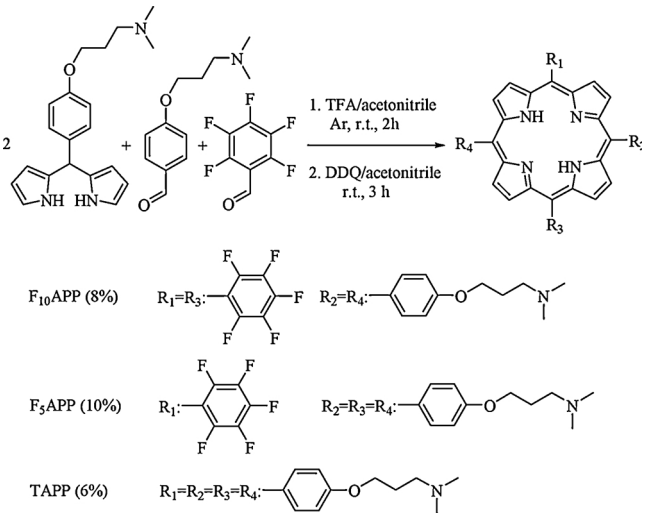
2.8. Control experiments and statistical analysis

Controls were achieved with cultures in the dark, with and without porphyrin, and irradiated cells in the absence of porphyrin. Three values were obtained per each condition and each experiment was repeated separately three times. The unpaired *t*-test was applied to found the significance of differences between groups. Differences between means were tested for significance by one-way ANOVA. Results were established as statistically significant with a confidence level of 95% ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

3. Results

3.1. Synthesis of porphyrins

The synthetic procedures to obtain porphyrins are shown in Scheme 1. Porphyrins with different symmetry patterns at the *meso* positions were obtained from a binary mixture of aldehydes and an appropriate dipyrromethane catalyzed by acid [18]. First, ADPM was synthetized by the condensation of 4-(3-*N*,*N*-dimethylaminopropoxy)benzaldehyde with pyrrole, catalyzed by TFA at room temperature [14]. Acid-catalyzed condensation of ADPM with 4-(3-*N*,*N*-dimethylaminopropoxy) benzaldehyde and pentafluorobenzaldehyde was used to obtain a mixture of three porphyrins (Scheme 1). The reaction was performed using a molar relationship (0.8:1.0:2.0) of 4-(3-*N*,*N*-dimethylaminopropoxy)benzaldehyde, pentafluorobenzaldehyde and ADPM, respectively. The use of dipyrromethanes requires the reaction to proceed



Scheme 1. Synthetic procedure of F_5APP, F_{10}APP and TAPP.

with minimal acidolysis. However, the acidolysis of dipyrromethanes in the reaction conditions appears to be difficult to suppress because the recombination of the resulting dipyrromethane fragments, which can yield undesired porphyrin products [19,20]. Therefore, a lower proportion of 4-(3-*N*,*N*-dimethylaminopropoxy)benzaldehyde than pentafluorobenzaldehyde was used to promote the formation of porphyrin A_3B. This was because some undesired fragment containing 4-(3-*N*,*N*-dimethylaminopropoxy)phenyl group can be formed from the acidolysis of ADPM. This scrambling effect produced a lower yield of the desired porphyrin. Therefore, the condensation was performed using TFA as catalyst in acetonitrile at room temperature, followed by oxidative treatment with DDQ to afford a mixture of porphyrins. The products were purified by flash column chromatography (silica gel) using gradient elution of dichloromethane/methanol, containing a basic medium provided by TEA. Under these conditions, F_{10}APP was obtained in 8% yield, followed by F_5APP and TAPP in 10% and 6% yield, respectively.

On the other hand, partition coefficients for these porphyrins were determined between *n*-octanol/water. Values of log P_{ow} of 0.75, 1.95 and 1.67 were obtained for F_5APP, F_{10}APP and TAPP, respectively. The lipophilic character was slightly larger for F_{10}APP than TAPP because the presence of two pentafluorophenyl groups. The lower partition coefficient found to F_5APP can be due to the formation of aggregated in aqueous medium.

3.2. Absorption and fluorescence spectroscopic properties

The absorption spectra of F_5APP, F_{10}APP and TAPP in DMF showed the characteristic Soret band at ~420 nm and the four Q-bands between 515–650 nm (Figure S1A) [10]. The Q-bands of porphyrins involved the transitions $Q_x(0,0)$, $Q_x(1,0)$, $Q_y(0,0)$ and $Q_y(1,0)$, which were associated with a D_{2h} symmetry [21,22]. Spectroscopic properties are summarized in Table 1. The maximum of the Soret band of F_{10}APP presented a ~3 nm hypsochromic shift whit respect to TAPP, which showed a major auxochromic effect due to the four ether groups. Also, the spectra exhibited sharp absorption bands, indicating that these porphyrins were not aggregated in DMF.

The fluorescence emission spectra of the porphyrins in DMF presented two bands typical of porphyrin derivatives, which were centered around 650 and 715 nm (Figure S1B). These emission bands correspond to $Q_x(0\text{-}0)$ and $Q_x(0\text{–}1)$ transitions [10]. Stokes shifts of ~2 were calculated from the absorption and fluorescence maxima of the $Q_x(0\text{-}0)$ band. The values of fluorescence quantum yields (Φ_F) determined for these porphyrins are shown in Table 1. Moreover, the fluorescence excitation spectra of the porphyrins were determined in DMF (Figure

Table 1
Spectroscopic and photodynamic properties of F_{10}APP, F_5APP and TAPP in DMF.

Photosensitizer	λ_{abs}^{Soret}	ε^{Soret}[a]	λ_{em}^{max}	Φ_F[b]	k_{obs}^{DMA} (s^{-1})[c]	Φ_Δ[d]
F_{10}APP	418	1.68×10^5	649	0.09 ± 0.01	(2.4 ± 0.1)x10^{-4}	0.33 ± 0.01
F_5APP	420	1.67×10^5	651	0.11 ± 0.01	(2.6 ± 0.1)x10^{-4}	0.36 ± 0.01
TAPP	421	1.64×10^5	655	0.15 ± 0.01[e]	(3.8 ± 0.2)x10^{-4}	0.53 ± 0.02[e]

[a] Molar absorption coefficient ($Lmol^{-1}\,cm^{-1}$).
[b] Fluorescence quantum yield.
[c] Observed rate constants for the photooxidation reaction of DMA.
[d] Quantum yield of $O_2(^1\Delta_g)$ production.
[e] From Ref. [10].

S1C), following the emission at 715 nm. As can be observed, the excitation spectra of these porphyrins were similar to the absorption spectra (Figure S1A and C). Therefore, these photosensitizers were mainly dissolved as monomers in DMF.

3.3. Photooxidized decomposition of DMA

The generation of $O_2(^1\Delta_g)$ induced by F_5APP, F_{10}APP and TAPP was evaluated using DMA as a molecular probe in DMF. Photooxidation of DMA was detected by the decay in the absorption at 378 nm. Fig. 1 shows the first-order kinetic behavior found for the decomposition of DMA. The values of k_{obs}^{DMA} are given in Table 1. The kinetic data of DMA photooxidation were used to determine the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) since DMA mainly quenches $O_2(^1\Delta_g)$ by chemical reaction [23]. Similar values of Φ_Δ were obtained for F_{10}APP and F_5APP in DMF (Table 1). The production of $O_2(^1\Delta_g)$ sensitized by these porphyrins was consistent with those previously reported for similar photosensitizers [24].

3.4. Binding of photosensitizers to microbial cells

The capacity of these porphyrins to bind to microbial cells was investigated in *S. aureus*, *E. coli* and *C. albicans* cell suspensions in PBS. Cells were treated with different porphyrin concentrations in the dark at 37 °C. The amount of photosensitizer bound to the cells was determined by fluorescence. Fig. 2 shows the quantity of F_5APP recovered from cells after different incubation time (2, 5 and 15 min). Similarly, Figures S2 and S3 show the results of binding for F_{10}APP y TAPP, respectively. These porphyrins were rapidly bound to cells, reaching highest values after 2 min incubation. The binding of F_5APP, F_{10}APP and TAPP was very similar in *S. aureus* and the amount of recovered molecules was ~0.02, ~0.05 and ~0.09 nmol/10^8 cells for cells treated with 1.0, 2.5 and 5.0 μM porphyrin, respectively. In the case of *E. coli*, the binding of these porphyrins was lower than those obtained in *S. aureus*. The amount of TAPP bound to *E. coli* cells was slightly higher

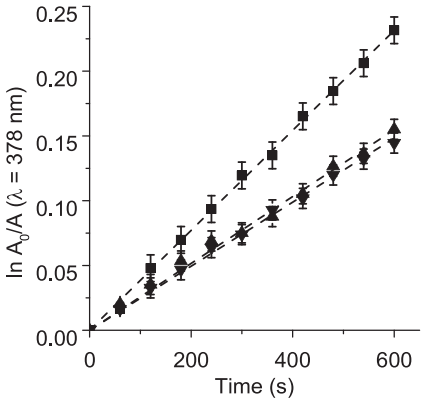


Fig. 1. First-order plots for the photooxidation of DMA photosensitized by F_5APP (▲), F_{10}APP (▼) and TAPP (■) (λ_{irr} = 420 nm) in DMF.

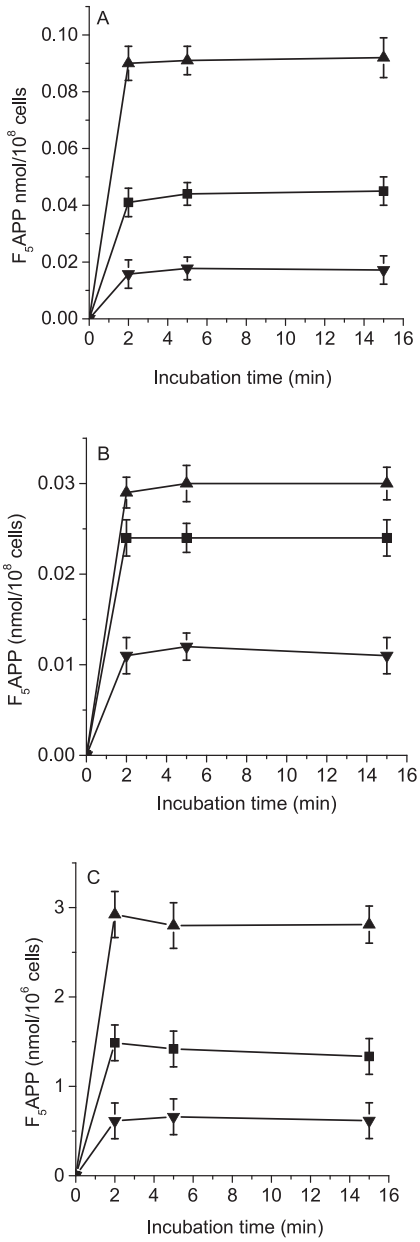


Fig. 2. Amount of F_5APP recovered from (A) *S. aureus* (~10^8 CFU/mL) treated with 1.0 μM (▼), 2.5 μM (■) and 5.0 μM (▲) photosensitizer, (B) *E. coli* (~10^8 CFU/mL) treated with 2.5 μM (▼), 5.0 μM (■) and 7.5 μM (▲) photosensitizer and (C) *C. albicans* (~10^6 CFU/mL) treated with 2.5 μM (▼), 5.0 μM (■) and 7.5 μM (▲) photosensitizer for different incubation times at 37 °C in dark.

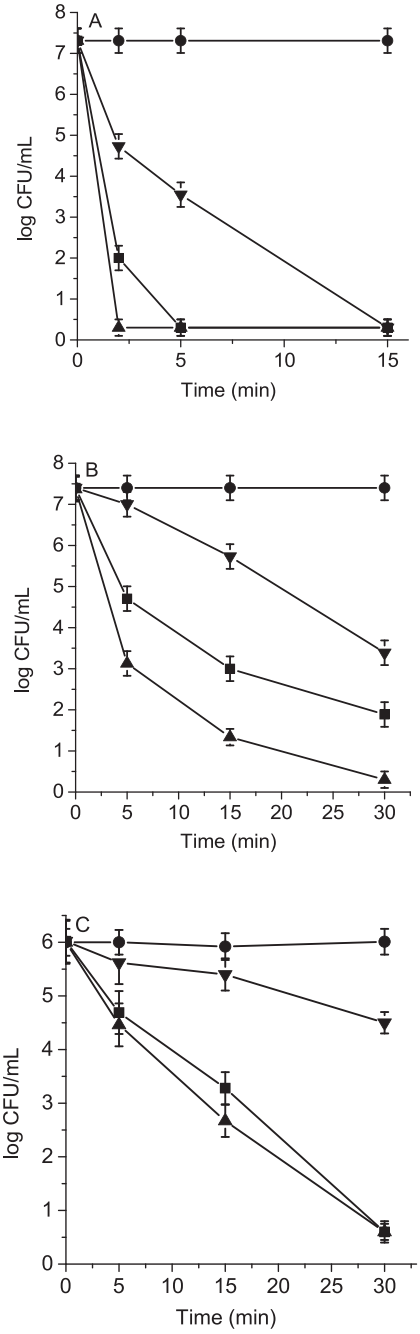


Fig. 3. Survival curves of cells sensitized by F_5APP; (A) *S. aureus* (~10^8 CFU/mL) incubated with 1.0 μM (▼), 2.5 μM (■) and 5.0 μM (▲), (B) *E. coli* (~10^8 CFU/mL) incubated with 2.5 μM (▼), 5.0 μM (■) and 7.5 μM (▲) and (C) *C. albicans* (~10^6 CFU/mL) incubated with 2.5 μM (▼), 5.0 μM (■) and 7.5 μM (▲) for 15 min at 37 °C in dark and irradiated with visible light for different times. Control culture (●) of cells untreated with the photosensitizer and irradiated.

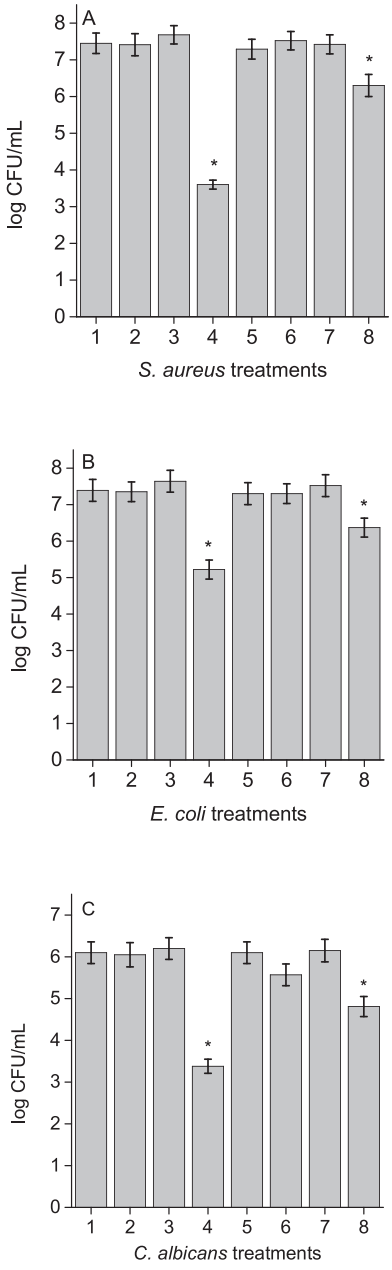


Fig. 4. Survival of (A) *S. aureus* (~10^8 CFU/mL) treated with 1 μM F_5APP and 5 min irradiation (B) *E. coli* (~10^8 CFU/mL) treated with 5 μM F_5APP and 5 min irradiation and (C) *C. albicans* (~10^6 CFU/mL) treated with 5 μM F_5APP and 15 min irradiation; (1) cells in dark; (2) irradiated cells; (3) cells treated with F_5APP in dark; (4) irradiated cells treated with F_5APP; (5) irradiated cells containing 50 mM sodium azide; (6) irradiated cells treated with F_5APP containing 50 mM sodium azide; (7) irradiated cells containing 50 mM D-mannitol; (8) irradiated cells treated with F_5APP containing 50 mM D-mannitol (*$p < 0.05$, compared with control).

than the fluorinated porphyrin derivatives, reaching a value of ~0.04 nmol/10^8 cells for cells incubated with 7.5 μM porphyrin. Under the same conditions, ~0.03 and ~0.025 nmol/10^8 cells were determined for F_5APP and F_{10}APP, respectively. The amount of porphyrin recovered from *C. albicans* cells was similar for F_5APP y TAPP, reaching values of ~0.6, ~1.4 and ~2.8 nmol/10^6 cells for *C. albicans* treated with 2.5, 5.0 and 7.5 μM porphyrin, respectively. In contrast, slight lower values were found using F_{10}APP. Moreover, the amount of cell-bound porphyrin was not affected after longer times of incubations with the yeast cells.

3.5. PDI of microbial cells

Photoinactivation of *S. aureus*, *E. coli* and *C. albicans* was investigated incubating the cultures with different porphyrin concentrations. Fig. 3 shows the cell survival of microorganisms photosensitized by F_5APP. Photoinactivation results mediated by F_{10}APP and TAPP are exposed in Figure S4 and S5, respectively. No toxicity was found for the cells treated with these concentrations of porphyrin for 30 min in dark (result no shown). Moreover, the viability of microbial cells was not changed only by irradiation (Figs. 4, S4 and S5). PDI of microbial cells

was dependent on photosensitizer concentrations and irradiation times. *S. aureus* was the most susceptible microorganism, attaining a complete photokilling after 15 min irradiation with 1 μM porphyrin. Similar PDI was also found using 5 μM porphyrin and a short irradiation period of 2 min. The different photokilling activity of these porphyrins was mainly found at this short irradiation times. F_5APP produced a photoinactivation of *S. aureus* similar to those found for TAPP, while a slight lower photokilling effect was found using 1 μM F_{10}APP. As expected, *E. coli* cells were the more difficult to photoinactivate than the Gram-positive bacterium. After 30 min irradiation, a reduction of 4.5 and 5.5 log in the survival was found for cells treated with 1.0 μM F_5APP or TAPP, respectively (Fig. 3B and Figure S5B). It was necessary 7.5 μM porphyrin and 30 min irradiation to obtain complete eradication of *E. coli*. Under this condition, the photocytotoxic effect mediated by F_5APP and TAPP was very similar. In contrast, F_{10}APP was considerably less effective and the photoinactivation mediated by 7.5 μM F_{10}APP induced a 5.5 log decrease in *E. coli* survival after 30 min irradiation (Figure S4B). On the other hand, similar photokilling of *C. albicans* was observed using F_5APP and TAPP (Fig. 3C and Figure S5C). For cells treated with 5 μM F_5APP or TAPP, a fast decrease of *C. albicans* survival (~3 log) was detected after 15 min irradiation. Using this concentration, no cell survival was detected after 30 min irradiation. On the other hand, photocytotoxic activity mediated by 5 μM F_{10}APP produced a 1.5 log reduction in the survival of the yeast cells after 30 min irradiation (Figure S4C).

3.6. Photodynamic mechanism in microorganisms

The PDI of microorganisms was studied using different conditions to have a clearer vision of the photodynamic action mechanism in the microbial cells. Thus, *S. aureus* was treated with 1.0 μM porphyrin and irradiated for 5 min, while *E. coli* and *C. albicans* were incubated with 5 μM porphyrin and irradiated for 5 and 15 min, respectively. These concentrations and irradiation times were chosen to not produce a complete eradication of microorganisms. In this way, it would be possible to observe photoprotective effects or increases in the photokilling of cells. The results for F_5APP are shown in Fig. 4, while for F_{10}APP and TAPP are exposed in Figures S6 and S7, respectively.

PDI of microorganisms was evaluated by the addition of two suppressors of ROS, sodium azide and D-mannitol. Azide anions was used as a quencher of $O_2(^1\Delta_g)$ that was generated in the cells [25]. Thus, microbial cells were treated with 50 mM sodium azide. No toxicity was detected using this azide concentration under irradiation without porphyrin (Figures 5, S6 and S7, line 5) or in the dark containing the photosensitizer. A considerable reduction in the cell photokilling mediated by porphyrins was observed in the medium containing the azide ions (Figures 5, S6 and S7, line 6). Therefore, sodium azide was able to quench the $O_2(^1\Delta_g)$, producing a protective effect on microbial cells. Similarly, the photoinactivation of microorganisms mediated by porphyrins was examined after incubation with 50 mM D-mannitol. This compound can be used as a scavenger of the superoxide anion radical and hydroxyl radical (type I reaction) [26]. The addition of 50 mM D-mannitol was not toxic to irradiated cells without porphyrin (Figures 5, S6 and S7, line 7). Also, D-mannitol was not toxic for microbial cells treated with porphyrin in dark. After irradiation, cell inactivation exhibited a photoprotection of over 2 log in suspensions containing D-mannitol (Figures 5, S6 and S7, line 8). This effect was mainly observed in *S. aureus* cells and it was lower for *E. coli* and *C. albicans* sensitized by F_{10}APP. Moreover, photoinactivation of microorganisms sensitized by porphyrins was performed in cells suspended in D_2O. No toxicity was detected in the presence of D_2O under irradiation without porphyrin (Figure S8, line 1) and porphyrins were not toxic for cells in dark (Figure S8, lines 2, 4 and 6). In the three microorganisms, a higher cell photoinactivation was observed for cells incubated with porphyrin in D_2O (Figure S8, lines 3, 5 and 7).

4. Discussion

Porphyrins substituted by intrinsic cationic groups have attracted significant interest due to their prominent ability as phototherapeutic agents against several microorganisms [6,7]. Another possibility is to obtain porphyrins substituted by precursor groups of positive charges, whose formation depends on the pH of the medium. Therefore, in this study were synthesized porphyrins with different substitution patterns on the tetrapyrrolic macrocycle. Porphyrins with symmetries ABAB (F_{10}TPP), A_3B (F_5TPP) and A_4 (TAPP) were obtained with basic amine groups as precursors of cationic centers at physiological pH. These substituents were separated from the tetrapyrrolic macrocycle by an aliphatic spacer that provides greater mobility. Also, the cationic groups present minimal effect on photophysical properties of the macrocycle. Therefore, the formation of positive charges may allow a better interaction between the photosensitizer and the cellular envelope of the microorganisms [10]. In the fluorinated porphyrins, the precursor of the cationic charge was combined with a highly lipophilic pentafluorophenyl group, which increases the amphiphilic properties of the structure. In addition, the pentafluorophenyl group can be used to attach the photosensitizer to other molecular structure by nucleophilic aromatic substitution reactions, including magnetic nanoparticles [27].

These porphyrin derivatives exhibited the typical Soret and Q absorption bands in the visible region [10]. Also, F_5TPP and F_{10}TPP showed two red emission bands and the Φ_F values were similar to that of TAPP. Small Stokes shifts indicated that the spectroscopic energy of these porphyrins was similar to the relaxed energies of the singlet state. Thus, only minor structural changes take place between its ground and excited states, as expected due to the rigid planar structure of the tetrapyrrolic macrocycle [15]. On the other hand, photodynamic activity mediated by porphyrins can occur *via* two mechanisms [7]. After light activation of the porphyrin, this photosensitizer can react with compounds from the media by electron or hydrogen transfer, producing radicals (type I reaction). Instead, porphyrin excited triplet state can transfer its energy to oxygen, forming $O_2(^1\Delta_g)$ (type II reaction). In the present study, the production of $O_2(^1\Delta_g)$ of F_5APP was very similar to that of F_{10}APP in DMF. Also, the Φ_Δ values of these photosensitizers agrees with those previously reported for free-base porphyrins [24]. However, the results of the photodynamic properties obtained in solution may not be directly extrapolated to those produced in a cellular microenvironment of microorganisms [28].

The cell envelope of microbial cells represents the main barrier to the binding of the porphyrins. Therefore, the number and distribution of charges may play a predominant role on the interaction between photosensitizer and cells [17]. *In vitro* studies with microorganisms showed that these porphyrins were rapidly bound to cells in a short incubation period. The binding of the porphyrins to cells was comparable in *S. aureus*, whereas slightly higher amount of F_5APP than F_{10}APP was found in *E. coli*. In addition, the amount of porphyrins bound to *S. aureus* cells was greater than those obtained in *E. coli*. In *C. albicans* cells, the binding of F_5APP and TAPP was similar, whereas a lower amount was found using F_{10}APP. The amount of F_5APP bound to *C. albicans* was comparable to that previously obtained for 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$, 1.4 nmol/10^6 cells) [16]. Also, a similar value of binding was determined using 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$) (1.7 nmol/10^6 cells) as a photosensitizer [29]. Therefore, the amount of F_5APP bound to *C. albicans* was comparable to those obtained for porphyrins, which are substituted by intrinsic cationic groups. These results showed a high affinity between F_5APP and the microbial cells.

PDI of microorganisms sensitized by T_5APP, T_{10}APP and TAPP was compared varying the porphyrin concentrations and irradiation times. *S. aureus* incubated with 5 μM porphyrin induced an over 7 log decrease in the viability after 2 min irradiation, which indicates an inactivation greater than 99.9999%. Moreover, a similar result was obtained with 1

μM porphyrin but after 30 min irradiation. A slightly higher photokilling was observed for F_5APP and TAPP than F_{10}APP. On the other hand, *E. coli* was more difficult to photoinactivate than *S. aureus* due to the nature of the envelope of Gram-negative bacteria [6]. When the *E. coli* cells were treated with porphyrin, an increase in the cell photokilling was obtained increasing the irradiation periods. After 30 min irradiation, decreases of 4.5 and 5.5 log in cell survival were reached for *E. coli* treated with 2.5 μM F_5APP and TAPP, respectively. In contrast, F_{10}APP produced a 1.5 log of photoinactivation. At a concentration of 7.5 μM, PDI of *E. coli* treated F_5APP or TAPP induced over 7 log (99.9999%) decrease. Also, the photocytotoxic activity was elevated using a shorter irradiation time of 5 min, which produced a 5 log decrease of survival. Thus, the photoinactivation of *E. coli* cells incubated with F_5TAPP was very similar to that of TAPP. Comparing the three microorganisms, the susceptibility of *C. albicans* was higher than the Gram-negative bacteria, but lower than the Gram-positive ones. *C. albicans* cells incubated with 2.5 μM F_5APP or TAPP induced a photoinactivation of 1.5 log decrease when cells were irradiated for 30 min. However, when 5 μM F_5APP or TAPP was used the photoinactivation remained elevated (> 99.999%). Even, the photocytotoxic activity mediated by F_5APP or TAPP was higher than those previously found using $TMAP^{4+}$ and $TMPyP^{4+}$ [17,29]. As opposed, a lower photoinactivation of *C. albicans* was obtained in presence of F_{10}APP.

The consequence of the basic amine substituents on the macrocycle periphery of these porphyrins was dependent on the microorganisms. In *S. aureus*, photoinactivation induced by F_5APP or F_{10}APP was very similar to that found for TAPP. Gram-positive bacteria are more susceptible to PDI and can be photoinactivate by neutral, anionic or cationic photosensitizers [30]. However, the photoinduced damages mediated by F_{10}APP considerably decrease in *E. coli* cells. It is known the importance of cationic groups on the photosensitizers to produce photoinactivation of Gram-negative bacteria [6]. The different permeability barriers between Gram-positive and Gram-negative bacteria were mainly involved in the observed efficiency of these porphyrins. Also, F_5APP was more effective than F_{10}APP to photoinactivate *C. albicans* cells. In general, the presence of cationic charges was required for an efficient photokilling of *C. albicans* [17]. Fungal cells have a layer of β-glucan and chitin in the cell wall, which produces a permeability barrier that can be found between those of Gram-positive and Gram-negative bacteria [31]. Thus, *S. aureus* was more susceptible to the photodynamic activity mediated by F_5APP, while longer radiation or a higher concentration of porphyrin was necessary to inactivate *E. coli*. On the other hand, a complete eradication of *C. albicans* sensitized by 5 μM of F_5APP was possible after 30 min of irradiation. In addition to the type of microbial cells, these porphyrins can be partitioned into different parts of the cell and the cell walls. Thus, the site of action can strongly influence in the efficiency of these photosensitizers because the generated ROS rapidly react with the biomolecules of their microenvironments [32]. Moreover, a redistribution of the porphyrin can take place during the treatment, probably due to a relocation of the molecules that were weakly bound to the cells or remained in the medium [33].

The photodynamic action mechanism sensitized by F_5APP, F_{10}APP and TAPP was investigated under different conditions. Thus, photoinactivation assays were carried out with the addition of sodium azide to obtain insights the participation of $O_2(^1\Delta_g)$. In cells, the lifetime of $O_2(^1\Delta_g)$ is delimited by quenching and reactions with biomolecules. Therefore, the cell damage mediated by $O_2(^1\Delta_g)$ occurs close to its site of generation [32]. The $O_2(^1\Delta_g)$ lifetime and the rate constant for $O_2(^1\Delta_g)$ quenching by azide ions may be determined by the location of the porphyrin in different components of the cells. Consequently, azide ions should be located in the subcellular compartments close to the photosensitizer [25]. In this study, the addition of sodium azide produced a complete photoprotection of cells survival. Therefore, the azide ions produced a considerable reduction of photodamage by the quenching of $O_2(^1\Delta_g)$. On the other hand, to examine the involvement of type I mechanism D-mannitol was added as a free-radical scavenger [26]. In presence of D-mannitol, photocytotoxicity of microbial cells sensitized by porphyrins was affected, producing a photoprotection mainly in *S. aureus* cells. The photoprotective results induced by D-mannitol was suggestive of some contribution of the type I pathway. In addition, photoinactivation of microbial cells was determined in D_2O to evaluate the $O_2(^1\Delta_g)$-mediated inactivation of microbial cells. An increase in the $O_2(^1\Delta_g)$ lifetime is expected when D_2O was used instead of water [32,34]. The lifetime of $O_2(^1\Delta_g)$ is 67 μs in D_2O but only 3.5 μs in water [35]. The photokilling activity mediated by porphyrins was increased in D_2O, indicating an involvement of type II pathway in the PDI of microorganisms. It was previously observed that the photokilling of *C. albicans* cells by cationic porphyrins was mostly mediated by $O_2(^1\Delta_g)$ [36]. Therefore, the photodamage produced to the microbial cells by these porphyrins appears to be mainly facilitated by the intermediacy of $O_2(^1\Delta_g)$. However, taking into account the studies with D-mannitol, the contribution of other ROS can not be completely ruled out in the PDI of these microorganisms.

5. Conclusions

New asymmetrically *meso*-substituted porphyrins containing basic amino groups were synthesized as photosensitizer to inactivate microbial cells. Similar spectroscopic and photodynamic properties of F_5APP and F_{10}APP were found in DMF. *In vitro* experiments showed that these porphyrins were rapidly bound to the microbial cells. Moreover, F_5APP and TAPP were efficient photosensitizers to kill microorganisms and the photoinactivation was mainly through the intermediacy of $O_2(^1\Delta_g)$. Although, a minor contribution of other ROS may also be involved in the photoinactivation of microbial cells. These porphyrins are photosensitizers not intrinsically charged. However, amino groups can obtain positive charges depending on the pH of the media, which in combination with the amphiphilic nature of F_5APP, make this porphyrin an effective photosensitizer for killing microorganisms by PDI.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2018.09.017.

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