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Original article

Mechanistic aspects of the photodynamic inactivation of *Candida albicans* induced by cationic porphyrin derivatives

Ezequiel D. Quiroga [a], M. Paula Cormick [a], Patricia Pons [b], M. Gabriela Alvarez [a], Edgardo N. Durantini [a,*]

[a] Departamento de Química, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X5804BYA Río Cuarto, Córdoba, Argentina
[b] Centro de Microscopía Electrónica, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

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ABSTRACT

Photodynamic inactivation of *Candida albicans* produced by 5-(4-trifluorophenyl)-10,15,20-tris(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TFAP^{3+}$), 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) and 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$) was investigated to obtain insight about the mechanism of cellular damage. In solution, absorption spectroscopic studies showed that these cationic porphyrins interact strongly with calf thymus DNA. The electrophoretic analysis indicated that photocleavage of DNA induced by $TFAP^{3+}$ took place after long irradiation periods (>5 h). In contrast, $TMAP^{4+}$ produced a marked reduction in DNA band after 1 h irradiation. In *C. albicans*, these cationic porphyrins produced a ~3.5 log decrease in survival when the cell suspensions (10^7 cells/mL) were incubated with 5 μM photosensitizer and irradiated for 30 min with visible light (fluence 162 J/cm^2). After this treatment, modifications of genomic DNA isolated from *C. albicans* cells were not found by electrophoresis. Furthermore, transmission electron microscopy showed structural changes with appearance of low density areas into the cells and irregularities in cell barriers. However, the photodamage to the cell envelope was insufficient to cause the release of intracellular biopolymers. Therefore, modifications in the cytoplasmic biomolecules and alteration in the cell barriers could be mainly involved in *C. albicans* photoinactivation.

1. Introduction

Fungal diseases represent a critical problem to health being one of the main reasons of morbidity and mortality worldwide [1]. *Candida albicans* is one of the most frequent species recovered from hospitalized patients. Therefore, *C. albicans* is considered as a frequent cause of hospital acquired and device-related infections [2]. In the hospital, *Candida* persists on colonized individuals and causes several different infections, depending on patient's immune system status [3]. Also, superficial skin mycosis produced by *Candida* species is one of the most frequent diseases in humans. Candidiasis is the most common type of yeast infection. Treatments for *Candida* infections are difficult due to the eukaryotic nature of fungal cells, which are similar to host cells. Only a few antifungal agents are in clinical use and the therapies are limited by drug-safety considerations. Although azoles have revolutionized the treatment of mycosis there are some disadvantages that remain unsolved. Azoles do not affect only lanosterol 14α-demethylase (cytochrome P450 51, CYP51) but also some mammalian xenobiotic-metabolizing CYP enzymes resulting in a wide range of interactions with other drugs [4]. Further increasing problem is the emergence of resistant yeast strains due to extensive and prolonged use of antifungal agents [5,6]. Consequently, the search for new effective antifungal treatments is awfully necessary. Photodynamic inactivation (PDI) of microorganisms has been proposed a new alternative to controlling *C. albicans* infections [7,8]. This approach combines a photosensitizer, visible light and oxygen to produce highly reactive oxygen species (ROS), which rapidly react with a variety of substrates inducing damage in biomolecules. These changes produce a loss of biological functionality leading to cell inactivation [9].

Previous studies have shown that cationic porphyrin derivatives are effective photosensitizers against fungi [10–13]. It was established that an amphiphilic tricationic porphyrin, 5-(4-trifluorophenyl)-10,15,20-tris(4-*N*,*N*,*N*-trimethylammonium phenyl)porphyrin ($TFAP^{3+}$, Scheme 1) is an effective photosensitizer for the eradication of *C. albicans* [12]. Moreover, 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$, Scheme 1)

* Corresponding author. Tel.: +54 358 4676157; fax: +54 358 4676233.
E-mail address: edurantini@exa.unrc.edu.ar (E.N. Durantini).

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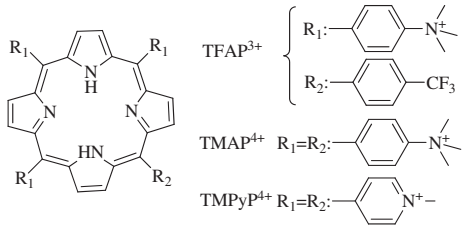

Scheme 1. Molecular structures of cationic porphyrins.

and 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$, Scheme 1) are recognized tetracationic macrocycles used to photoinactivate *C. albicans* cultures [12,13]. Studies of the photodynamic mechanism of action on *C. albicans* cells indicated that these cationic porphyrins appear to act as photosensitizers mainly via the intermediacy of singlet molecular oxygen, $O_2(^1\Delta_g)$ [14].

In the present work, we examined the photodynamic action of $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ *in vitro* to inactivate *C. albicans* cells. The aim was to characterize the photodynamic damages to yeast on the level of DNA by electrophoresis and the cell components observed by transmission electron microscopy (TEM). The hazard of DNA damages in eukaryotic fungi is further reduced by the presence of a membrane that envelopes the nucleus and may act as a barrier to the penetration of dyes or their high-energy photoproducts [15]. However, several cationic photosensitizers interact strongly with DNA and photosensitization of various types of eukaryotic cells could result in DNA lesions [16]. On the other hand, the fungal cell wall provides structure to the cell and protects the cell from the environment [17]. Therefore, the efficiency of the photosensitized process is markedly more pronounced by agents, which enhance their penetration into the inner cellular district. Thus, this investigation provides more insight into the specific mechanism of cellular photodamage, which causes the death of *C. albicans* after PDI treatment with these cationic porphyrins.

2. Materials and methods

2.1. General

Absorption spectra were recorded on an Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan) using 1 cm path length quartz cuvettes at room temperature. Irradiation experiments were performed using a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. Irradiation of the cultures was performed as previously described [18]. Experiments were performed at room temperature and there was no heating during irradiation. A wavelength range between 350 and 800 nm was selected by optical filters. The light fluence rate at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA) with a fluence of 162 J/cm^2 after 30 min irradiation. Electrophoresis was performed on a Shelton Scientific (Peosta, IA, USA) IBI MP-1015 Horizontal Gel Electrophoresis Unit and the electric field was generated by a power supply PS251-2 (Sigma–Aldrich Techware). Images of the stained gel were acquired using UV transillumination with an Alpha Innotech Corporation (San Leandro, CA, USA) Multi Image Light Cabinet with CCD camera. Chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Calf thymus double-stranded DNA was purchased from Sigma (St. Louis, MO, USA). Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Porphyrins

5,10,15,20-Tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl) porphyrin *p*-tosylate ($TMAP^{4+}$) and 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin *p*-tosylate ($TMPyP^{4+}$) were purchased from Aldrich. 5-(4-Trifluorophenyl)-10,15,20-tris(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin iodide ($TFAP^{3+}$) was synthesized as previously described [19]. Stock solutions (0.5 mM) of $TMAP^{4+}$ and $TMPyP^{4+}$ were prepared by dissolution in 1 mL of water, while $TFAP^{3+}$ was dissolved in *N*,*N*-dimethylformamide (DMF). The concentration was checked by spectroscopy, taking into account the value of molar absorptivity [19,20].

2.3. Porphyrin binding to calf thymus DNA

The concentration of calf thymus double-stranded DNA stock solution (1.3×10^{-4} M), calculated in base pairs, was determined spectrophotometrically using molar absorptivity $\varepsilon_{260} = 1.31 \times 10^4\ M^{-1}\ cm^{-1}$ [21]. Absorbance titrations were conducted by adding concentrated stock solution of DNA directly to a cuvette containing the porphyrin solution (2 mL, ~2 μM) in PBS. The apparent binding constants (K_{DNA}) for porphyrin–DNA complex were calculated from the absorbance changes at the *Soret*-band maximum (ΔA), assuming a 1:1 stoichiometry and that the DNA concentration is always significantly larger than the porphyrin concentration, using Equation (1), where ΔA_∞ represents the extrapolated absorbance change at [DNA] → ∞ [21,22].

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_\infty} + \frac{1}{\Delta A_\infty\ K_{DNA}\ [DNA]} \qquad [1]$$

A plot of $1/\Delta A$ *vs* 1/[DNA] was used to calculate the value of K_{DNA} from the ratio of the intercept to the slope.

2.4. Steady state photolysis of calf thymus DNA and electrophoresis

A mixture of 1.3×10^{-4} M calf thymus DNA and 20 μM sensitizer in 1 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) was irradiated in polypropylene 1.5 mL Eppendorf tubes with visible light (90 mW/cm^2) at room temperature. The tubes were placed vertically with the cap open in the path of the visible beam and irradiated for 1, 2, 3, 4 and 5 h. After irradiation, the samples were analyzed by electrophoresis. A sample of 15 μL was taken each time and gently mixed with 3 μL of Loading Buffer 6X (bromophenol blue 0.25% w/v, sucrose 40% w/v, acetic acid 1.15%, Tris 40 mM, EDTA 1 mM). DNA was analyzed by electrophoresis 1% agarose gel in TBE buffer (90 mM Tris–HCl, 90 mM boric acid and 2 mM EDTA, pH = 8) at 2.9 V/cm for 2 h. Ethidium bromide (1 mg/mL) was incorporated into the agarose gel. The Lamda DNA/*Hind* III (Promega, Madison, WI, USA) was used as molecular weight marker (MK) (0.5 μg/μL) with DNA fragments between 125 and 23,130 base pairs. The photographs were analyzed using ImageJ (National Institute of Health, USA) software to quantify the amount of DNA remaining after different treatments. Values of DNA photocleavage were determined by selecting an area between 4000 and 23,000 base pairs for each linea after different periods of irradiations.

2.5. Yeast strain and preparation of cultures

The strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified [12]. Yeast was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (3 mL) at 37 °C to stationary phase. An aliquot of this culture (1 mL) was dissolved in 3 mL Sabouraud broth. Then, 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. Cellular suspensions were serially diluted with PBS and each solution was plated in triplicate. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar plates after ~48 h incubation at 37 °C.

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

Cellular suspensions of *C. albicans* (~10^7 CFU/mL) in PBS were incubated with the 5 μM or 10 μM porphyrin for 30 min in the dark at 37 °C. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and the photosensitizer was added from the corresponding stock solution. After that, the cultures were exposed to visible light using the equipment aforementioned for 30 min. Cellular suspensions were serially diluted with PBS and the number of colonies formed was counted as described above. In all cases, control experiments were carried out in the absence of porphyrin with cellular suspensions irradiated and in the dark and in presence of porphyrin in the dark. Each experiment was repeated separately three times. To determine the half maximal inhibitory concentration (IC_{50}), the cell suspensions prepared as described above were treated with different concentrations of porphyrin (0.1–10 μM) for 30 min in dark at 37 °C and irradiated with visible light for 30 min.

2.7. Purification of C. albicans genomic DNA

Fungal cultures of *C. albicans* cells (~10^7 CFU/mL) were grown as described above. Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega). In PDI experiments, the cellular suspensions were treated as described above and genomic DNA was extracted from the cells immediately post irradiation. DNA samples were analyzed by electrophoresis as described above.

2.8. Transmission electron microscopy (TEM)

Cultures of *C. albicans* (~10^7 CFU/mL) were incubated with 5 and 10 μM photosensitizer by 30 min in dark. Then, cultures were irradiated as described above for 30 min. Samples were centrifuged (1000 rpm/10 min) and the cells were fixed in 4% w/v formaldehyde and 2% v/v glutaraldehyde mixture in cacodylate buffer 0.1 M for 2 h at room temperature. After that, the fungal cells were washed three times with cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer solution for 1–2 h at room temperature. The cells were dehydrated with gradients of acetone and embedded in Araldite epoxy resin and polymerized at 60 °C for 24 h. Thin sections (80–100 nm thick) were cut with a diamond knife on a JEOL JUM-7 ultramicrotome (Nikon, Tokyo, Japan). The samples were stained with uranyl acetate in alcoholic solution (2 min) and lead citrate (2 min). Finally, the samples were examined and digitally photographed with a transmission electronic microscopy Leo 906E equipped with a MegaView III camera.

2.9. Measurement of DNA and protein leakage

C. albicans cellular suspension (~10^7 CFU/mL) in PBS (2 mL) was obtained and treated as described above with 5 μM porphyrin. After that, the tubes were irradiated with visible light for 30 min. Each irradiated tube was centrifuged at 13,000 rpm for 5 min. The pellet was separated and the release of materials in the supernatant was monitored by UV–visible spectroscopy [23]. On the other hand, *C. albicans* cells were lysed by the freeze–thaw cycles in liquid nitrogen to obtain positive controls.

2.10. Statistical analysis

Data were depicted as the mean ± standard deviation of each group. Variation between each experiments was calculated using the Kruskal–Wallis test, with a confidence level of 95% ($p < 0.05$) considered statistically significant.

3. Results

3.1. Interaction of cationic porphyrins with calf thymus DNA

The affinity of cationic porphyrins for calf thymus double-stranded DNA was evaluated by absorption spectroscopic analysis in PBS. Titration of porphyrin with DNA produced mainly spectral changes in the *Soret* band. Typical results for $TFAP^{3+}$ and $TMPyP^{4+}$ in PBS are shown in Fig. 1A and B inset, respectively. It was assumed that spectral perturbations upon addition of DNA are due to association of the porphyrin with the DNA matrix. As can be seen in Table 1, these interactions are characterized by a bathochromic shift of the *Soret* maximum and by a hypochromicity (42–51%). These

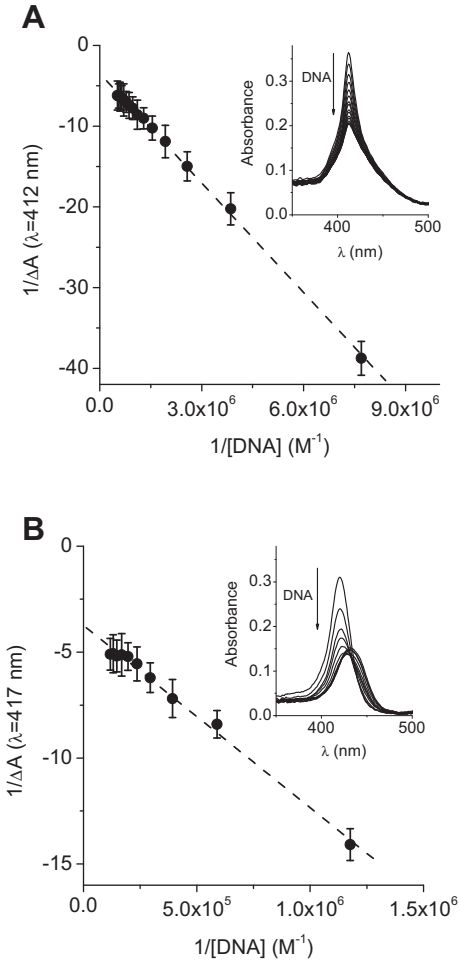

Fig. 1. Variation of 1/Δ*A vs* 1/[DNA] for spectral titration of (A) $TFAP^{3+}$ (DNA concentrations range 0.13–1.90 μM) and (B) $TMPyP^{4+}$ (DNA concentrations range 0.84–8.45 μM) with calf thymus DNA in PBS. Dashed line: linear fit by Equation (1). Inset: absorption spectra of porphyrin at different DNA concentrations. Values represent mean ± standard deviation of three separate experiments.

Table 1
Position of *Soret* band for complexes, bathochromic shift ($\Delta\lambda$), hypochromic effect (h) and apparent binding constant (K_{DNA}) of porphyrins with calf thymus DNA in PBS.

Porphyrin	*Soret* band (nm)	$\Delta\lambda$ (nm)	h (%)	K_{DNA} (M^{-1})
$TFAP^{3+}$	415	3	44	$(6.7 \pm 0.4) \times 10^5$
$TMAP^{4+}$	417	5	42	$(7.5 \pm 0.3) \times 10^5$[a]
$TMPyP^{4+}$	430	13	51	$(4.4 \pm 0.3) \times 10^5$

[a] From Ref. [12].

large hypochromicity suggests that porphyrin π electrons are perturbed by the association with DNA. Absorption spectra were analyzed through Equation (1) to obtain the values of apparent binding constant of porphyrin–DNA (K_{DNA}), as shown Fig. 1. The values of K_{DNA} are summarized in Table 1. In the three cases, high values of K_{DNA} were found indicating the formation of stable complexes with calf thymus DNA in PBS.

3.2. Photocleavage of calf thymus DNA sensitized by cationic porphyrins

Photoinduced damage of calf thymus DNA sensitized by these porphyrins was studied in TE buffer solution. Samples of DNA and photosensitizer were irradiated with visible light under aerobic condition for 1, 2, 3, 4 and 5 h. The DNA integrity was analyzed by agarose gel electrophoresis. The result for $TFAP^{3+}$ is shown in Fig. 2. No cleavage was observed without irradiation, indicating that cleavage of DNA take place by photodynamic action of the porphyrin. In general, DNA photodamage can be observed by the appearance of a remarkable smearing after electrophoresis. As can be observed, a slightly modified pattern for DNA with respect to control (Fig. 2, line 2) was found for samples photosensitized by $TFAP^{3+}$. The photocleavage is clearly evidenced after 5 h irradiation (Fig. 2, line 9). The photodynamic effect of $TMAP^{4+}$ on DNA was previously analyzed [24]. The comparison of the DNA cleaving efficiencies of $TFAP^{3+}$ and $TMAP^{4+}$ is shown in Fig. 3. The results indicate that $TMAP^{4+}$ produced a marked reduction (59.3%) in DNA band even after 1 h irradiation, while only 19.7% was calculated for $TFAP^{3+}$. Therefore, the photocleavage effect on DNA by $TFAP^{3+}$ was not very significant even after longer irradiation periods.

On the other hand, $TMPyP^{4+}$ bind strongly to DNA and this porphyrin co-electrophorese with the DNA reducing the electrophoretic mobility of the DNA (Fig. 4). This effect does not allow clearly observing the possible DNA photocleavage induced by $TMPyP^{4+}$ at different irradiation times. Despite this, it is possible to note that the DNA band disappeared after irradiation.

3.3. Photosensitized inactivation of C. albicans

Photoinactivation of *C. albicans* was evaluated in PBS cellular suspensions using $\sim 10^7$ cells/mL. The cultures were treated with 5 μM porphyrin for 30 min in dark at 37 °C and irradiated for 30 min with visible light. The results in Fig. 5 show that these cationic porphyrins exhibited a photosensitizing activity causing a ~3.5 log decrease of cell survival. On the other hand, control experiments indicated that the viability of *C. albicans* was unaffected by illumination alone or by dark incubation with 5 μM photosensitizer. Thus, under these experimental conditions these cationic porphyrins evidenced a comparable photodynamic activity to decrease the amount of viable *C. albicans* cells. Also, IC_{50} values were estimated for $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ under the above conditions. The susceptibility of *C. albicans* organism to the photodynamic activity of these cationic porphyrins depends of the light dose. Therefore, after 30 min of irradiation, the values of IC_{50} were 0.16, 0.23 and 0.41 μM for $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$, respectively. These results evidences a higher photoinactivation induced by the tricationic porphyrin derivative.

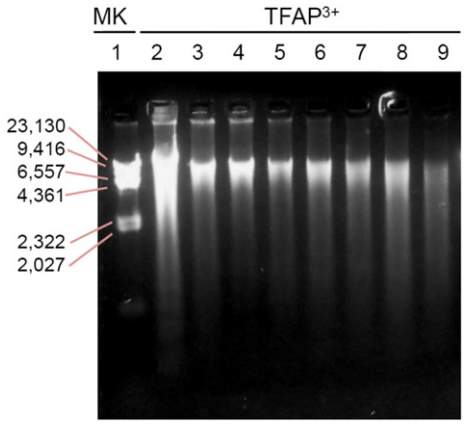

Fig. 2. Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated by 1, 2, 3, 4 and 5 h (lines 5–9, respectively) with visible light (90 mW/cm^2) in the presence of $TFAP^{3+}$ (20 μM). Line 1: DNA weight maker, line 2: DNA control, line 3: DNA control with $TFAP^{3+}$ (20 μM) in dark, line 4: irradiated DNA control.

3.4. PDI effect on C. albicans genomic DNA

The photodynamic activity of cationic porphyrins was evaluated *in vitro* to observe possible genomic DNA photodegradation. In these experiments, genomic DNA was extracted from *C. albicans* cells after PDI treatment with 5 μM sensitizer and exposed to visible light for 30 min. The extracts were analyzed by agarose gel electrophoresis as showed in Fig. 6. In all cases, an unmodified pattern of DNA was found for cells treated with porphyrin with respect to the control assay. Therefore, these cationic porphyrins did not cause any significant cleavage of genomic DNA even after 30 min exposure to visible light. However, it should be considered either the genomic DNA was not involved in the photodamage process or the overall DNA degradation of a few lesions was not sufficient to induce DNA strand breaks.

3.5. Photodynamic effect on cellular ultrastructure of C. albicans

In order to investigate at morphological level the photocytotoxic effect of the different porphyrins, the cell structure of *C. albicans*

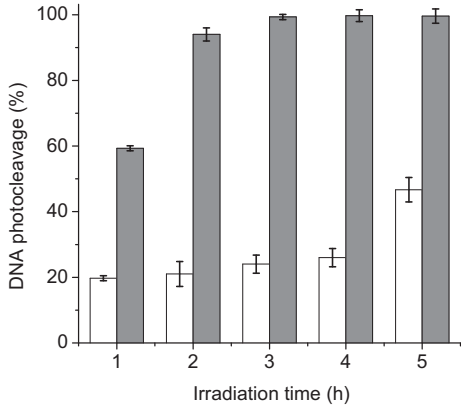

Fig. 3. Photocleavage of DNA (%) (fragments between 4000–23,000 base pairs) sensitized by $TFAP^{3+}$ (white bars) and $TMAP^{+4}$ (grey bars) porphyrins irradiated by 1, 2, 3, 4 and 5 h (lines 1–5, respectively) with visible light (90 mW/cm^2).

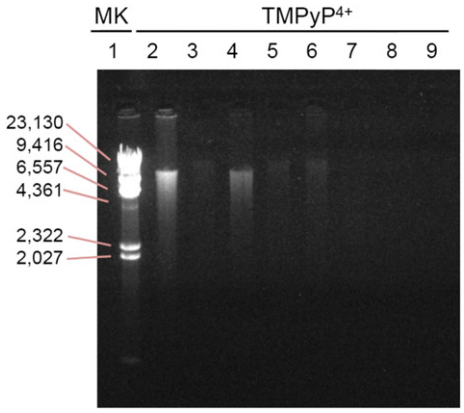

Fig. 4. Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated by 1, 2, 3, 4 and 5 h (lines 5–9, respectively) with visible light (90 mW/cm^2) in the presence of TMPyP^{4+} (20 μM). Line 1: DNA weight maker, line 2: DNA control, line 3: DNA control with TMPyP^{4+} (20 μM) in dark, line 4: irradiated DNA control.

cells were analyzed by transmission electron microscopy (TEM). For this purpose, *C. albicans* cells were treated with 5 and 10 μM porphyrin for 30 min in dark and the cultures irradiated with visible light for 30 min before the TEM images. The results for 5 and 10 μM porphyrin are shown in Figs. 7 and 8. As can be observed, at both concentration images manifested alterations at ultrastructural level of the treated *C. albicans* cells, which were not detected in the controls.

Untreated fungal cells showed typical morphology of *C. albicans* with an irregularly shaped nuclei limited by double membranes. Mitochondria containing many cristae were randomly distributed throughout the cytoplasm whose density depended largely upon the amount of ribosomes. Round shaped bodies, characterized by a single limiting membrane and a dense matrix, lipid-like droplets and several elements of endomembrane were seen in the cytoplasm. The cell wall was characteristically thick and the intact plasmalemma presented short invaginations into the cytoplasm (Figs. 7 and 8A). TEM images after PDI treatment are shown in Figs. 7 and 8B–D, for cells treated with TFAP^{3+}, TMAP^{4+} and

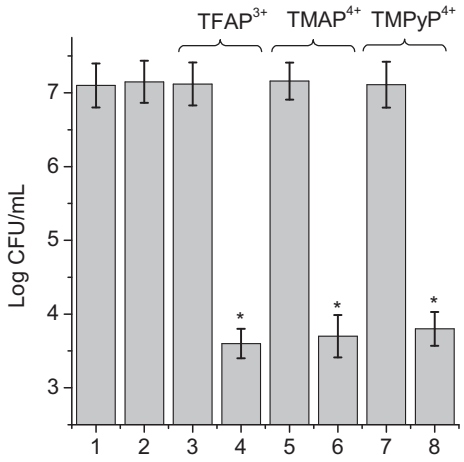

Fig. 5. Survival of *C. albicans* (~10^7 CFU/mL) incubated with 5 μM porphyrin for 30 min at 37 °C in dark and exposed to visible light for 30 min (fluence 162 J/cm^2); 1) control culture in dark; 2) control culture irradiated; 3) control culture treated with TFAP^{3+} and keeping in dark; 4) culture treated with TFAP^{3+} and irradiated; 5) control culture treated with TMAP^{4+} and keeping in dark; 6) culture treated with TMAP^{4+} and irradiated; 7) control culture treated with TMPyP^{4+} and keeping in dark; 8) culture treated with TMPyP^{4+} and irradiated. Values represent mean ± standard deviation of three separate experiments. $^*p < 0.05$, compared with untreated yeast.

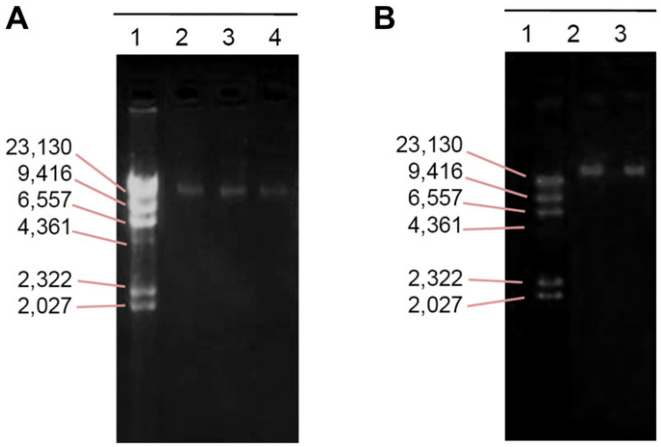

Fig. 6. Agarose gel electrophoresis of genomic DNA samples extracted from *C. albicans* cells after PDI treatment. The cultures were treated with 5 μM porphyrin for 30 min at 37 °C in dark and irradiated for 30 min with visible light (fluence 162 J/cm^2) (A) Line 1: DNA weight maker, line 2: irradiated DNA control, line 3: in presence of TMAP^{4+}, line 4: in presence of TFAP^{3+}; (B) Line 1: DNA weight maker, line 2: irradiated DNA control, line 3: in presence of TMPyP^{4+}.

TMPyP^{4+}, respectively. Representative results about the photocytotoxic effect produced by cationic porphyrin derivatives showed marked changes in the wall, membrane and cytoplasm in comparison to that for the control. An enlargement of TEM images of *C. albicans* treated with TMPyP^{4+} is shown in Fig. 9. The cell appeared very dense with the vesicles and membranous bodies disposed within the cell. Also, photoinactivation produced cells that present the appearance of low-density areas and the cytoplasmatic volume started to decrease.

3.6. Photosensitized effect on the leakage of intracellular DNA and proteins

The release of biopolymers was monitored by UV absorption at 260 nm as an indication of membrane damage. Thus, *C. albicans* cellular suspensions (~10^7 cells/mL) were treated with 5 μM porphyrin for 30 min in dark and irradiated with visible light for 30 min. The cultures were centrifuged and the supernatants analyzed by UV spectroscopy (Fig. 10). In all these cases, the absorbance at 260 nm was almost unchanged. Therefore, this PDI treatment of *C. albicans* cells did not induce fast release of λ ~260 nm absorbing materials from cells. This suggests that although the functionality of the membrane can be damaged, it was not a sufficient amount to produce release of higher molecular weight species.

4. Discussion

The tetracationic porphyrins, TMAP^{4+} and TMPyP^{4+}, are among the most active water-soluble photosensitizers. These porphyrin derivatives contain four cationic groups in the periphery of the macrocycle. In contrast, TFAP^{3+} bears three cationic charges and a highly lipophilic trifluoromethyl group that increases the amphiphilic character of the structure (Scheme 1). These porphyrins present a high efficiency in the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) of 0.77 for TMAP^{4+} and 0.74 for TMPyP^{4+} in water [25,26],while a value of 0.44 was previously calculated for TFAP^{3+} in DMF [27].Also, TFAP^{3+} is an efficient photosensitizer to produce photooxidation of biological substrates, such as the amino acid L-tryptophan and the nucleotide guanosine 5′-monophosphate [27,28].

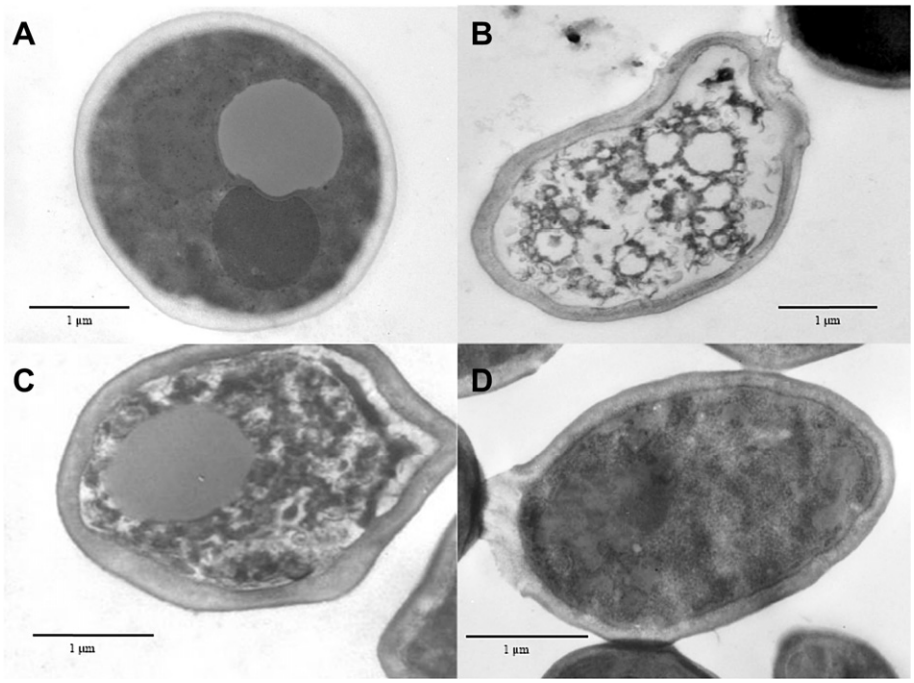

Fig. 7. Transmission electron microscopy (TEM) of *C. albicans* treated with 5 µM porphyrin for 30 min at 37 °C in dark and irradiated for 30 min with visible light (fluence 162 J/cm^2). (A) Control, (B) TFAP^{3+}, (C) TMAP^{4+} and (D) TMPyP^{4+} (magnification: 21,560).

The studies in presence of calf thymus DNA showed that TFAP^{3+} strongly interacts with the double-stranded DNA in PBS solution (Fig. 1). The value of K_{DNA} for TFAP^{3+} is similar to that previously reported for a tricationic 5-[4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl]-10,15,20-tris(4-trifluoromethylphenyl)porphyrin [24] . Moreover, comparable K_{DNA} value was reported for a tricationic *meso*-5,10,15-tris(*N*-methyl-4-pyridiniumyl)-20-(4-perfluorocapromidophenyl)porphyrin, bearing a hydrophobic fluorinated alkyl chain [29]. On the other hand, spectroscopic studies showed a high affinity of TMAP^{4+} and TMPyP^{4+} towards DNA (Table 1). Spectral features of TMPyP^{4+}–DNA complexes have been reported [21,30]. We observed a similar bathochromic shift of the *Soret* band concomitant with a large hypochromicity (Fig. 1B). Comparing the two tetracationic porphyrins, a higher value of K_{DNA} was found for

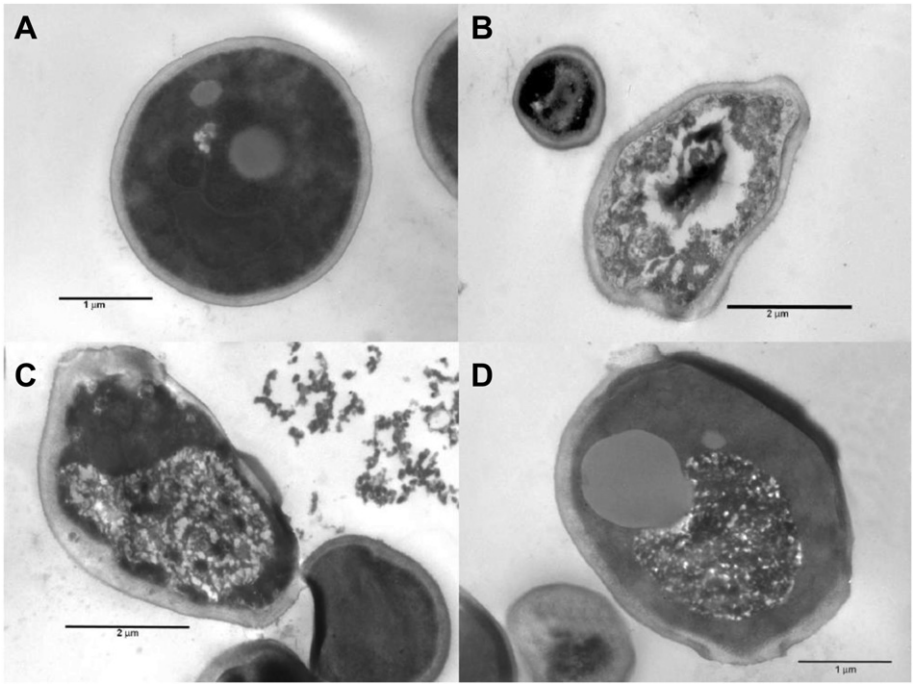

Fig. 8. Transmission electron microscopy (TEM) of *C. albicans* treated with 10 µM porphyrin for 30 min at 37 °C in dark and irradiated for 30 min with visible light (fluence 162 J/cm^2). (A) Control, (B) TFAP^{3+}, (C) TMAP^{4+} and (D) TMPyP^{4+} (magnification: 21,560).

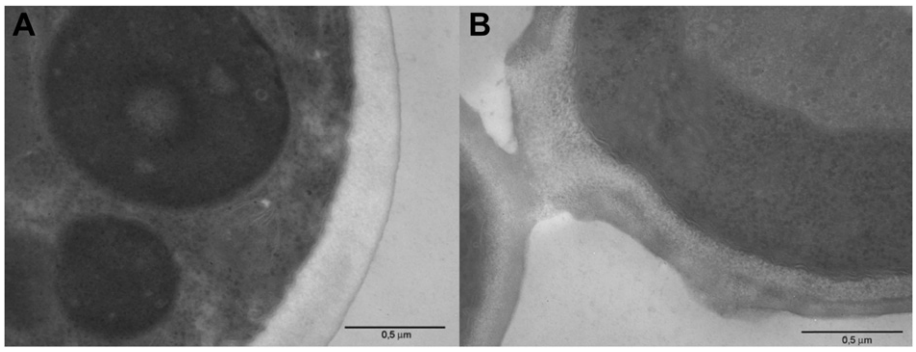

Fig. 9. Transmission electron microscopy (TEM) of *C. albicans* treated with 10 μM $TMPyP^{4+}$ for 30 min at 37 °C in dark and irradiated for 30 min with visible light (fluence 162 J/cm^2). (A) Control, (B) $TMPyP^{4+}$ (magnification: 46,000).

$TMAP^{4+}$ with respect to $TMPyP^{4+}$. Although, $TFAP^{3+}$ has three cationic groups, the K_{DNA} value is comparable with those for $TMAP^{4+}$ and $TMPyP^{4+}$. Possibly, the amphiphilic structure of $TFAP^{3+}$ could promote the binding with DNA.

Photoinduced damage of calf thymus DNA in TE buffer solution indicated that although $TMAP^{4+}$ and $TFAP^{3+}$ have similar values of K_{DNA}, the photodynamic effect on DNA is quite different. DNA cleaving efficiencies of $TFAP^{3+}$ and $TMAP^{4+}$ (Fig. 3) showed a reduction of >90% for $TMAP^{4+}$ after 2 h irradiation, while the DNA photodamage sensitized by $TFAP^{3+}$ reached a ~50% after a long period of irradiation of 5 h. In presence of $TMPyP^{4+}$ was observed a reduction in the migration rate of DNA, preceding at least a partial reversal of the direction of migration. This delay in the DNA migration can be explained by the charge effect of the cationic porphyrin [31]. It was previously demonstrated the co-electrophoresis of DNA and $TMPyP^{4+}$ [32]. This tetracationic porphyrin migrates to the cathode when subjected to electrophoresis, while DNA, being negatively charged, moves in the opposite direction. As $TMPyP^{4+}$ bind strongly to DNA it is expected to co-electrophorese with the DNA and it reduces the electrophoretic mobility of the DNA. Although the binding constants of the three cationic porphyrins present high values (Table 1), however, these K_{DNA} values do not take into account the type of binding. In general, three binding models have been described for the interaction of cationic porphyrins with DNA, which involve intercalation, outside groove binding and outside binding with porphyrins self-stacking [33]. It was previously established that $TMPyP^{4+}$ is capable of intercalating in calf thymus DNA and the binding of the porphyrin results in an unwinding of covalently closed superhelical DNA [30,34]. Also, it was found that the photodynamic effect produced the conversion of pBR322 from its closed circular to open circular form upon visible light irradiation in the presence of $TMPyP^{4+}$ [32].

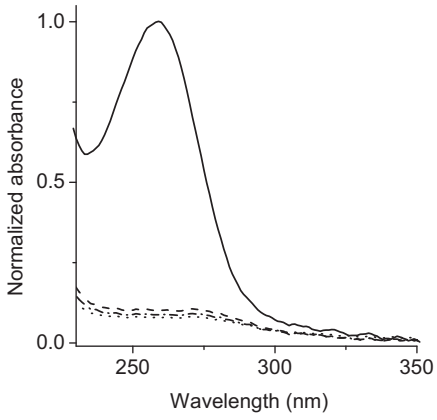

Fig. 10. Release of absorbing material from *C. albicans* cells treated with 5 μM $TFAP^{3+}$ (dashed line), $TMAP^{4+}$ (dotted line) and $TMPyP^{4+}$ (dashed-dotted line) porphyrins for 30 min in dark and irradiated for 30 min with visible light (fluence 162 J/cm^2). *C. albicans* cells subjected to the freeze–thaw cycles.

In vitro investigations showed that $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ are active photosensitizers to inactivate *C. albicans* cells. After 30 min incubation with 5 μM porphyrin, the PDI observed for the three compounds was similar in PBS cell suspensions of 10^7 CFU/mL (Fig. 5). A photoinactivation greater than 99.95% of cell death was found after 30 min irradiation. Under similar condition but using 10^6 CFU/mL, these cationic porphyrins exhibited a photosensitizing activity causing a ~5 log decrease of cell survival. These results represent a value greater than 99.999% of cellular inactivation [12,13]. Thus, the effectiveness of PDI decreases significantly with an increase in the cell density. Since the vast majority of the cationic porphyrin is bound to the cells, when the cell number is increased the amount of photosensitizer bound to each cell decreases producing a lower cell death. This behaviour was also observed with other photosensitizers, such as hypericin on various *Candida* spp. [35]. Moreover, the photoinactivation of *C. albicans* cells induced by these photosensitizers was studied under different experimental conditions to elucidate the oxidative processes that occur during the killing of yeast [14]. It was observed that an oxygen atmosphere is necessary for an efficient photoinactivation. Moreover, photooxidative cell killing was further enhanced in D_2O due to a prolonged lifetime of $O_2(^1\Delta_g)$. Phototoxicity efficacy was not affected when mannitol was used as a type I scavenger, in contrast photoprotection was found using sodium azide as type II scavengers. Also, the chemical $O_2(^1\Delta_g)$ quencher consumption inside living cells was demonstrated via time resolved $O_2(^1\Delta_g)$ detection. In the presence of these cationic porphyrins the time-resolved luminescence of $O_2(^1\Delta_g)$ showed a short decay time, indicating that it is strongly quenched by the biomolecules. Thus, the killing of *C. albicans* cells by these cationic porphyrins and visible light irradiation seem to be mediated mainly by $O_2(^1\Delta_g)$ [14].

The photodynamic activity produced by $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ was analyzed exploring the effect on *C. albicans* DNA and cellular ultrastructure incurred during PDI. Even when these porphyrins showed different photocleavage ability in solution, it was found that genomic DNA isolated from *C. albicans* was not significantly cleaved after PDI treatment (Fig. 6). However, after 30 min irradiation practically all yeast cells were photoinactivated. Therefore, the results of the present study about DNA integrity extracted from *C. albicans* indicated that genomic DNA remained practically intact. Also, the samples of DNA are moved in the same way than the control (Fig. 6), indicating that $TMPyP^{4+}$ is not interacting with the DNA extracted from the cells.

On the other hand, TEM analysis showed alterations in the ultrastructure of the *C. albicans* cells treated with these porphyrins after PDI treatment (Figs. 7–9). After 30 min of irradiation before the TEM images, the cells exhibited notable structural disorganization within the cell cytoplasm with alterations in the cell envelope. A certain number of cells apparently underwent shape changes characterized by same cell wall swelling. In Fig. 9, the control cells showed round bodies, characterized by a single limiting membrane, a moderately dense matrix with a central electron-lucid core. In contrast, cells treated with $TMPyP^{4+}$ and irradiated for 30 min were typified by cytoplasm densely packed ribosomes and smooth endoplasmic reticulum. Therefore, oxidative reactions involving $O_2(^1\Delta_g)$ may be mainly responsible for the effects on cytoplasmatic and cell wall components stimulating the accumulation of macromolecules and conducing to alterations of the cell envelope structure. This process can lead to the inability to multiply and to produce viable *C. albicans* cells. Differences on the cellular surface were also observed via freeze-fracture electron microscopy images for *C. albicans* following PDI with 5-phenyl-10,15,20-tris(*N*-methyl-4-pyridyl)porphyrin [10]. The cytoplasmic cell membrane is the target for many antifungal agents. Also, the TEM examinations indicated some evident membrane damages with clear swelling of the cell wall for PDI of *C. albicans* in presence of 5-aminolaevulinic acid [36]. The damage of fungi membrane can be first accompanied by leached out low molecular weight species and followed by nucleotides and other materials [23]. However, in the present investigation the release of intracellular biopolymers upon photodynamic action was not detected. Therefore, modifications in the cytoplasmic macromolecules and alteration in the cell barriers induced by the photodynamic action of these cationic porphyrins play a major role in *C. albicans* inactivation.

5. Conclusions

The present study provides knowledge about the cell damage photosensitized by $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ after the PDI of *C. albicans* cells. Even though these porphyrins strongly interact with DNA, photocleavage of genomic DNA was not found after PDI. This may be because DNA damage becomes visible when the electrophoretic pattern of the genomic DNA is strongly modified and this could occur only after a long period of irradiation. However, after an exposure to visible light for 30 min the amount of cellular inactivation was practically complete. Thus, DNA damage does not contribute to the cytotoxicity of the cells at least under these conditions of PDI treatment. In contrast, the photooxidative processes in the cell led to alterations in the cytoplasmatic components and in the cellular barrier. Although this damage did not conduce to a release of intracellular biomolecules, it can interfere with membrane functions and causes the inactivation of *C. albicans* cells.

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