European Journal of Medicinal Chemistry 44 (2009) 1592–1599

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Photodynamic inactivation of *Candida albicans* sensitized by tri- and tetra-cationic porphyrin derivatives

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ARTICLE INFO

Article history:
Received 7 April 2008
Received in revised form 16 July 2008
Accepted 17 July 2008
Available online 26 July 2008

Keywords:
Cationic porphyrin
Photodynamic inactivation
Photosensitizers
Candida albicans
Antifungal

ABSTRACT

The photodynamic action of 5-(4-trifluorophenyl)-10,15,20-tris(4-trimethylammoniumphenyl)porphyrin iodide ($TFAP^{3+}$) and 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammonium phenyl)porphyrin *p*-tosylate ($TMAP^{4+}$) has been studied *in vitro* on *Candida albicans*. The results of these cationic porphyrins were compared with those of 5,10,15,20-tetra(4-sulphonatophenyl)porphyrin ($TPPS^{4-}$), which characterizes an anionic sensitizer. *In vitro* investigations show that these cationic porphyrins are rapidly bound to *C. albicans* cells, reaching a value of ~1.4 nmol/10^6 cells, when the cellular suspensions were incubated with 5 μM sensitizer for 30 min. In contrast, $TPPS^{4-}$ is poorly uptaken by yeast cells. The fluorescence spectra of these sensitizers into the cells confirm this behaviour. The amount of porphyrin binds to cells is dependent on both sensitizer concentrations (1–5 μM) and cells densities (10^6–10^8 cells/mL). Photosensitized inactivation of *C. albicans* cellular suspensions increases with sensitizer concentration, causing a ~5 log decrease of cell survival, when the cultures are treated with 5 μM of cationic porphyrin and irradiated for 30 min. However, the photocytotoxicity decreases with an increase in the cell density, according to its low binding to cells. Under these conditions, the photodynamic activity of $TFAP^{3+}$ is quite similar to that produced by $TMAP^{4+}$, whereas no important inactivation effect was found for $TPPS^{4-}$. The high photodynamic activity of cationic porphyrins was confirmed by growth delay experiments. Thus, *C. albicans* cell growth was not detected in the presence of 5 μM $TFAP^{3+}$. Photodynamic inactivation capacities of these sensitizers were also evaluated on *C. albicans* cells growing in colonies on agar surfaces. Cationic porphyrins produce a growth delay of *C. albicans* colonies and viability of cells was not observed after 3 h irradiation, indicating a complete inactivation of yeast cells. Therefore, these results indicate that these cationic porphyrins are interesting sensitizers for photodynamic inactivation of yeasts in liquid suspensions or in localized foci of infection.

1. Introduction

Oral candidiasis is a significant infection in patients being treated with chemotherapy and radiotherapy for cancer, and in patients who are immunocompromised because of HIV infection and AIDS. *Candida albicans* is the most common fungal pathogen and has developed an extensive array of recognized virulent mechanisms that allow successful colonization and infection of the host under suitable predisposing conditions [1]. Antimicrobial resistance is a growing problem that complicates the treatment of important nosocomial and community-acquired infections. In the last years, resistance of *C. albicans* is increasing against traditional antifungal, such as fluconazole [2–4].

Therefore, the search for new therapeutic approaches is stimulated by the fact that standard antifungal treatments are prolonged and expensive and the appearance of drug resistant strains is more frequent in patients [5]. In this way, photodynamic inactivation (PDI) represents an interesting alternative to inactive microorganisms [6–8]. This methodology is based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. The subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms. Different oxidative mechanisms can occur after photoactivation of the photosensitizer. In the type I photochemical reaction, the photosensitizer interacts with a biomolecule to produce free radicals, while in the type II mechanism, singlet molecular oxygen, $O_2(^1\Delta_g)$, is produced as the main species responsible for cell inactivation [9,10].

There is much less systematic study, compared to those carried out with bacteria, on effective photosensitizers to inactivate various

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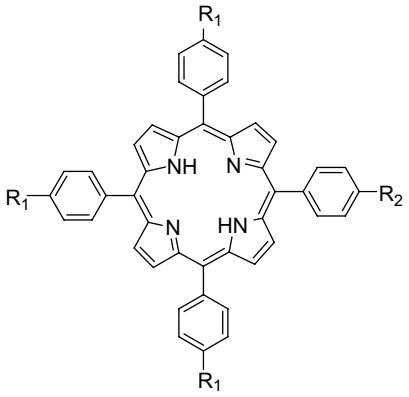
doi:10.1016/j.ejmech.2008.07.026

species of yeast and fungi [6]. Antifungal properties of hematoporphyrin have been investigated on yeast cells and its photocytotoxic activity is mainly promoted by unbounded dye molecules in the bulk aqueous medium [11]. After irradiation, this sensitizer causes an initial limited alteration of the cytoplasmic membrane that allows for the penetration of the dye into the cell, the translocation to the inner membrane and the consequent photodamage of intracellular targets [12]. Internalization of Photofrin by *C. albicans* was confirmed by confocal fluorescence microscopy. Uptake of Photofrin by cells and subsequent sensitivity to irradiation were influenced by culture conditions [13]. The fungal cell wall provides structure to the cell and protects the cell from the environment. It is primarily composed of polysaccharide polymers, like chitin, β-glucans and glycoproteins. Therefore, the efficiency of the photosensitized process is markedly more pronounced by agents, which enhance their penetration into the inner cellular district. Recently, porphyrin and phthalocyanine derivatives have been investigated for PDI applications in the treatment and control of yeast [14,15]. Cellular suspensions of *C. albicans* were successfully inactivated by a tricationic porphyrin and the target organelle of PDI activity was the cytoplasmic membrane [14]. Thus, the interest in this field and the need for new molecules with improved characteristics are always high owing to the large variety of therapeutic applications.

In previous work, the photodynamic activity of cationic porphyrin derivatives with different patterns of substitution was investigated *in vitro* as sensitizers to eradicate Gram-negative bacteria [16]. An amphiphilic porphyrin bearing three cationic charges and a highly lipophilic trifluoromethyl group, $TFAP^{3+}$ (Scheme 1), showed to be active photosensitizers to inactivate *Escherichia coli* cells. The tight binding of $TFAP^{3+}$ to cells is accompanied by a high PDI of *E. coli* cultures in liquid cellular suspensions. On the other hand, $TMAP^{4+}$ represents a standard active sensitizer established to eradicate microorganisms, while $TPPS^{4-}$ is used as an anionic photosensitizer model (Scheme 1) [17].

In the present study, we have investigated the susceptibility of *C. albicans* to the phototoxic effect produced by cationic and anionic porphyrin derivatives. Thus, PDI action of tri-, $TFAP^{3+}$, and a tetracationic porphyrin, $TMAP^{4+}$, derivatives was compared with that obtained for a tetra-anionic porphyrin, $TPPS^{4-}$. The photoinactivation efficiencies of these sensitizers were evaluated under different conditions to eradicate *C. albicans* in cellular suspensions and in colonies immobilized on surfaces. This investigation provides information that can be used to establish conditions for the photoinactivation of yeast cells growing in liquid medium or in a localized focus of infection on accessible area to be irradiated with visible light.



$TFAP^{3+}$ R_1: $-N^+(CH_3)_3\ I^-$ R_2: $-CF_3$

$TMAP^{4+}$ $R_1=R_2$: $-N^+(CH_3)_3\ p\text{-}Tos^-$

$TPPS^{4-}$ $R_1=R_2$: $-SO_3^-Na^+$

Scheme 1. Molecular structures of porphyrins.

2. Materials and methods

2.1. General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. 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. A wavelength range between 350 and 800 nm was selected by optical filters. The light intensity at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA).

All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Sensitizers

5-(4-Trifluorophenyl)-10,15,20-tris(4-trimethylammoniumphenyl)porphyrin iodide ($TFAP^{3+}$) was synthesized as previously described [18]. 5,10,15,20-Tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin *p*-tosylate ($TMAP^{4+}$) and 5,10,15,20-tetra(4-sulphonatophenyl)porphyrin ($TPPS^{4-}$) sodium salt were purchased from Aldrich. A porphyrin stock solution (~0.5 mM) was prepared by dissolution in 1 mL of water. The concentration was checked by spectroscopy, taking into account the value of molar coefficient [18,19].

2.3. Microorganism and growth conditions

C. albicans strain PC31, recovered from human skin lesion, was previously characterized and identified. The fungal strain was identified according to conventional procedures [20]. Primary classification of colonies from plates was based on colony characteristics (pigmentation and shape), mode of vegetative reproduction, formation of pseudohyphae and ascopore production. Identification of the yeast isolates to species level was done using the API 20C AUX (BioMérieux, Marcy ÍEtoile, France) system of carbohydrate assimilation profiles. Strain of *C. albicans* was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (4 mL) at 37 °C to stationary phase. Cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0), corresponding to ~10^7 colony forming units (CFU)/mL. The cells were appropriately diluted to obtain ~10^6 CFU/mL or concentrated to get ~10^8 CFU/mL in PBS. In all the experiments, 2 mL of the cell suspensions in Pirex brand culture tubes (13 × 100 mm) were used and the sensitizer was added from a stock solution ~0.5 mM. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar plates and ~48 h incubation at 37 °C. Fungal cultures grown under the same conditions with and without photosensitizers kept in the dark and illuminated cultures without sensitizer served as controls.

2.4. Fluorescence spectra in cells

Suspensions of *C. albicans* (2 mL, ~10^6 CFU/mL) in PBS were incubated with 5 μM sensitizer in dark at 37 °C for 30 min. The cultures were centrifuged (3000 rpm for 15 min) and the cell pellets were re-suspended in 2 mL PBS. Spectra were recorded

using 1 cm path length quartz cuvettes at room temperature. Emission spectra were recorded exciting the samples at 420 nm, while the emission of porphyrin at 720 nm was used to obtain excitation spectra.

2.5. Photosensitizer binding to yeast cells

Suspensions of *C. albicans* (2 mL, $\sim 10^6$–10^8 CFU/mL) in PBS were incubated in dark at 37 °C with a determined concentration (1 and 5 μM) of sensitizer for different times. The cultures were centrifuged (3000 rpm for 15 min) and the cell pellets 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 ($\lambda_{exc} = 418$ nm, $\lambda_{em} = 650$ nm) in solution of 2% SDS in PBS. The fluorescence values obtained from each sample were referred to the total number of cells contained in the suspension. The concentration of the porphyrin in this sample was estimated by comparison with a calibration curve obtained with standard solutions of the sensitizer in 2% SDS ([sensitizer] ~0.005–0.1 μM).

2.6. Photosensitized inactivation of *C. albicans* cells in PBS

Cellular suspensions of *C. albicans* (2 mL, $\sim 10^6$–10^8 CFU/mL) in PBS were incubated with an appropriate concentration of porphyrin for 30 min in the dark at 37 °C. After that, the cultures were exposed to visible light for different time intervals. 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 Sabouraud agar and the number of colonies formed after ~48 h incubation at 37 °C was counted. Each experiment was repeated separately 3 times.

2.7. Growth delay of *C. albicans*

Cultures of *C. albicans* cells were grown overnight as described above. A portion (1 mL) of this culture was transferred to 20 mL of fresh Sabouraud broth medium. The suspension was homogenized and aliquots of 2 mL were incubated with 5 μM of sensitizer at 37 °C. The culture grown was measured by turbidity at 660 nm using a Tuner SP-830 spectrophotometer. Then the flasks were irradiated with visible light at 37 °C, as described above. In all cases, control experiments were carried out without illumination in the absence and in the presence of sensitizer. Each experiment was repeated separately 3 times.

2.8. Photosensitization of *C. albicans* on Sabouraud agar

Suspensions of *C. albicans* ($\sim 10^2$ CFU/mL) in PBS were spread on 10 cm diameter plates containing Sabouraud agar and they were grown at 37 °C by 24 h. About 30 small colonies per plate were obtained using this procedure. The sensitizers were added from a ~0.5 mM stock solution in water. The colonies were spread with different amounts of sensitizers (2.2–8.8 nmol). The cultures were kept in dark for 15 min at 37 °C to allow the binding of sensitizer to *C. albicans* cells and irradiated with visible light as described above. Then, the plates were incubated in dark at 37 °C and the variation in the area of *C. albicans* colonies was determined after different times. Viability of cells after PDI treatment was evaluated by transferring samples of these colonies to fresh Sabouraud broth and plating on new Sabouraud agar plate. Controls with and without photosensitizers kept in the dark as well as illuminated controls without porphyrin were carried out. Each experiment was repeated separately 3 times.

2.9. Statistical analysis

All data were presented as the mean ± standard deviation of each group. Variation between groups was evaluated using the Student *t*-test, with a confidence level of 95% ($p < 0.05$) considered statistically significant.

3. Results and discussion

3.1. Spectroscopic and photochemical properties of porphyrins

The absorption spectra of $TFAP^{3+}$, $TMAP^{4+}$ and $TPPS^{4-}$ porphyrins in DMF show the typical *Soret* and *Q*-bands characteristic of free-base porphyrin derivatives. The absorption maxima at *Soret* band are summarized in Table 1. The relative intensities of the *Q*-bands for these porphyrins show an *etio*-type spectrum ($\varepsilon_{VI} > \varepsilon_{III} > \varepsilon_{II} > \varepsilon_{I}$) [21]. The steady-state fluorescence emission spectra of these porphyrins in DMF show two bands in the red spectral region (Table 1). Moreover, the fluorescence quantum yield (ϕ_F) values of these compounds (Table 1) are appropriated for detection and quantification of the sensitizer in the biological media [16]. In this way, the fluorescence spectra of these porphyrins were analyzed in cells suspended in PBS. Thus, *C. albicans* cultures were treated with 5 μM of sensitizer for 30 min at 37 °C in dark. After one washing step, cellular suspensions in PBS showed fluorescence emission spectra for $TFAP^{3+}$ and $TMAP^{4+}$ porphyrins that present a shape close to that of the fluorescence emission in homogeneous media, with maxima at ~650 and 714 nm (Fig. 1A). This result indicates that these cationic porphyrins are binding to yeast cells and the photosensitizer remains mainly dissolved as monomer into the cells, possibly favoured by the cellular microenvironment where the sensitizer is localized. These results were also confirmed by fluorescence excitation studies. As can be observed in Fig. 1B, the shape and intensity of the bands of the excitation spectra of cationic porphyrins performed in cellular suspensions closely match the corresponding absorption spectra of these sensitizers in DMF (Fig. 1B, inset). In contrast, fluorescence was not detected in cells treated with $TPPS^{4-}$ (Fig. 1), indicating that this anionic porphyrin is very weakly bound to *C. albicans* and, therefore, it is removed after one washing step. This result was also established below in the quantification of the amount of porphyrin binds to cells.

On the other hand, the cationic sensitizers evaluated in this work present a high efficiency in the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) (Table 1) [22,23]. Also, efficient photochemical activity was previously found for $TPPS^{4-}$ in water [24]. This is an important photosensitizer property because the reactions induced by $O_2(^1\Delta_g)$ are believed to be the main cause of cellular damage sensitized by porphyrins under aerobic conditions [9]. However, the values of Φ_Δ can significantly change according to the medium, diminishing when the sensitizer is partially aggregated. Also, the biological microenvironment of the sensitizer can induce important modifications in the photophysics of the porphyrin established

Table 1
Absorption and fluorescence emission maxima, fluorescence quantum yield (ϕ_F) and singlet molecular oxygen quantum yield (Φ_Δ) of porphyrins in DMF

Porphyrin	Absorption λ_{max} (nm)					Emission λ_{max} (nm)		ϕ_F	Φ_Δ
$TFAP^{3+}$	417	513	551	587	650	656	714	0.08	0.42[a]
$TMAP^{4+}$	417	513	549	587	646	649	713	0.12	0.65[b]
$TPPS^{4-}$	416	513	549	590	647	652	716	0.10	0.71[c]

[a] From Ref. [22].
[b] From Ref. [23].
[c] From Ref. [24] in water.

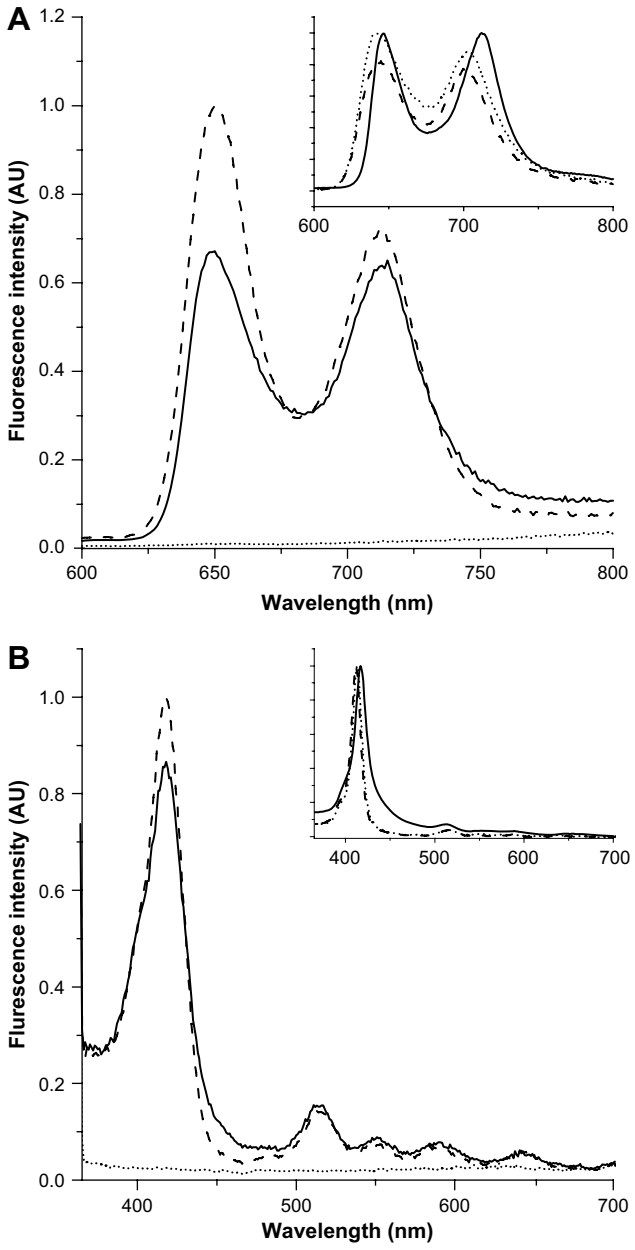


Fig. 1. Fluorescence spectra of porphyrins in *C. albicans* cellular suspension (10^6 CFU/mL) treated with 5 μM of sensitizer for 30 min at 37 °C in dark, washed once and re-suspended in PBS, $TFAP^{3+}$ (solid line), $TMAP^{4+}$ (dashed line) and $TPPS^{4-}$ (dotted line). (A) Emission spectra of porphyrins ($\lambda_{exc} = 420$ nm). Inset: emission spectra of $TFAP^{3+}$ in *N*,*N*-dimethylformamide, $TMAP^{4+}$ in water and $TPPS^{4-}$ in water ($\lambda_{exc} = 515$ nm). (B) Excitation spectra of porphyrins ($\lambda_{em} = 720$ nm). Inset: absorption spectra of $TFAP^{3+}$ in *N*,*N*-dimethylformamide, $TMAP^{4+}$ in water and $TPPS^{4-}$ in water.

in solution [25]. In consequence, there are limitations to predict photodynamic efficiencies of sensitizers in biological systems on the basis of photophysical investigations in homogeneous solution.

3.2. Binding of porphyrin to C. albicans cells

The capacity of these porphyrins to bind to *C. albicans* cells was first compared in cellular suspension of ~10^6 cells/mL in PBS. In this way, the *C. albicans* cultures were incubated with 5 μM porphyrin for different times at 37 °C in the dark. In each case, the sensitizer associated with cells was determined by fluorescence emission analysis (see Section 2). The amounts of porphyrin recovered after different incubation periods are shown in Fig. 2.

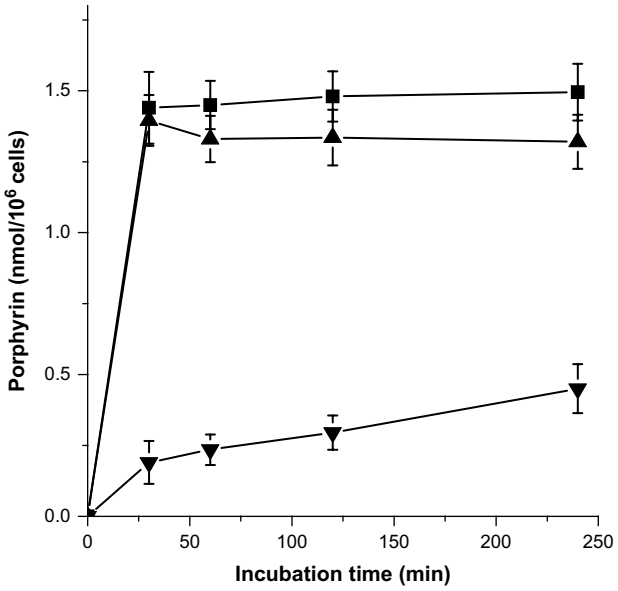


Fig. 2. Amount of $TFAP^{3+}$ (▲), $TMAP^{4+}$ (■) and $TPPS^{4-}$ (▼) recovered from *C. albicans* cells (~10^6 CFU/mL) treated with 5 μM of sensitizer for different incubation times at 37 °C in dark. Values represent mean ± standard deviation of three separate experiments.

Under these conditions, both cationic porphyrins reach the highest value of cell-bound sensitizer at short time (<30 min). Prolonging the incubation time does not cause appreciable increase in the amount of photosensitizer bound to *C. albicans* cells. The results show that the binding of tricationic $TFAP^{3+}$ porphyrin tends to a saturation value of ~1.48 nmol/10^6 cells, while a slightly smaller value (~1.35 nmol/10^6 cells) was obtained with $TMAP^{4+}$. These cationic porphyrins are higher bound to cells in comparison with the anionic porphyrin $TPPS^{4-}$ (Fig. 2). These results are in agreement with those found above by fluorescence spectral analysis and they suggest that these cationic porphyrins have particularly high binding affinity for *C. albicans* cells. Also, binding of sensitizer to cells was analyzed using 1 μM porphyrin. Under this condition, the behaviour was similar to that shown in Fig. 2 but both cationic porphyrins reach a value ~0.32 nmol/10^6 cells, whereas for $TPPS^{4-}$ the value is ~0.03 nmol/10^6 cells. By comparison with the results using 5 μM porphyrin, it can be noted that apparently non-saturation in the intracellular photosensitizer concentration occurs, at least over this concentration range.

In addition, the binding of these porphyrins was analyzed incubating different cellular densities of *C. albicans* (10^6–10^8 cells/mL) with 5 μM sensitizer for 30 min at 37 °C in the dark. The uptake of $TFAP^{3+}$ by cells was 0.12 and 0.01 nmol/10^6 cells for cultures of 10^7 and 10^8 cells/mL, respectively. Also, similar behaviour was found for the binding of $TMAP^{4+}$, reaching values of 0.11 and 0.01 nmol/10^6 cells for cultures of 10^7 and 10^8 cells/mL. Under this condition, porphyrin molecules are distributed in different numbers of cells and, therefore, it is expected that the amount of sensitizer by cell diminishes at the same time as the cellular density increases. Consequently, the cationic sensitizer bound to the fungi cells decreased almost proportional with increasing cell density.

3.3. Photosensitized inactivation of C. albicans cellular suspensions in PBS

PDI of *C. albicans* was first evaluated in PBS cellular suspensions treated with different concentrations (1–5 μM) of sensitizer for 30 min at 37 °C in dark and irradiated for 15 min with visible light (Fig. 3A). Control experiments showed that the viability of *C. albicans* was unaffected by

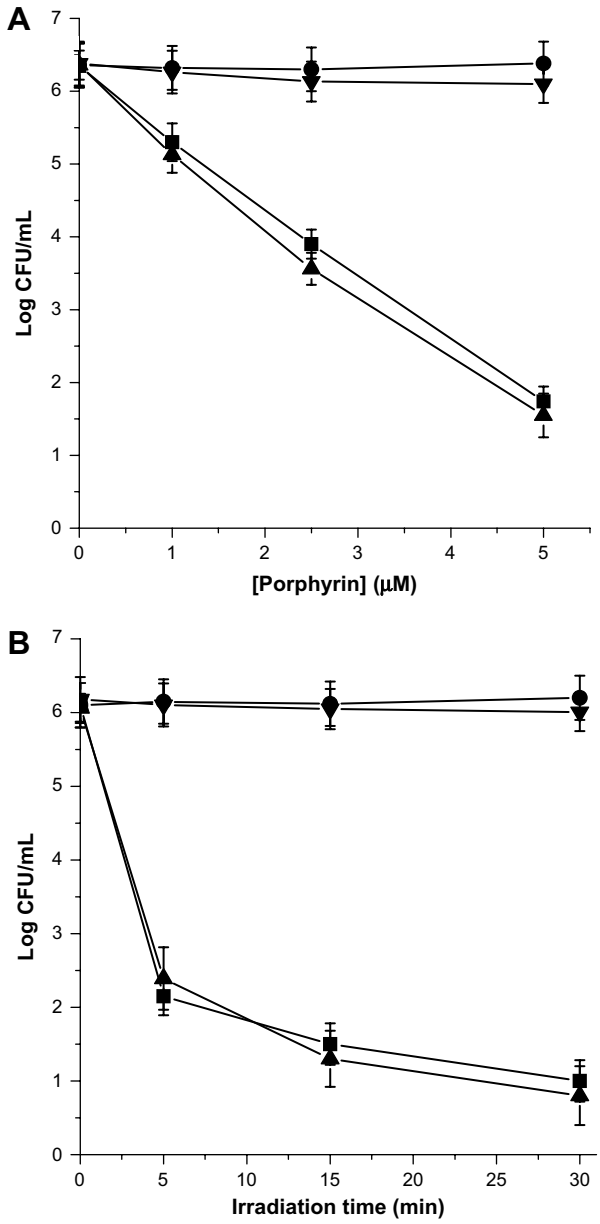


Fig. 3. (A) Survival curves of *C. albicans* (10^6 CFU/mL) incubated with different porphyrin concentrations for 30 min at 37 °C in dark and exposed to visible light for 15 min; (B) photoinactivation of *C. albicans* incubated with 5 μM of $TFAP^{3+}$ (▲), $TMAP^{4+}$ (■) and $TPPS^{4-}$ (▼) for 30 min at 37 °C in dark and exposed to visible light for different irradiation times. Control culture untreated (●). Values represent mean ± standard deviation of three separate experiments.

illumination alone or by dark incubation with 5 μM of the photosensitizer. This indicates that the cell mortality obtained after irradiation of the cultures treated with the porphyrin is due to the photosensitization effect of the agent, produced by visible light. The viability of *C. albicans* cells after irradiation was dependent upon porphyrin concentrations used in the treatment (Fig. 3A). Under these conditions, an increase in the sensitizer concentration is accompanied by an enhancement in the PDI efficiency. The photoinactivation of yeast cells is almost proportional to the amount of sensitizer solution in this range of concentrations. This is in accordance with the amount of sensitizer binding to *C. albicans* cells, indicating that nonsaturation of the intracellular concentration of sensitizer takes place under these experimental conditions.

Moreover, the PDI of *C. albicans* treated with 5 μM of sensitizer was investigated using different irradiation periods. As seen in Fig. 3B, the *C. albicans* cells are rapidly photoinactivated when the cultures treated with tricationic $TFAP^{3+}$ and tetra-cationic $TMAP^{4+}$ porphyrins are exposed to visible light. These cationic porphyrins exhibit a photosensitizing activity causing a ~5 log decrease of cell survival, when the cultures are treated with 5 μM of sensitizer and 30 min of irradiation. These results represent a value greater than 99.9994% of cellular inactivation. Although, a short irradiation time (5 min) is sufficient to produce a high photoinactivation (~99.99%) of *C. albicans* cells treated with cationic porphyrins, the efficiency in the photodynamic activity of these cationic porphyrins is not very different even at longer irradiation time used (30 min, Fig. 3B). Comparable behaviour was previously reported for *C. albicans* cells treated with different concentrations of 5-phenyl-10,15,20-tris(*N*-methyl-4-pyridyl)porphyrin chloride [26]. Also, effective photoinactivation of *C. albicans* cells was found for cultures incubated with zinc(II) tetrakis-(3-methylpyridyloxy)phthalocyanine and irradiated with different doses of light [15].

In contrast, no significant inactivation effect was found for cultures treated with 5 μM of anionic $TPPS^{4-}$ porphyrin still after 30 min of irradiation. This result is expected due to the low binding of $TPPS^{4-}$ to *C. albicans* cells. This behaviour is in agreement with that reported before for anionic photosensitizers, which are unsuccessful sensitizers for inactivation of yeast and Gram-negative bacteria cells [15,16].

Moreover, photoinactivation of *C. albicans* induced by these porphyrins was analyzed using different cellular densities. The results for 10^7 and 10^8 cells/mL densities treated with 5 μM sensitizer are shown in Fig. 4. The effectiveness of PDI decreases significantly with an increase in the cell density. Thus, the cultures incubated with cationic porphyrin require over 30 min of irradiation to photoinactivate ~3 log (99.9%) of *C. albicans* at a cellular density of 10^7 cells/mL. Similar effect of cell density on PDI was previously observed for different microorganisms [27]. In particular, cellular survival was practically not changed, when 10^8 cells/mL of *C. albicans* was incubated with 5 μM of cationic porphyrins and irradiated for 30 min. The high level of dependence of cell density observed with cationic porphyrins reflects the strength and avidity of the binding of these sensitizers to the cells. Since the vast

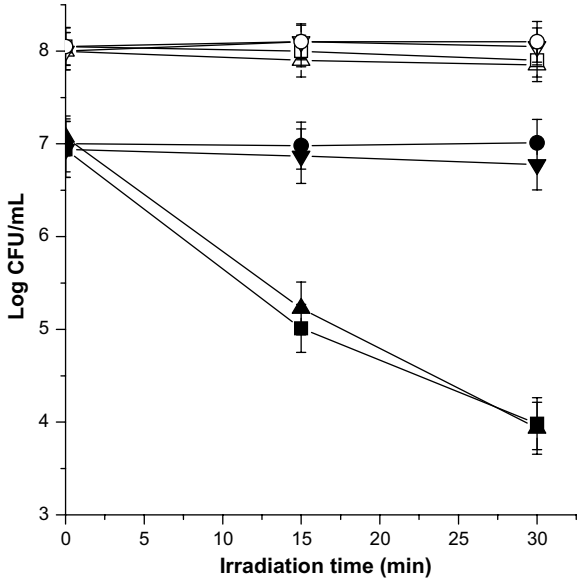


Fig. 4. Survival curves of *C. albicans* cells 10^7 CFU/mL, incubated with 5 μM of $TFAP^{3+}$ (▲), $TMAP^{4+}$ (■) and $TPPS^{4-}$ (▼), and 10^8 CFU/mL, incubated with 5 μM of $TFAP^{3+}$ (△), $TMAP^{4+}$ (□) and $TPPS^{4-}$ (▽), for 30 min at 37 °C in dark and exposed to visible light for different irradiation times. Control culture untreated 10^7 CFU/mL (●) and 10^8 CFU/mL (○). Values represent mean ± standard deviation of three separate experiments.

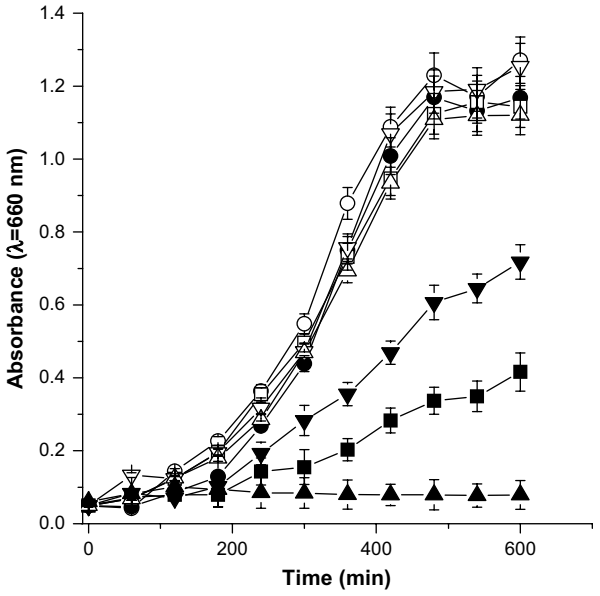


Fig. 5. Growth delay curves of *C. albicans* cells incubated with 5 μM of $TFAP^{3+}$ (▲), $TMAP^{4+}$ (■) and $TPPS^{4-}$ (▼) porphyrins and exposed to different irradiation times with visible light in Sabouraud broth at 37 °C. Control cultures: cells treated 5 μM of $TFAP^{3+}$ (△), $TMAP^{4+}$ (□) and $TPPS^{4-}$ (▽) porphyrins in dark, cells untreated irradiated (●) and in dark (○). Values represent mean ± standard deviation of three separate experiments.

majority of the $TFAP^{3+}$ and $TMAP^{4+}$ in the incubation mixture is bound to the cells, when the cell number is increased the amount of photosensitizer bound to each cell decreases. This effect is mainly evidenced when the cell density increases from 10^6 to 10^8 cells/mL and, therefore, the ratio porphyrin/cell diminishes by three orders of magnitude. This effect is clearly confirmed by the amount of sensitizer bound to cells at different cellular densities.

3.4. Photosensitized growth delay of *C. albicans* cultures

Growth delay of *C. albicans* cultures sensitized by porphyrins was carried out in Sabouraud medium. These experiments were performed to ensure that PDI of cells is still possible when the cultures were not under starvation conditions or the potential damaging effects of phosphate buffer washing. Thus, 5 μM sensitizer was added to fresh cultures of *C. albicans* reaching the log phase and the flasks were irradiated with visible light at 37 °C. As can be observed in Fig. 5, growth was suppressed when *C. albicans* cultures were treated with cationic porphyrins and illuminated. After 30 min of irradiation in the presence of 5 μM $TFAP^{3+}$, the cells no longer appeared to be growing as measured by turbidity at 660 nm. Under these conditions, the effect of $TFAP^{3+}$ porphyrin is faster than that of tetra-cationic porphyrins, $TMAP^{4+}$. On the other hand, *C. albicans* cells incubated with porphyrins 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 photodynamic effect of the sensitizers on the cells.

Furthermore, a minor effect in the growth delay was found for cells treated with 5 μM of anionic $TPPS^{4-}$ porphyrin. These results agree with those observed above, and also under these conditions $TPPS^{4-}$ does not produce significant cellular inactivation.

3.5. Photosensitization of *C. albicans* cells growing in colonies

Taking into account the result in PBS solution, the photodynamic activity of these photosensitizers was evaluated in *C. albicans* cells

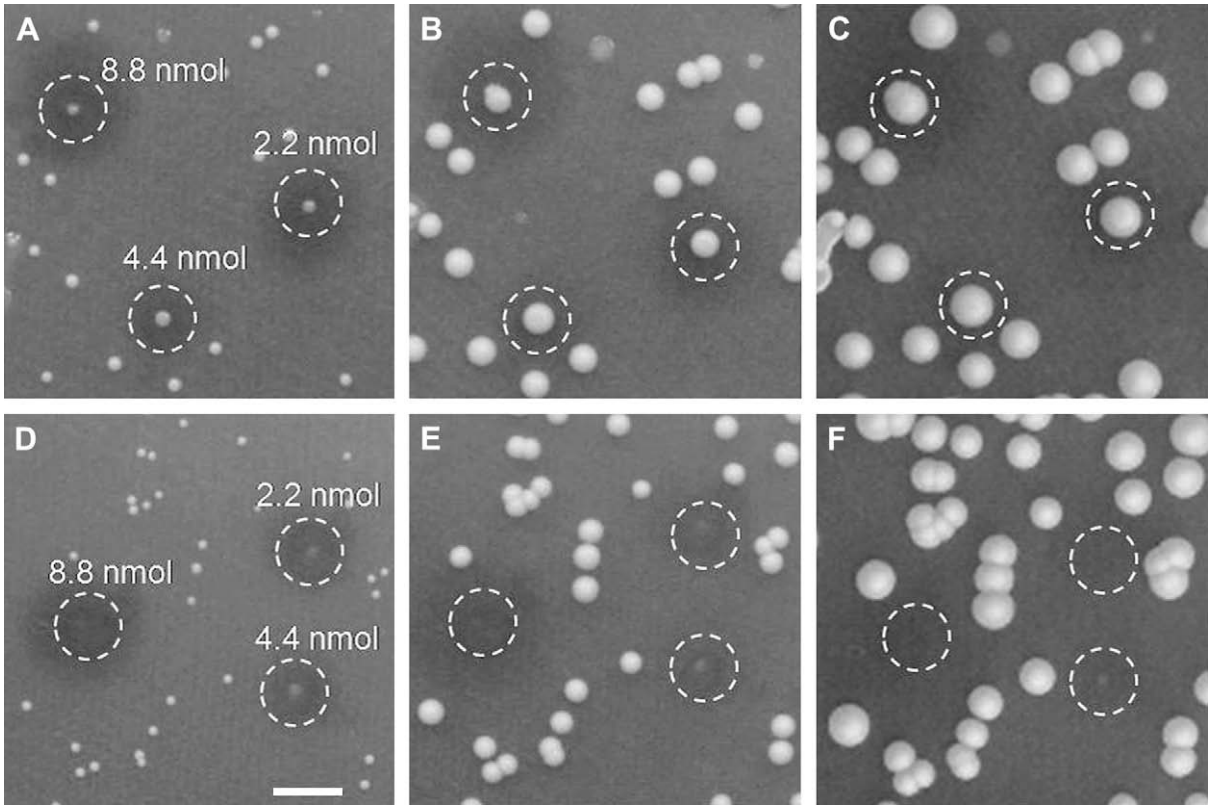


Fig. 6. Growth delay of *C. albicans* colonies on Sabouraud agar treated with different amounts of $TFAP^{3+}$. Sensitizer was spread on the colony from a stock solution (~0.5 mM) and the culture incubated for 10 min at 37 °C before irradiation. Control colonies were incubated at 37 °C in dark for 0 (A), 24 (B) and 48 h (C). Colonies were irradiated with visible light (90 mW/cm^2) for 3 h and incubated in dark for 0 (D), 24 (E) and 48 h (F). Scale bars: 5 mm.

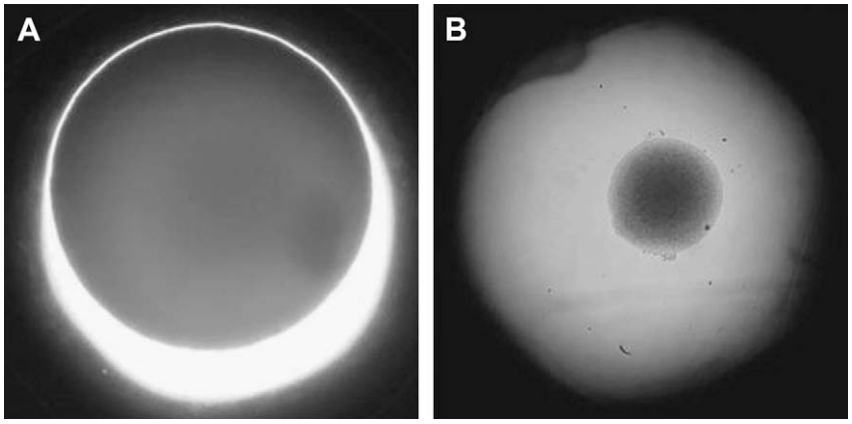


Fig. 7. Microscopic visualization (5×) of *C. albicans* colonies: (A) colony control without sensitizer, (B) colony treated with 8.8 nmol $TFAP^{3+}$. Colonies were irradiated with visible light for 3 h and incubated at 37 °C in dark for two days.

immobilized on Sabouraud agar. This approach can be used to inactivate *C. albicans* cells growing *in vivo* as localized foci of infection, on skin or on accessible mucous membrane [28,29]. Also, photodynamic treatment has been proposed as a new possibility for protecting foods from microbial spoilage [30].

Appropriated dilution of *C. albicans* cellular suspensions in PBS was spread on Sabouraud agar plates to obtain about 30 separated colonies. The cultures were incubated overnight at 37 °C to form colonies of ~1 mm of diameter. The colonies were treated with different amounts of porphyrin (2.2–8.8 nmol), which was homogeneously distributed on the colony from a stock solution in water. After that, the plates were irradiated with visible light for 3 h and they were incubated at 37 °C in dark. Characteristic results for $TFAP^{3+}$ porphyrin are shown in Fig. 6. As can be observed, growth delay of *C. albicans* colonies on Sabouraud agar was clearly evidenced for colonies treated with $TFAP^{3+}$ (Fig. 6D–F) with respect to dark control (Fig. 6A–C) or control without sensitizer. Comparable increase in the area size was also obtained for a control containing porphyrin but without irradiation. The colony area of the control increased ~5 times after one overnight and 12 times in a second overnight incubation at 37 °C, in contrast changes were not observed in colonies with PDI treatments. Thus, the growth delay obtained after irradiation of the cultures treated with the porphyrin is due to the photosensitization effect of the agent produced by visible light. Similar behaviour was observed for tetra-cationic $TMAP^{4+}$ porphyrin. In both cases, growth of the colonies was not detected even after six days post-PDI treatment. To confirm the results, samples of these colonies treated with sensitizer and irradiated were aseptically transferred to fresh Sabouraud broth medium and transferred to a new Sabouraud agar plate. After additional overnight incubation at 37 °C, the viability of the *C. albicans* cells was not detected by the formation of colonies, indicating a complete inactivation of yeast cells.

Both sensitizers produce similar photosensitization effect and the increase in the colony size was suppressed in a similar way using these cationic porphyrins. Thus, these results indicate a high efficiency in the growth delay of colonies for cells treated with $TFAP^{3+}$ or $TMAP^{4+}$ porphyrins.

Morphological changes of colonies were observed during the photodynamic experiments (Fig. 7). Control colonies are characterized by convex appearance and glossy colonies (Fig. 7A). In contrast, colonies under PDI treatment and after incubation in dark at 37 °C showed the appearance of Fig. 7B. As can be observed, the colony does not show evidence of growth and no viable cells remain after the PDI procedure. Therefore, the results indicated that under these experimental conditions *C. albicans* cells growing in small colonies (~1 mm diameter) can be efficiently controlled by PDI treatment with these cationic porphyrins.

4. Conclusions

Two cationic porphyrins were evaluated as photosensitizers to inactivate *C. albicans* cells in suspension of PBS and localized on agar surface. The studies indicate that the cationic porphyrins are tightly bound to *C. albicans* cells in comparison with anionic sensitizer, $TPPS^{4-}$. *In vitro* studies show that *C. albicans* cellular suspensions in PBS treated with 5 μM sensitizer are efficiently photoinactivated by these cationic porphyrins, whereas a negligible effect was found for $TPPS^{4-}$. An increase in the cellular photodamage was obtained enhancing the sensitizer concentration in the range 1–5 μM, which is mainly accompanied by a higher intracellular concentration of the porphyrin. In the other hand, PDI of *C. albicans* cell suspensions considerably diminishes at the same time as cellular densities increase. This effect is mainly produced by a major distribution of sensitizer between the number of cells. Thus the amount of sensitizer per cell is smaller at high densities and consequently it is accompanied by a minor cytotoxicity [15,27]. The photodamage induced by these cationic porphyrins was also confirmed for the growth delay studies, indicating a faster inactivation of the *C. albicans* cultures by tricationic $TFAP^{3+}$ porphyrin.

Photoinactivation activities of these sensitizers were analyzed in *C. albicans* cells growing in colonies immobilized on Sabouraud agar surfaces. The experiments with small colonies show that the growth delay of the colonies can be suppressed and a complete eradication of *C. albicans* cells is possible under these conditions. Thus, the photocytotoxic capacity of these cationic porphyrins remains even high for immobilized colonies of cells spread with the sensitizer. Therefore, these studies indicate that the $TFAP^{3+}$ and $TMAP^{4+}$ porphyrins are interesting sensitizers to be applied in treatment and control of *C. albicans* cells growing in cellular suspensions and as localized foci of infections by PDI. Further investigations of photosensitization *in vitro* are presently in progress in our laboratory to establish mechanistic aspects of *C. albicans* photoinactivation sensitized by these cationic porphyrins.

Acknowledgments

Authors are grateful to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina and Agencia Nacional de Promoción Científica y Tecnológica (FONCYT) for financial support. M.G.A. and E.N.D. are Scientific Members of CONICET. M.P.C. thanks CONICET for a research fellowship.

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