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Photodynamic Action Mechanism Mediated by Zinc(II) 2,9,16,23-Tetrakis [4-(*N*-methylpyridyloxy)]phthalocyanine in *Candida albicans* Cells†

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ABSTRACT

The photoreaction type I/type II pathways mediated by zinc (II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine ($ZnPPc^{4+}$) was studied in *Candida albicans* cells. This photosensitizer was strongly bound to *C. albicans* cells at short times. After 30 min irradiation, 5 μM $ZnPPc^{4+}$ produced ~5 log decrease in cell viability. Different probes were used to detect reactive oxygen species (ROS) in cell suspensions (~10^6 CFU mL^{-1}). Singlet molecular oxygen, $O_2(^1\Delta_g)$, was observed by the reaction with 9,10-dimethylanthracene (DMA) and tetrasodium 2,2-(anthracene-9,10-diyl)bis(methylmalonate) (ABMM), whereas the nitro blue tetrazolium (NBT) method was used to sense superoxide anion radical ($O_2^{\cdot-}$). Moreover, the effects produced by an anoxic atmosphere and cell suspensions in D_2O, as well as the addition of sodium azide and mannitol as ROS trapping were evaluated in the PDI of *C. albicans*. These investigation indicates that $O_2(^1\Delta_g)$ is generated in the cells, although a minor extension other radical species can also be involved in the PDI of *C. albicans* mediated by $ZnPPc^{4+}$.

INTRODUCTION

In recent years, the rising incidence of *Candida* infections is the consequence of many factors, most remarkably the rise of severely immunocompromised patients (1). *Candida albicans* yeast is known as an opportunistic fungal microorganism, which can cause infections of the skin, mucosal or systemic. The interaction between *C. albicans* and its host is dynamic and complex. This pathogen exhibits multifaceted strategies for growth, proliferation and survival within the host (2). Novel antifungal agents have recently contributed to the effective therapies. However, inadequate dosing may contribute to treatment failure and the emergence of resistance (3). In the search for new alternative therapies, the photodynamic inactivation (PDI) of microbes has been suggested to controlling fungal diseases (4,5). PDI involves the addition of a phototherapeutic agent, which preferentially binds to the microorganisms. After irradiation of the affected area with visible light, microbial cells are inactivated (6). In presence of molecular oxygen, the triplet excited state of the photosensitizer (^{3}Sens*) can produce reactive oxygen species (ROS). This process can involve energy transfer to form singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II pathway) or electron transfer to generate superoxide anion radical ($O_2^{\cdot-}$) (type I pathway). Also, $O_2^{\cdot-}$ can produce hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) (7,8). The electron transfer type of reaction mainly occurs in a polar microenvironment in the presence of reducing agents.

It was observed that the type II mechanism played the most important role on the PDI process of a bioluminescent recombinant *Escherichia coli* by the cationic porphyrins (9). It was concluded that photoinactivation of *Streptococcus mitis* induced by a tetracationic phtalocyanine mainly involved a type II pathway (10). Also, the photodynamic mechanism of mammalian viruses and bacteriophage photosensitization involved $O_2(^1\Delta_g)$ with a slight influence of free radical species (8). Type II mechanism appears to be the main ROS involved in nonenveloped DNA and RNA bacteriophages photoinactivation by cationic photosensitizers (11). In yeast, the photokilling of *C. albicans* cells by cationic porphyrins seem to be mediated mainly by $O_2(^1\Delta_g)$ (12). However, even in the same nature of microorganisms, photodynamic mechanism can be considerably influenced by the photosensitizer with a significant contribution of type I process (13,14).

It was previously established that zinc(II) 2,9(10),16(17),23(24)-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine ($ZnPPc^{4+}$, Scheme 1) is an active photosensitizer for the eradication of bacteria, such as *E. coli* and *S. mitis* (15,16). Moreover, optimal conditions for cellular uptake of $ZnPPc^{4+}$ were determined in *C. albicans* cell suspensions to obtain maximal yeast photoinactivation (17). Therefore, in this study we are interested to obtain mechanistic insight about type I/type II processes involved in the photoinactivation of *C. albicans* cells mediated by $ZnPPc^{4+}$. In this way, PDI of this yeast was investigated in presence of specific molecular probes to sense the presence of ROS, such as $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$. Also, photoinactivation was studied under different experimental conditions and in presence of scavengers of ROS to find information about the photoprocesses that produce the cell death.

MATERIALS AND METHODS

General. UV-visible absorption spectra were performed in a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Steady-state fluorescence emission spectra were recorded using a Spex FluoroMax

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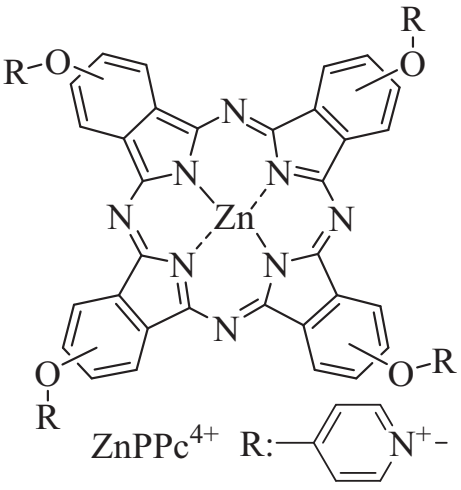

Scheme 1. Chemical structure of phthalocyanine $ZnPPc^{4+}$.

spectrofluorometer (Horiba Jobin Yvon Inc, Edison, NJ). Fluence rates were obtained with a Radiometer Laser Mate-Q (Coherent, Santa Clara, CA). All chemicals were purchased from Aldrich (Milwaukee, WI) and used without further purification. 9,10-Dimethylanthracene (DMA) was purchased from Aldrich. Tetrasodium 2,2′-(anthracene-9,10-diyl)bis (methylmalonate) (ABMM) was prepared as previously described (18).

Photosensitizer. $ZnPPc^{4+}$ was obtained as previously reported (15). A stock solution 0.5 mM $ZnPPc^{4+}$ was obtained in 1 mL of *N,N*-dimethylformamide (DMF). $ZnPPc^{4+}$ concentration was established by absorption taking into account the molar extinction coefficient, $\varepsilon = 1.1 \times 10^5\ M^{-1}\ cm^{-1}$ at 678 nm in DMF (15).

Strain and preparation of cultures. Candida albicans PC31 cells were identified and characterized as previously reported (19). Fungal cells were cultivated in Sabouraud (Britania, Buenos Aires, Argentina) broth under aerobic condition at 37°C. After overnight cultures, cells were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in phosphate-buffered saline (PBS, 10 mM, pH = 7.0) solution. Cells were diluted in PBS to obtain ~10^6 colony forming units (CFU) mL^{-1}. Cell suspensions were quantified by the spread plate technique on Sabouraud agar plates after ~48 h incubation at 37°C in triplicate (17).

Steady-state photolysis. Photooxidation of substrates were carried out in a quartz cell of 1 cm path length using a Cole-Parmer illuminator 41720-series (Cole-Parmer, Vernon Hills, IL) with a 150 W halogen lamp. A wavelength range between 455 and 800 nm (30 mW cm^{-2}) was selected using an optical filter (GG455 cutoff filter). Cell suspensions of *C. albicans* (2 mL, ~10^6 CFU mL^{-1}) were treated with DMA or ABMM (10 μM) for 30 min in dark at 37°C. Cells were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in 2 mL PBS. Then, different protocols were followed: (1) cells were incubated with 5 μM $ZnPPc^{4+}$ for 10 min in dark at 37°C; (2) the treated cells as described in a) were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in 2 mL PBS; and (3) cells were incubated with 5 μM $ZnPPc^{4+}$ and 100 mM NaN_3 for 10 min in dark at 37°C. This incubation time of only 10 min was chosen because the same uptake was obtained between 5 and 30 min (17). Photooxidation of DMA and ABMM were studied by following the decrease in the fluorescence intensity at λ = 403 nm and 405, respectively, exciting the samples at λ_{exc} = 378 nm. Control experiments showed that under these conditions the fluorescence intensity correlates linearly with substrate concentrations. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of semilogarithmic plots of ln (I_0/I) *vs*. time (18). The nitro blue tetrazolium (NBT) analysis was performed incubating *C. albicans* (2 mL, ~10^6 CFU mL^{-1}) with NBT (0.2 mM) for 30 min in dark at 37°C. Cells were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in 2 mL PBS. Then, cells were incubated with 5 μM and 0.5 mM NADH for 10 min in dark at 37°C. Cells were irradiated for different periods as described for DMA or ABMM. The formation of diformazan was observed by the increase in absorbance at λ = 560 nm (13).

Photosensitized inactivation of C. albicans. Yeast cells in PBS (2 mL, ~10^6 CFU mL^{-1}) were traded with different concentration of $ZnPPc^{4+}$ (2.5 and 5.0 μM) in Pyrex culture tubes (13 × 100 mm) in the dark for 30 min at 37 °C. Then, the culture tubes were irradiated for 30 min (54 J cm^{-2}) using a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector with a 150 W lamp and a wavelength range between 350 and 800 nm (30 mW cm^{-2}). The heat from the lamp was removed with a 2.5 cm thick glass cuvette filled with water. Irradiation of cells in presence of anthracene derivative was performed using the GG455 cutoff filter as described above. Experiments in anoxic conditions were carried out with argon to remove the oxygen. The culture tube was resealed with a rubber septum in the dark. The septum was pierced with a hollow needle connected to argon line and samples were bubbled with this gas for 15 min before irradiation. An argon atmosphere was keeping in the sample during the irradiation. Experiments in D_2O were performed using cell suspensions (2 mL) in PBS, which were centrifuged (3000 rpm for 15 min) and re-suspended in D_2O (2 mL). Then the cell suspensions in D_2O were incubated with 2.5 μM $ZnPPc^{4+}$ as described above. Stock solutions (2.5 M) of sodium azide and mannitol were prepared in water. Cells were incubated with 100 mM sodium azide or mannitol in dark for 30 min at 37°C previous to the addition of $ZnPPc^{4+}$. This concentration was chosen to obtain a more significant effect as previously found in PDI of cell suspensions (12,16).

Controls and statistical analysis. Control experiments were carried out in presence and absence of $ZnPPc^{4+}$ in the dark and in the absence of the phthalocyanine with cells irradiated. The amount of DMF (<1% v/v) used in each experiment was not toxic to *C. albicans* cells. Data were obtained from three repetitions and the values were given as the mean ± standard deviation of each group of results. The unpaired *t*-test was used to establish the significance of differences between groups. The significance level was set at $P < 0.05$ when compared between different results.

RESULTS

Photoinactivation of *C. albicans* in presence of anthracene derivatives

To evaluate possible effects induced by DMA and ABMM on the photosensitized inactivation of *C. albicans* by $ZnPPc^{4+}$, experiments were performed treating the cell suspensions with 10 μM anthracene. After that, the cells were once washed and irradiated. Also, assays were carried out using a further incubation with 5 μM $ZnPPc^{4+}$ and without anthracene derivative. The results are shown in Fig. 1. No toxicity was detected for the cells treated with DMA or ABMM after 30 min irradiation. Moreover, the presence of these anthracenes no significantly affects the PDI mediated by $ZnPPc^{4+}$, which inactivates 5 log of *C. albicans* cells after 30 min irradiation (54 J cm^{-2}).

Photooxidation of anthracene derivatives in *C. albicans* cells

Photooxidation of DMA or ABMM induced by $ZnPPc^{4+}$ was compared in *C. albicans* cell suspensions under aerobic conditions. In all cases, cells were first treated with 10 μM anthracene derivative followed by a washing step and incubation with 5 μM $ZnPPc^{4+}$. Photooxidation of these substrates were observed by the decay in the fluorescence intensity after irradiation using light doses between 0 and 1.8 J cm^{-2}. Typical results are shown in Figs. 2 and 3 for DMA and ABMM, respectively. The values of the observed rate constant (k_{obs}) were calculated from first-order kinetic plots of the anthracene derivative emission intensities with time (Table 1). It was found that in presence of $ZnPPc^{4+}$, the photodecomposition of DMA and ABMM considerably increase (~10 times) with respect to the cells in absence of phthalocyanine (Table 1, exp. 1 and 2 for DMA, exp. 5 and 6 for ABMM). Also, photooxidation of DMA sensitized by $ZnPPc^{4+}$, was about three times faster than that of ABMM (Table 1, exp. 2 and 6). After a

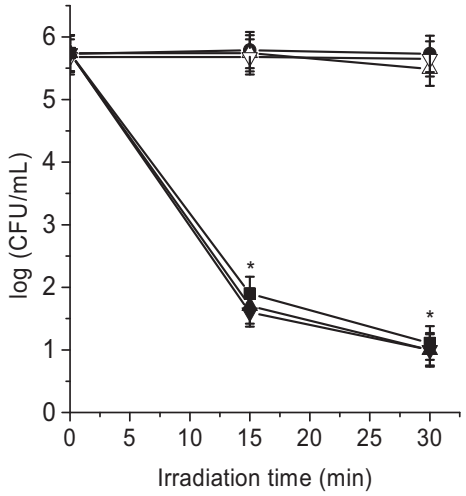

Figure 1. Photoinactivation of *Candida albicans* cells (~10^6 CFU mL^{-1}) incubated with: (■) 5 μM $ZnPPc^{4+}$ for 10 min, (▲) 10 μM DMA for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ for 10 min, (▼) 10 μM ABMM for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ for 10 min, (△) 10 μM DMA for 30 min followed by a washing step, (▽) 10 μM ABMM for 30 min followed by a washing step and exposed to light for different irradiation times. Control untreated cells and irradiated (●) (*$P < 0.05$, compared with control).

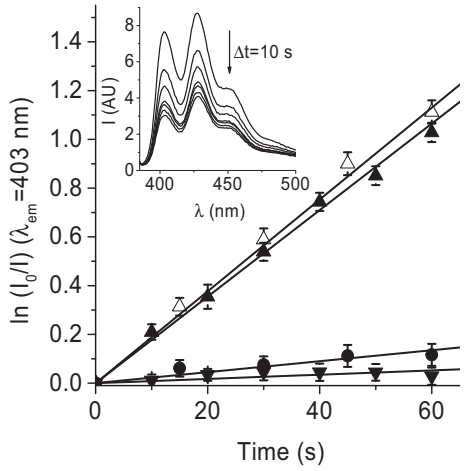

Figure 2. Photooxidation of DMA in *Candida albicans* cells (~10^6 CFU mL^{-1}) incubated with: (●) 10 μM DMA for 30 min followed by a washing step, (▲) 10 μM DMA for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ for 10 min, (△) 10 μM DMA for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ for 10 min followed by a washing step, (▼) 10 μM DMA for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ and 100 mM sodium azide for 10 min. Inset: fluorescence emission spectra changes of DMA photooxidation mediated by $ZnPPc^{4+}$ in *C. albicans* cells after different irradiation times (Δt = 10 s).

washing step to eliminate the photosensitizer that was not bound to cells, the value of k_{obs} for DMA was very similar to that unwashed (Table 1, exp. 2 and 3), whereas photooxidation of ABMM decreased after a washing step (Table 1, exp. 6 and 7). Moreover, photooxidation of both substrates induced by $ZnPPc^{4+}$ in *C. albicans* cells were evaluated in suspensions containing 100 mM sodium azide, which was added together with the phthalocyanine. For both anthracenes, the values of k_{obs} (Table 1, exp. 4 and 8) decreased in presence of azide ion. However, a higher photoprotective effect was found for DMA than that obtained for ABMM.

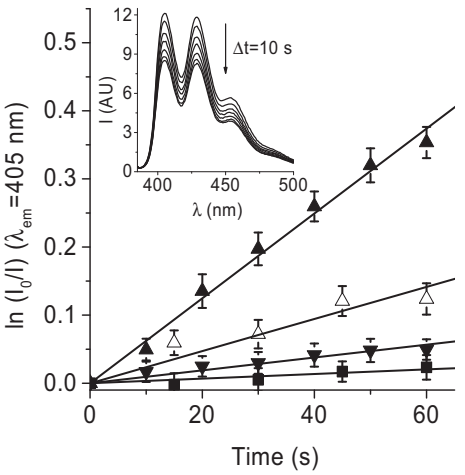

Figure 3. Photooxidation of ABMM in *Candida albicans* cells (~10^6 CFU mL^{-1}) incubated with: (●) 10 μM ABMM for 30 min followed by a washing step, (▲) 10 μM ABMM for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ for 10 min, (△) 10 μM ABMM for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ for 10 min followed by a washing step, (▼) 10 μM ABMM for 30 min followed by a washing step and treated with 5 μM $ZnPPc^{4+}$ and 100 mM sodium azide for 10 min. Inset: fluorescence emission spectra changes of ABMM photooxidation mediated by $ZnPPc^{4+}$ in *C. albicans* cells after different irradiation times (Δt = 10 s).

Table 1. Kinetic parameters for the photooxidation reaction of anthracene derivatives, DMA and ABMM, (k_{obs}) in *Candida albicans* cell suspensions (~10^6 CFU mL^{-1}).

Exp.	Conditions	k_{obs}^{DMA} (s^{-1})
1	DMA*	$(0.22 \pm 0.02) \times 10^{-2}$
2	DMA* + $ZnPPc^{4+}$†	$(1.77 \pm 0.09) \times 10^{-2}$
3	DMA* + $ZnPPc^{4+}$†·‡	$(1.73 \pm 0.08) \times 10^{-2}$
4	DMA* + ($ZnPPc^{4+}/N_3^-$)§	$(0.08 \pm 0.01) \times 10^{-2}$
5	ABMM*	$(0.61 \pm 0.04) \times 10^{-3}$
6	ABMM* + $ZnPPc^{4+}$†	$(5.51 \pm 0.08) \times 10^{-3}$
7	ABMM* + $ZnPPc^{4+}$†·‡	$(2.11 \pm 0.09) \times 10^{-3}$
8	ABMM* + ($ZnPPc^{4+}/N_3^-$)§	$(0.92 \pm 0.06) \times 10^{-3}$

*10 μM DMA, 30 min incubation in dark at 37°C followed by a washing step. †5 μM $ZnPPc^{4+}$, 10 min incubation in dark at 37°C. ‡Followed by a washing step. §5 μM $ZnPPc^{4+}$, 100 mM NaN_3, 10 min incubation in dark at 37°C.

Photosensitized reduction in NBT

The reduction in NBT to diformazan was examined following the increase in absorption at 560 nm (Fig. 4, inset). First, the reaction of NBT was studied in DMF solution. The diformazan absorption was investigated at different periods of irradiation (0–9.0 J cm^{-2}). The results shown in Fig. 4 indicate that in the presence of $ZnPPc^{4+}$ and absence NADH was not observed the formation of formazan after irradiation. In the presence of NADH decomposition of NBT was observed. Moreover, when the photosensitizer was added, a small change was found in diformazan generation with respect to solutions without $ZnPPc^{4+}$.

On the other hand, formation of diformazan was investigated in *C. albicans*. Cell suspensions were treated with 0.2 mM NBT. Then, 5 μM $ZnPPc^{4+}$ and 0.5 mM NADH was added and the

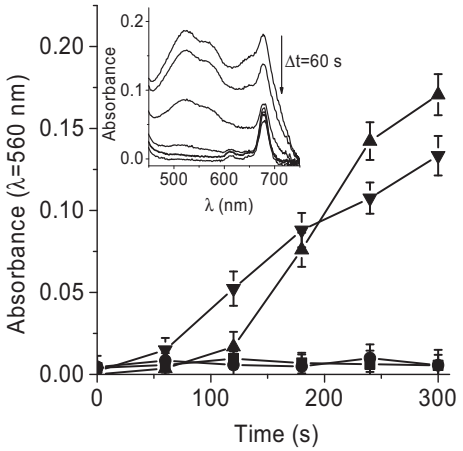

Figure 4. Detection of $O_2^{\cdot-}$ by the NBT method following the changes in the absorption at 560 nm, for: (▲) NBT + β-NADH + $ZnPPc^{4+}$, (▼) NBT + β-NADH, (■) NBT + $ZnPPc^{4+}$, (●) NBT in DMF irradiated with light, [$ZnPPc^{4+}$] = 1 μM, [NBT] = 0.2 mM and [NADH] = 0.5 mM. Inset: absorption spectra modifications of NTB in DMF bearing $ZnPPc^{4+}$ and NADH after different irradiation times (Δt = 60 s).

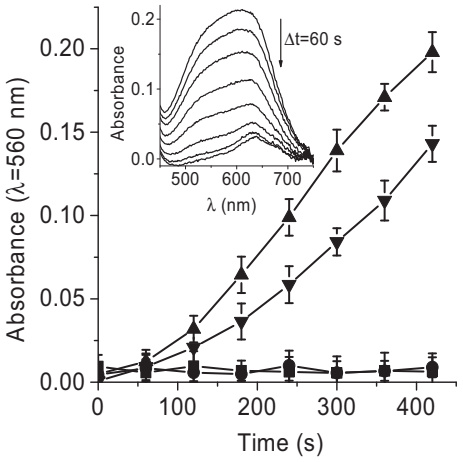

Figure 5. Formation of $O_2^{\cdot-}$ detected by the reaction with NBT as an increase in the absorption at 560 nm, for: (▲) NBT + NADH + $ZnPPc^{4+}$, (▼) NBT + NADH, (■) NBT + $ZnPPc^{4+}$, (●) NBT in *Candida albicans* (~10^6 CFU mL^{-1}) after different irradiation periods. Cells were treated with 0.2 mM NBT for 30 min followed by a washing step and incubated with 5 μM $ZnPPc^{4+}$ and 0.5 mM NADH for 10 min. Inset: absorption spectra modifications of NTB in *C. albicans* cells bearing $ZnPPc^{4+}$ and NADH after different irradiation times (Δt = 60 s).

samples were incubated for further 10 min. After irradiation (0–12.7 J cm^{-2}), diformazan absorption was observed in presence of NADH (Fig. 5 inset). As can be observed in Fig. 5, reduction in NBT was not found in the photoirradiated samples without NADH. Therefore, decomposition of NBT increases in presence of $ZnPPc^{4+}$ and NADH after irradiation with respect to solution without the phthalocyanine.

Effect of media on the photoinactivation of *C. albicans*

Photosensitized inactivation of *C. albicans* cells by $ZnPPc^{4+}$was studied under anoxic conditions and cells suspended in D_2O. First, fungal cells were treated with 2.5 μM $ZnPPc^{4+}$ and irradiated for 30 min (54 J cm^{-2}). This concentration was chosen to not produce a complete eradication of *C. albicans*. PDI mediated by $ZnPPc^{4+}$ showed a photosensitizing activity causing a ~4 log decrease in cell survival (Fig. 6, exp. 4). Furthermore, no toxicity was detected for *C. albicans* within $ZnPPc^{4+}$ and irradiated for 30 min (Fig. 6, exp. 2). Similar result was found for cells incubated with the photosensitizer and kept in dark (Fig. 6, exp. 3).

Yeast cells incubated with $ZnPPc^{4+}$ were irradiated under an argon atmosphere. This anoxic condition allows evaluating the significance of oxygen on the PDI of *C. albicans*. Cell viability was unaffected for cell suspensions irradiated under an argon atmosphere (Fig. 6, exp. 5) or cells treated with $ZnPPc^{4+}$ under an argon atmosphere in the dark (result not shown). Photosensitized inactivation of *C. albicans* was dependent on the amount of oxygen in the medium (Fig. 6, exp. 6). Only about 1 log decrease in cell survival was found after 30 min irradiation. This low PDI effect can be attributed to some oxygen remaining in the cell suspension.

Moreover, photoinactivation of *C. albicans* mediated by $ZnPPc^{4+}$ was carried out in cells suspended in D_2O. Cell viability was not changed in presence of D_2O under irradiation without photosensitizer (Fig. 6, exp. 7) nor treated with $ZnPPc^{4+}$ in dark for 30 min in (result not shown). In contrast, a higher cell photoinactivation was observed for *C. albicans* cells incubated with $ZnPPc^{4+}$ in D_2O than that obtained in PBS cell suspension after 30 min irradiation (Fig. 6, exp. 8).

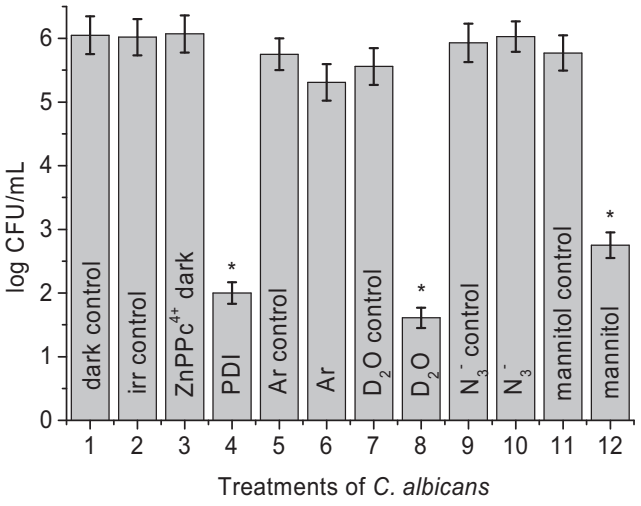

Figure 6. Photoinactivation of *Candida albicans* cell suspensions (~10^6 CFU mL^{-1}) treated with 2.5 μM $ZnPPc^{4+}$ for 30 min in dark at 37°C and irradiated for 30 min; (1) cells kept in dark; (2) cells irradiated; (3) cells treated with $ZnPPc^{4+}$ and kept in dark; (4) cells treated with $ZnPPc^{4+}$ and irradiated; (5) cells under Ar and irradiated; (6) cells treated with $ZnPPc^{4+}$ under Ar and irradiated; (7) cells in D_2O and irradiated; (8) cells treated with $ZnPPc^{4+}$ in D_2O and irradiated; (9) cells containing 100 mM azide and irradiated; (10) cells treated with $ZnPPc^{4+}$ containing 100 mM azide and irradiated; (11) cells containing 100 mM mannitol and irradiated; (12) culture treated with $ZnPPc^{4+}$ containing 100 mM mannitol and irradiated (*$P < 0.05$, compared with control).

Effect of scavengers of ROS on the photoinactivation of *C. albicans*

The effect of two suppressors of ROS, azide ion and mannitol, were investigated with the purpose of clarify the photodynamic mechanism involved in the photosensitized inactivation of *C. albicans* cells. Yeast cells were treated with 100 mM sodium azide and 2.5 μM $ZnPPc^{4+}$. Cells were irradiated for 30 min with

visible light (54 J cm^{-2}). No toxicity was detected using this concentration of azide ion under irradiation without $ZnPPc^{4+}$ (Fig. 6, exp. 9) or in the dark containing the phthalocyanine (result not shown). A reduction in the cell inactivation photosensitized by $ZnPPc^{4+}$ was obtained in the medium containing the azide ion (Fig. 6, exp. 10). Thus, the addition of azide ion quenched the photocytotoxic species, producing a complete protective effect on microbial cells.

Likewise, the photoinactivation of *C. albicans* was examined after incubation with 100 mM mannitol and 2.5 μM $ZnPPc^{4+}$. The presence of 100 mM mannitol was not toxic to irradiated cells without $ZnPPc^{4+}$ (Fig. 6, line 11). Also, it was not toxic for *C. albicans* cells treated with $ZnPPc^{4+}$ in dark (result not shown). However, cell inactivation mediated by $ZnPPc^{4+}$ exhibited a slight photoprotective effect of about 0.7 log in suspensions bearing mannitol after irradiation (Fig. 6, line 12).

DISCUSSION

The phthalocyanine $ZnPPc^{4+}$ is substituted by *N*-methylpyridyloxy groups in the periphery of the macrocycle through ether bridges (Scheme 1). Thus, the mobility of these cationic charges can facilitate the interaction with the microbial cell envelope (20). Previous studies in *C. albicans* cells indicated that this phthalocyanine reached the highest value of cell-bound photosensitizer at short time (<5 min) (17). A value of 1.8 nmol/10^6 cells was obtained for the binding of $ZnPPc^{4+}$ to *C. albicans* incubated with 5 μM phthalocyanine. Moreover, a 90% of the initial $ZnPPc^{4+}$ still remains bound to the cells after one washing steps, indicating a strong interaction between this phthalocyanine and *C. albicans*.

In the present investigation, the treatment of *C. albicans* cells with 5 μM $ZnPPc^{4+}$ produced a photoinactivation of 99.999% (~5 log decrease) after 30 min irradiation. This phototoxic activity remained high, even using a shorter irradiation time of 15 min. Moreover, PDI of yeast cells mediated by $ZnPPc^{4+}$ was evaluated in presence of DMA and ABMM. These anthracene derivatives can themselves act as photosensitizers producing $O_2(^1\Delta_g)$ (21). However, these substrates essentially do not absorb in the wavelength range used to irradiate (455 to 800 nm) in comparison with the intense absorption *Q*-band of $ZnPPc^{4+}$ at 678 nm (15). Therefore, PDI of *C. albicans* was not affected by the addition of DMA or ABMM. Furthermore, shorter irradiation times were used in kinetic studies, with a total time of about 1 min. Considering that DMA quenches $O_2(^1\Delta_g)$ by means of chemical reaction to form the corresponding endoperoxide, it is used as a method to evaluate the ability of $ZnPPc^{4+}$ to produce $O_2(^1\Delta_g)$ in *C. albicans* cells (22). Also, anthracene derivatives substituted by hydrophilic groups has been used as efficient traps of $O_2(^1\Delta_g)$ in aqueous solution (23). In this way, ABMM contains four carboxylate salts per anthracene molecule and it can be dissolved in polar media (18). The ABMM substrate acts as rapid trapping reactant of $O_2(^1\Delta_g)$, leading to the formation of 9,10-endoperoxide product (24). Consequently, the salt derivative ABMM was used as a molecular probe to sense $O_2(^1\Delta_g)$ formation in an more polar cell microenvironment. Thus, both hydrophobic (DMA) and hydrophilic (ABMM) anthracenes were chosen, expecting that these molecules can sense the $O_2(^1\Delta_g)$ production in different domains of the *C. albicans* cell.

It is known that $ZnPPc^{4+}$ presents a high $O_2(^1\Delta_g)$ quantum yield of 0.59 in DMF (15). However, the photodynamic activity of this phthalocyanine determined in an organic solution can change within the cellular environment. In *C. albicans* cells, photooxidation rates of DMA and ABMM significantly increased in presence of $ZnPPc^{4+}$, indicating the generation of $O_2(^1\Delta_g)$. A lower photodecomposition rate was observed for ABMM with respect to DMA. Photooxidation of ABMM can be influenced by an electrostatic interaction between this anionic substrate and the cationic phthalocyanine. This effect can affect the capacity of ABMM to detect $O_2(^1\Delta_g)$ production by the photosensitizer. Similar behavior was previously observed for ABMM and cationic porphyrins, such as 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) in aqueous solutions (18). Therefore, a disadvantage of the ABMM anionic trap is the interaction with cationic photosensitizers, mainly when the positive charges are closely linked to the tetrapyrrolic macrocycle.

The effectiveness of $ZnPPc^{4+}$ to produce $O_2(^1\Delta_g)$ in *C. albicans* cells was also evaluated after a washing step. Under this condition, the photodynamic activity was mainly related with the $ZnPPc^{4+}$ molecules that were tightly bound to cells (17). The results showed no changes in the values of k_{obs} for DMA unwashed or washed cells, indicating a strong interaction between $ZnPPc^{4+}$ and *C. albicans*. Therefore, DMA was detecting the $O_2(^1\Delta_g)$ production in the cell environment. This was in agreement with a low loss of the amount of cell-bound photosensitizer. Also, a minor difference in the photoinactivation of *C. albicans* was found with a successive washing step (17). However, photooxidation of ABMM decreased after a washing step. This water soluble substrate can be involved in electrostatic interaction with $ZnPPc^{4+}$, facilitating the elimination of the photosensitizer after a washing step. Moreover, the azide ion was used to establish that presence of $O_2(^1\Delta_g)$, preventing type II photoprocess (25,26). The photooxidation of both anthracenes decreased in presence of azide ion in the cell suspensions. Thus, the deactivation of $O_2(^1\Delta_g)$ by physical quenching decreases the formation of endoperoxide in the cells.

On the other hand, formation of $O_2^{\cdot-}$ by $ZnPPc^{4+}$ in *C. albicans* cells was analyzed using NBT approach. Photodecomposition of NBT can occur through a type I photodynamic mechanism (27,28). In DMF containing NADH, the generation of diformazan was similar in presence or absence of the photosensitizer. Thus, the involvement of $ZnPPc^{4+}$ to reduce NBT was not remarkable in solution, mainly in comparison with other photosensitizers (13,29). In *C. albicans* cells containing NADH, the photosensitizing effect of $ZnPPc^{4+}$ showed a contribution to the formation of diformazan, indicating the presence of $O_2^{\cdot-}$. Therefore, even though $O_2(^1\Delta_g)$ can be generated effectively by photoexcited triplet state of $ZnPPc^{4+}$, it was observed that $O_2^{\cdot-}$ can also be produced in the presence of NADH.

The photodynamic mechanism was also investigated evaluating different effects on the photoinactivation of *C. albicans*. Cells treated with 2.5 μM $ZnPPc^{4+}$ produced a photoinactivation of 99.99% (~4 log decrease) after 30 min irradiation. However, under an anoxic atmosphere the photodamage of yeast cells mediated by $ZnPPc^{4+}$ was negligible. The presence of oxygen is crucial for the formation of $O_2(^1\Delta_g)$ through the type II photosensitization mechanism through a triplet energy transfer reaction from $ZnPPc^{4+}$. Moreover, oxygen molecules are involved in the type I mechanism (7). In this process, the light-excited photosensitizer can react with substrates to yield radical ions in a hydrogen atom or electron transfer reaction. These radicals can interact with oxygen in the ground state to form highly reactive oxygen intermediates. The generation of $O_2^{\cdot-}$ can occur by the reaction

of molecular oxygen with the photosensitizer radical anion (7,8). Photodamage of *C. albicans* induced by $ZnPPc^{4+}$ was studied in D_2O to evaluate the $O_2(^1\Delta_g)$-mediated inactivation of microbial cells. Thus, D_2O was used instead of water in order to increase the $O_2(^1\Delta_g)$ lifetime (25). Photocytotoxic effect sensitized by $ZnPPc^{4+}$ was higher in D_2O than in aqueous medium. These results revealed a contribution of type II photosensitization in the PDI of *C. albicans*. Moreover, experiments were performed in the presence of sodium azide to determinate the participation of $O_2(^1\Delta_g)$. Quenching experiments have shown the benefit of its use to characterize the effects of intracellular inhomogeneity on the reactivity of $O_2(^1\Delta_g)$ (30). In a cellular environment, the lifetime of $O_2(^1\Delta_g)$ is limited by quenching and reactions with cell constituents. The diffusion distance of $O_2(^1\Delta_g)$ in eukaryotic cells was estimated in 155 nm and the cell damage mediated by $O_2(^1\Delta_g)$ occurs close to its site of generation (31). The addition of sodium azide to the *C. albicans* cell suspensions produced a high photoprotection of the microorganism. The presence of azide ion caused a significant decrease in the photodynamic activity of the $ZnPPc^{4+}$ by quenching of $O_2(^1\Delta_g)$. It was demonstrate that intracellular quenching by azide ion of $O_2(^1\Delta_g)$ sensitized by the tetracationic 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21H,23H-porphine (TMPyP) occurs at the diffusion-controlled limit (30). Therefore, azide ion should be located in the subcellular compartments close to the photosensitizer because $O_2(^1\Delta_g)$ does not diffuse a great distance from its site of production. Also, to investigate the contribution of type I mechanism, mannitol was used as a free radical scavenger (32,33). Photocytotoxicity of *C. albicans* cells sensitized by $ZnPPc^{4+}$ was slight affected when mannitol was added to cells. In previous studies, the inhibitory effect of the photodynamic process in the presence of sodium azide or mannitol was evaluated by exposing *E. coli* bacterial suspensions with cationic porphyrins to white light (9). Strong bacterial protection was observed with sodium azide, whereas a small protection was detected in the presence of mannitol. Similar behavior was found in the PDI of *S. mitis* mediated by a cationic phtalocyanine (10). Moreover, the effect of these $O_2(^1\Delta_g)$ oxygen and free radical scavenger were investigated in the photoinactivation of *C. albicans* cells sensitized by different types of photosensitizers (12–14). A type II photoprocess was mainly found in presence of cationic porphyrins (12). However, the addition of mannitol produced a photoprotective effect on the cellular survival, when cationic fullerene or triarylmethane derivatives were used as photosensitizers (13,14). In these cases, the photoprotective effect caused by mannitol was indicative of a contribution of type I pathway. Therefore, the photodamage produced to the *C. albicans* cells by $ZnPPc^{4+}$ appears to be facilitated by $O_2(^1\Delta_g)$, even though considering the results with NBT and mannitol, the participation of other ROS cannot be completely discarded in the PDI of this yeast.

CONCLUSIONS

This study provides evidence on the photodynamic mechanism that occurs in the PDI of *C. albicans* cells mediated by $ZnPPc^{4+}$. Both photoprocesses can occur simultaneously and the ratio between the type I and type II can depend on the nature of the photosensitizer, the PDI treatment and the kind of microbial cells. In particular, the main mechanism can be influenced by the location of the photosensitizer in the different domains of microbial cells. To elucidate the photodynamic processes, DMA and ABMM were used as a sensitive probe of $O_2(^1\Delta_g)$. Both substrates detected the generation of $O_2(^1\Delta_g)$ in the yeast cells. Moreover, the formation of $O_2^{\cdot-}$ was sensed using NBT. However, the addition of extracellular NADH was necessary to detect the formation of diformazan. The effect of the media on PDI of *C. albicans* showed that an oxygen atmosphere was required for an efficient photokilling. Also, photoinactivation of yeast was increased in D_2O due to a longer lifetime of $O_2(^1\Delta_g)$. When ROS scavengers were added, photoprotection was observed with azide ion as $O_2(^1\Delta_g)$ quencher, whereas a minor effect was found using mannitol as a free radical trapping. Therefore, this investigation indicates that $ZnPPc^{4+}$ appears to act as photosensitizers mainly via the intermediacy of $O_2(^1\Delta_g)$, although a minor contribution of other ROS cannot be absolutely ruled out in the photoinactivated *C. albicans* cells.

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