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**PAPER**

# Mechanistic insight of the photodynamic effect induced by tri- and tetra-cationic porphyrins on *Candida albicans* cells

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The photodynamic mechanism of action induced 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 on *Candida albicans* cells. These cationic porphyrins are effective photosensitizers, producing a ~5 log decrease of cell survival when the cultures are incubated with 5 μM photosensitizer and irradiated for 30 min with visible light. Studies under anoxic conditions indicated that oxygen is necessary for the mechanism of action of photodynamic inactivation of this yeast. Furthermore, photoinactivation of *C. albicans* cells was negligible in the presence of 100 mM azide ion, whereas the photocytotoxicity induced by these porphyrins increased in $D_2O$. In contrast, the addition of 100 mM mannitol produced a negligible effect on the cellular phototoxicity. On the other hand, *in vitro* direct observation of singlet molecular oxygen, $O_2(^1\Delta_g)$ phosphorescence at 1270 nm was analyzed using *C. albicans* in $D_2O$. A shorter lifetime of $O_2(^1\Delta_g)$ was found in yeast cellular suspensions. These cationic porphyrins bind strongly to *C. albicans* cells and the $O_2(^1\Delta_g)$ generated inside the cells is rapidly quenched by the biomolecules of the cellular microenvironment. Therefore, the results indicate that these cationic porphyrins appear to act as photosensitizers mainly *via* the intermediacy of $O_2(^1\Delta_g)$.

## Introduction

Superficial skin mycosis, either caused by *Candida* species or dermatophytes, is one of the most frequent diseases in human beings and animals. The search for new therapeutic approaches is stimulated by the fact that standard drug treatments are prolonged and the appearance of drug resistant strains is more frequent in high risk groups.[1,2] In this way, photodynamic inactivation (PDI) is a new promising approach to treat microbial infections.[3] Essentially, PDI is based on the administration of a photosensitizer, which is preferentially accumulated in microbial cells. Subsequent irradiation with visible light, in the presence of oxygen, specifically generates a cascade of biochemical events that produce cell damages that inactivate the microorganisms.[4,5]

Basically two oxidative mechanisms can occur after photoactivation of the photosensitizer.[6] In the type I photosensitization pathway, the photosensitizer interacts with biomolecules 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. Depending on the experimental conditions, these mechanisms can take place simultaneously and the ratio between the two processes is influenced by the photosensitizer, substrate and the nature of the medium.[7] However, under aerobic condition it is accepted that $O_2(^1\Delta_g)$ is the main species responsible for cell inactivation.[8] Furthermore, even if an excited photosensitizer reacts with a given substrate by type I photoprocess, the final result is also the oxidation of essential biomolecules.[9] Thus, the reactive $O_2(^1\Delta_g)$ and other highly reactive oxygen species (ROS) rapidly react with a variety of substrates inducing damage in biomolecules. These changes lead to a loss of appropriate biological functionality producing cell inactivation.

In previous studies, cationic porphyrin derivatives have been investigated for PDI applications in the treatment and control of yeast.[10–13] Tetracationic porphyrins (Scheme 1), such as 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) and 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$), are standard active photosensitizer known to eradicate *C. albicans*.[12,13] Recently, an amphiphilic porphyrin bearing three cationic charges and a highly lipophilic trifluoromethyl group, 5-(4-trifluorophenyl)-10,15,20-tris(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TFAP^{3+}$, Scheme 1), was shown to be an effective photosensitizer for the inactivate of *C. albicans*.[12] In the present work, we examined the photoinactivation of *C. albicans* cells induced by $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ under different experimental conditions, such as an argon atmosphere, cellular suspensions in $D_2O$, addition of azide ion and presence of mannitol. Also, the detection of $O_2(^1\Delta_g)$ luminescence was analyzed *in vitro* on yeast cells. Thus, the current study is intended to provide insights into the specific mechanism of the

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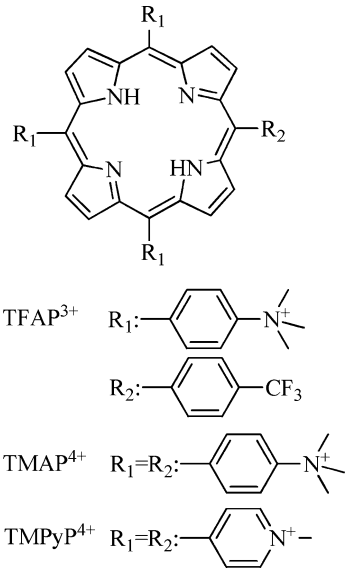


**Scheme 1** Molecular structures of cationic porphyrins.

photoreaction process which cause the cellular death of *C. albicans* after PDI treatment with these cationic porphyrins.

## Materials and methods

### General

The light source used was a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) 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 350–800 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.

### Photosensitizers

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.[14] 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 absorption coefficient.[14,15]

### Microorganism and growth conditions

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 (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 in PBS. 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. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar plates after ~48 h incubation at 37 °C. Each suspension was plated in triplicate and each experiment was repeated separately three times.

### Photosensitized inactivation of *C. albicans* cells in PBS and in $D_2O$

Cellular suspensions of *C. albicans* (2 mL, ~$10^6$ CFU/mL) in PBS were incubated with 5 μM porphyrin for 30 min in the dark at 37 °C. After that, the cultures were exposed to visible light for 30 min, using the irradiation system described above. Studies in the absence of oxygen were performed displacing the oxygen with argon in the cultures flasks for 15 min before irradiation and maintaining argon atmosphere during the illumination. Sodium azide (100 mM) or mannitol (100 mM) were added to yeast suspensions from 2.5 M stock solutions in water and the cells were incubated for 30 min at 37 °C in dark previous to the treatment with the photosensitizer. For photoinactivation assays in $D_2O$, cells were centrifuged (3000 rpm for 15 min) and re-suspended in 2 mL $D_2O$, then the cell suspensions were incubated with the porphyrin as described above. Fungal cultures grown under the same conditions with and without photosensitizers, kept in the dark, and illuminated cultures without photosensitizer served as controls. Each experiment was repeated separately three times.

### Detection of singlet molecular oxygen in *C. albicans*

Photosensitization of $O_2(^1\Delta_g)$ was measured by using a time-resolved phosphorescence detection method (TRPD).[16] A Q-switched Nd:YAG laser (Spectron Laser Systems, Rugby, Warwickshire, England) was used as the excitation source, operating at 532 nm (18 ns halfwidth, 10 mJ $cm^{-2}$, diameter area 8 mm) in order to excite the porphyrins. The emitted phosphorescence radiation (mainly 1270 nm) was detected at right angles with an amplified Judson J16/8Sp germanium detector, after passing through appropriate filters. The output of the detector was coupled to a digital oscilloscope (Hewlett-Packard HP-54504A, Santa Rosa, CA, USA). In the dynamic determinations the solvent was $D_2O$ instead of water, in order to enlarge the $O_2(^1\Delta_g)$ lifetime.[17] About five shots were usually needed for averaging decay times, in order to get a good signal to noise ratio. No change in the porphyrin absorption spectrum was observed after these experiments, indicating no significant photodegradation of the photosensitizer. The averaged signals were analyzed as single exponential decays.[16] Air-saturated *C. albicans* cellular suspensions in $D_2O$ were employed in all cases. Two protocols were followed: (a) to a solution of porphyrin (5 μM) in $D_2O$ was added a pellet of cells (~$10^6$ CFU/mL), which was obtained by centrifugation (3000 rpm for 15 min) of cells in $D_2O$. The porphyrin was incubated with the pellet cells for 15 min before irradiation. In this experiment, the photosensitizer can interact with cells but a fraction of the porphyrin molecules remain in the $D_2O$. (b) A

cell suspension (~$10^6$ CFU/mL) was treated with the porphyrin for 30 min as described above, then the culture was centrifuged (3000 rpm for 15 min) and the cells were resuspended in $D_2O$. This procedure was used to analyze only the effect of photosensitizer molecules tightly bound to cells.

### Statistical analysis

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

## Results

### Photosensitized inactivation of *C. albicans*

Photoinactivation of *C. albicans* was evaluated in PBS cellular suspensions treated with 5 μM photosensitizer for 30 min in dark at 37 °C and irradiated for 30 min with visible light. Under these conditions, the cationic porphyrins exhibited a photosensitizing activity causing a ~5 log decrease of cell survival (Fig. 1–3, line 4). On the other hand, control experiments showed that the viability of *C. albicans* was unaffected by illumination alone (Fig. 1–3, line 2) or by dark incubation with 5 μM photosensitizer (Fig. 1–3, line 3), demonstrating that the cell inactivation obtained after irradiation was induced by the photosensitizing activity of the porphyrin. Thus, under these experimental conditions the photodynamic efficiencies of these cationic porphyrins against *C. albicans* was similar.

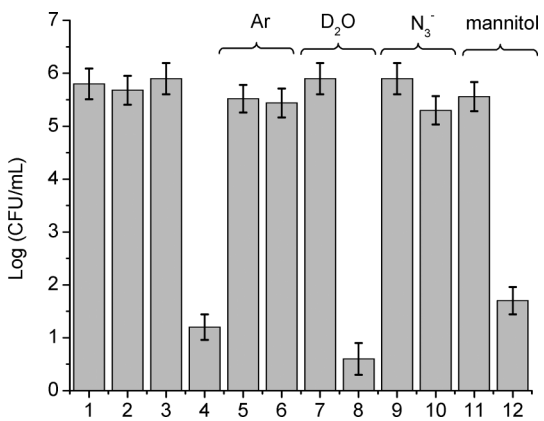


**Fig. 1** Survival of *C. albicans* (~$10^6$ CFU/mL) incubated with 5 μM $TFAP^{3+}$ in dark for 30 min at 37 °C and exposed to visible light for 30 min (90 mW $cm^{-2}$): (1) control culture in dark; (2) control culture irradiated; (3) control culture treated with $TFAP^{3+}$ and keeping in dark; (4) culture treated with $TFAP^{3+}$ and irradiated; (5) control culture under argon and irradiated; (6) culture treated with $TFAP^{3+}$ under argon and irradiated; (7) control culture in $D_2O$ and irradiated; (8) culture treated with $TFAP^{3+}$ in $D_2O$ and irradiated; (9) control culture containing 100 mM azide and irradiated; (10) culture treated with $TFAP^{3+}$ containing 100 mM azide and irradiated; (11) control culture containing 100 mM mannitol and irradiated; (12) culture treated with $TFAP^{3+}$ containing 100 mM mannitol and irradiated. Values represent mean ± standard deviation of three separate experiments.

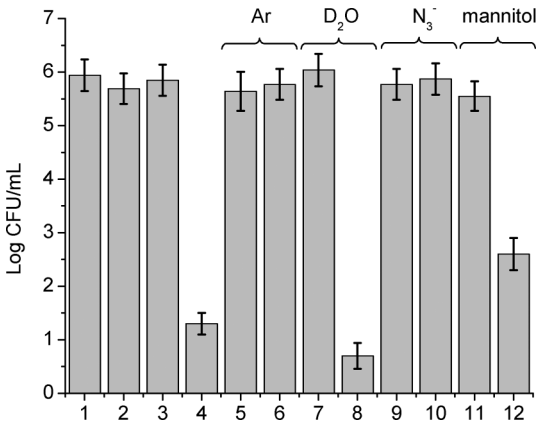


**Fig. 2** Survival of *C. albicans* (~$10^6$ CFU/mL) incubated with 5 μM $TMAP^{4+}$ in dark for 30 min at 37 °C and exposed to visible light for 30 min (90 mW $cm^{-2}$): (1) control culture in dark; (2) control culture irradiated; (3) control culture treated with $TMAP^{4+}$ and keeping in dark; (4) culture treated with $TMAP^{4+}$ and irradiated; (5) control culture under argon and irradiated; (6) culture treated with $TMAP^{4+}$ under argon and irradiated; (7) control culture in $D_2O$ and irradiated; (8) culture treated with $TMAP^{4+}$ in $D_2O$ and irradiated; (9) control culture containing 100 mM azide and irradiated; (10) culture treated with $TMAP^{4+}$ containing 100 mM azide and irradiated; (11) control culture containing 100 mM mannitol and irradiated; (12) culture treated with $TMAP^{4+}$ containing 100 mM mannitol and irradiated. Values represent mean ± standard deviation of three separate experiments.

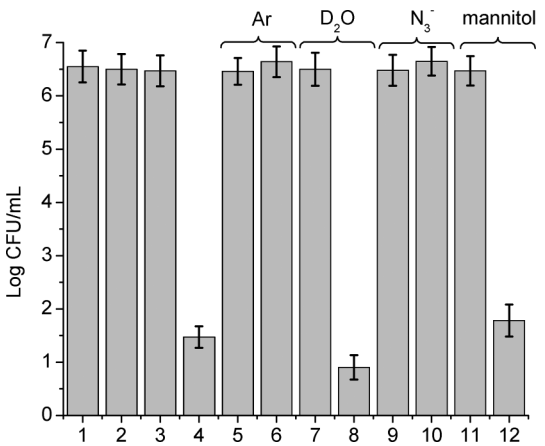


**Fig. 3** Survival of *C. albicans* (~$10^6$ CFU/mL) incubated with 5 μM $TMPyP^{4+}$ in dark for 30 min at 37 °C and exposed to visible light for 30 min (90 mW $cm^{-2}$): (1) control culture in dark; (2) control culture irradiated; (3) control culture treated with $TMPyP^{4+}$ and keeping in dark; (4) culture treated with $TMPyP^{4+}$ and irradiated; (5) control culture under argon and irradiated; (6) culture treated with $TMPyP^{4+}$ under argon and irradiated; (7) control culture in $D_2O$ and irradiated; (8) culture treated with $TMPyP^{4+}$ in $D_2O$ and irradiated; (9) control culture containing 100 mM azide and irradiated; (10) culture treated with $TMPyP^{4+}$ containing 100 mM azide and irradiated; (11) control culture containing 100 mM mannitol and irradiated; (12) culture treated with $TMPyP^{4+}$ containing 100 mM mannitol and irradiated. Values represent mean ± standard deviation of three separate experiments.

### Photoinactivation under anoxic atmosphere

To evaluate the influence of oxygen atmosphere, the photoinactivation of *C. albicans* cells was evaluated under anoxic conditions.

 

Thus, the irradiation of the cellular suspension treated with 5 μM of photosensitizer was performed under argon atmosphere. Cellular survival was compared under both air and argon experimental conditions (Fig. 1–3, lines 4 and 6). As can be observed, the loss of viability was highly oxygen dependent for the three cationic porphyrins. Practically no cell inactivation occurred using these porphyrin derivatives under a low oxygen concentration. On the other hand, no toxicity was observed for cell suspensions irradiated under an argon atmosphere (Fig. 1–3, lines 5) or cells treated with the porphyrins under an argon atmosphere in the dark (result not shown).

### Photosensitization of *C. albicans* in $D_2O$

To evaluate the $O_2(^1\Delta_g)$-mediated photoinactivation of *C. albicans* by porphyrins, the reaction was performed in $D_2O$. This solvent was used to increase the lifetime of $O_2(^1\Delta_g)$.[17] Cell viabilities of *C. albicans* treated with 5 μM porphyrin derivative were not affected in $D_2O$ for 60 min in dark nor in $D_2O$ under irradiation without photosensitizer (Fig. 1–3, line 7). However, irradiation of *C. albicans* cells incubated with the photosensitizers in $D_2O$ produced a higher photoinactivation than that observed in PBS (Fig. 1–3, line 8).

### Effect of azide ion on PDI of *C. albicans*

The photoinactivation of *C. albicans* incubated with 5 μM porphyrin was studied in presence of 100 mM azide ion. This concentration of sodium azide was not toxic in the dark or under irradiation without porphyrin (Fig. 1–3, line 9). The azide ion is a quencher of $O_2(^1\Delta_g)$ but it also can deactivate compounds in their triplet excited state, thus preventing both type I and type II photoprocesses.[6] The resulting photoinactivation was greatly affected by the azide ion, the addition of which produced a high reduction in the inactivation of *C. albicans* sensitized by porphyrins (Fig. 1–3, line 10). Under this condition, no more than a 0.5 log decrease was observed in the survival of the yeast treated with $TFAP^{3+}$ after 30 min irradiation, while no difference with the control was found for $TMAP^{4+}$ and $TMPyP^{4+}$. Therefore, the azide ion quenched the photocytotoxic species, producing a protective effect on *C. albicans*.

### Effect of mannitol on PDI of *C. albicans*

The photoinactivation of *C. albicans* was investigated in the presence of 100 mM mannitol. This compound acts as scavenger of the superoxide anion radical and hydroxyl radical.[18] The addition of 100 mM mannitol was not cytotoxic for cells treated with 5 μM porphyrin in dark. Also, no toxicity was found for cells irradiated in the presence of mannitol (Fig. 1–3, line 11). After illumination, cell viability increased slightly to ~1.5 log for cells sensitized by $TFAP^{3+}$ and $TMPyP^{4+}$ (Fig. 1 and 3, line 12), while the photoprotective effect reached ~2.2 log for cell suspensions incubated with $TMAP^{4+}$ (Fig. 2, line 12), after 30 min irradiation.

### Sensitized $O_2(^1\Delta_g)$ production in *C. albicans* cultures

Time-resolved detection of $O_2(^1\Delta_g)$-luminescence sensitized by porphyrins was performed in *C. albicans* cells suspended in $D_2O$. This solvent was used to increase the lifetime of $O_2(^1\Delta_g)$ outside of the cells.[19] First, the $O_2(^1\Delta_g)$-luminescence was carried out in solution of porphyrin in $D_2O$ and after addition of a pellet, which produced a cell suspension of ~$10^6$ cells/mL. Representative results of direct measurements of $O_2(^1\Delta_g)$-phosphorescence in $D_2O$ sensitized by $TMAP^{4+}$ after exposure of the cells to laser light are shown in Fig. 4A. The signals were analyzed by a single exponential fit and the lifetime of $O_2(^1\Delta_g)$ ($\tau$) sensitized by these porphyrins are summarized in Table 1. $TFAP^{3+}$ was not soluble enough as a monomer in $D_2O$ to get an appropriate absorption at 532 nm. The values of $\tau$ obtained in $D_2O$ containing $TMAP^{4+}$ and $TMPyP^{4+}$ were consistent with that expected for $O_2(^1\Delta_g)$ in this homogenic medium. This value is a very similar to that of $O_2(^1\Delta_g)$ reported in bulk solution of $D_2O$ ($\tau$ = 65 μs).[20] The addition of a pellet of cells, which produced a suspension of ~$10^6$ cell/mL, was accompanied by a considerably decrease in the value of $\tau$ (~30 μs). Moreover, the values of $\tau$ were considerably shorted (Fig. 4B), when the experiments were performed with *C. albicans* cellular suspensions in $D_2O$ containing only the bound porphyrin.

**Table 1** Singlet molecular oxygen quantum yield ($\Phi_\Delta$) of porphyrins and lifetime of $O_2(^1\Delta_g)$ ($\tau$) in $D_2O$ and cell suspensions sensitized by cationic porphyrins

| Porphyrin | $\Phi_\Delta$ | $\tau$ (μs)[c] | $\tau$ (μs)[d] | $\tau$ (μs)[e] |
|---|---|---|---|---|
| $TFAP^{3+}$ | 0.42[a] | — | — | 8 ± 1 |
| $TMAP^{4+}$ | 0.77[b] | 60 ± 5 | 27 ± 3 | 7 ± 1 |
| $TMPyP^{4+}$ | 0.74[b] | 61 ± 5 | 30 ± 3 | 9 ± 1 |

[a] From ref. 12 in *N*,*N*-dimethylformamide. [b] From ref. 13 in water. [c] Porphyrin in $D_2O$. [d] Porphyrin in $D_2O$ treated with a pellet of cells (~$10^6$ cell/mL). [e] Porphyrin bound to cells in $D_2O$.

## Discussion

Of the porphyrin derivatives used in the present study, $TMAP^{4+}$ and $TMPyP^{4+}$ contain four cationic groups in the periphery of the macrocycle, whereas $TFAP^{3+}$ bears three cationic charges and a highly lipophilic trifluoromethyl group that increases the amphiphilic character of the structure (Scheme 1). In solution, these porphyrins were highly efficient in the quantum yield of $O_2(^1\Delta_g)$ production ($\Phi_\Delta$) (Table 1).[12,13] Also, they were efficient in producing the photooxidation of biological substrates, such as the amino acid L-tryptophan and guanosine 5′-monophosphate.[21–24] Under aerobic conditions the decomposition of these substrates occurs predominantly through a type II photoreaction process. However, the photophysical properties of the porphyrins determined in homogenic solution can significantly change in a biological medium. The values of $\Phi_\Delta$ can be modified depending where the photosensitizer is localized, diminishing when the photosensitizer forms aggregates.[16]

*In vitro* investigations have shown that $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ are active photosensitizers in the inactivation of *C. albicans* cells.[12,13] The binding to the cells indicated that these porphyrins have particularly high affinity for *C. albicans*. After 30 min incubation with 5 μM porphyrin, the tendency observed for the three compounds was similar, reaching values of 1.48, 1.35 and 1.70 nmol/$10^6$ cells for $TFAP^{3+}$, $TMAP^{+4}$ and $TMPyP^{+4}$, respectively. The PDI of *C. albicans* evaluated in PBS cell suspensions showed that an exposure to visible light of 30 min produced a photoinactivation greater than 99.999% of cell death.

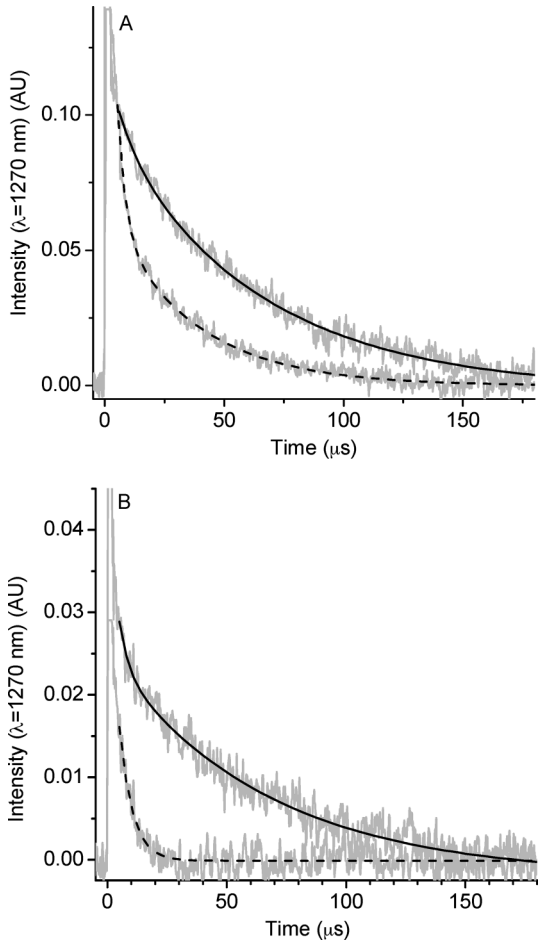


**Fig. 4** Decay traces of $O_2(^1\Delta_g)$-luminescence at 1270 nm sensitized by $TMAP^{4+}$, (A) $TMAP^{4+}$ in $D_2O$ (solid line) and after the addition of a pellet of cells to obtain a cell suspension of ~$10^6$ cell/mL (dashed line) and (B) $TMAP^{4+}$ in $D_2O$ (solid line) and $TMAP^{4+}$ bound to *C. albicans* in a cell suspension of ~$10^6$ cell/mL in $D_2O$.

The photoreaction mechanisms by which light and photosensitizers cause biological damage have been the subject of many studies.[6] Singlet oxygen is a highly reactive and toxic intermediate and it is often postulated to be a major mediator of cellular damage. The cytotoxic effects induced by these cationic photosensitizers were almost negligible under anoxic conditions in comparison with an aerobic atmosphere. The presence of oxygen is essential for the generation of $O_2(^1\Delta_g)$ through the type II photosensitization mechanism that involves a triplet energy transfer reaction.[6] However, oxygen also plays a major role in the type I mechanism by adding to biochemical radicals. In a type I process, the light-excited photosensitizers directly interact with substrate to yield radical ions in a hydrogen atom or electron transfer reaction. The majority of these radicals instantaneously reacts with oxygen and generates a complicated mixture of highly reactive oxygen intermediates, which can oxidize a wide variety of biomolecules.[6] Oxygen is also necessary for the formation of superoxide anion radical that can occur as the result of the reaction of molecular oxygen with the radical anion of the photosensitizer. The studies under anoxic conditions indicate that oxygen is necessary for the mechanism of action in the PDI of *C. albicans*. However, these experiments are not decisive in establishing the predominant photoreaction process involved in the cytotoxicity.

To evaluate the $O_2(^1\Delta_g)$-mediated photoinactivation of *C. albicans*, the PDI was performed in $D_2O$. Under this condition, the photocytotoxic effect was higher in $D_2O$ than in aqueous solution. The results reveal a contribution of type II photosensitization in the yeast inactivation induced by these cationic photosensitizers. Also, in order to assess the involvement of $O_2(^1\Delta_g)$, experiments were carried out in the presence of sodium azide, a known quencher of $O_2(^1\Delta_g)$.[25] The presence of sodium azide produced almost complete photoprotection of *C. albicans*. This suggests that azide ions cause significant lowering of the photodynamic effects of the cationic porphyrins by quenching $O_2(^1\Delta_g)$. In general, the involvement of $O_2(^1\Delta_g)$ in several photosensitized processes *in vivo* is accepted by the observed $D_2O$ enhancement and azide inhibition of diverse oxidative reaction rates.[18,26–28] On the other hand, to investigate the predominant mechanisms of action mannitol was used as a type I scavenger.[18] The results indicated that phototoxicity efficacy was not significantly affected when mannitol was added to *C. albicans* cells sensitized by $TFAP^{3+}$, and $TMPyP^{4+}$. However, although small, a photoprotective effect was found with $TMAP^{4+}$, indicating some contribution of type I reactions in the inactivation process.

Direct measurements of $O_2(^1\Delta_g)$-phosphorescence at 1270 nm in biological systems have been possible using bulk suspensions where the signal observed derives from a large ensemble of cells.[17,20,29] In the present study, when a pellet of *C. albicans* cells was added to a porphyrin solution in $D_2O$, the lifetime of $O_2(^1\Delta_g)$ decreased compared with that of the photosensitizer in pure $D_2O$ (Table 1). The shorter lifetime may be due to the $O_2(^1\Delta_g)$-luminescence was strongly quenched by the biomolecules in the cellular microenvironment. It was previously found that $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$ are highly bound to *C. albicans* cells.[12,13] However, a small fraction of porphyrin molecules remains in the $D_2O$, producing $O_2(^1\Delta_g)$ outside the cells. Thus, both species may be contributing to lifetime of $O_2(^1\Delta_g)$ observed under these experimental conditions. On the other hand, after a washing step of cells only the photosensitizer tightly bound to the cell remains in the cell suspension. Therefore, $O_2(^1\Delta_g)$ is generated inside the *C. albicans* cells and the quenching of $O_2(^1\Delta_g)$ in cells is very efficient producing a shorter $O_2(^1\Delta_g)$ lifetimes. Similar results were previously observed for photosensitizers associated to membrane or included inside the inner volume of red cell ghosts in suspensions.[20,30] The chemical $O_2(^1\Delta_g)$ quencher consumption inside living cells was demonstrated *via* time resolved $O_2(^1\Delta_g)$ detection.[31] Therefore, the $O_2(^1\Delta_g)$ generated inside de cells can diffuse into the solvent or it can stay in the interior of the cell. However, a shorter $\tau$ of $O_2(^1\Delta_g)$ in *C. albicans* cellular suspension than in $D_2O$ solution can be attributed to a higher quenching rate inside de cells. This effect is especially caused by biological substrates, which can produce physical deactivation of $O_2(^1\Delta_g)$ or chemical reaction.[28]

## Conclusions

The present study provides knowledge about the photodynamic mechanism that takes place in the PDI of *C. albicans* cells sensitized by $TFAP^{3+}$, $TMAP^{4+}$ and $TMPyP^{4+}$. To elucidate the oxidative processes that occur during the killing of yeast, first,

 

the effect of the media was analyzed on cell photoinactivation. 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. In the presence of cationic porphyrins, which are tightly bound to cells, 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, in the present *in vitro* experiments, the killing of *C. albicans* cells by these cationic porphyrins and visible light irradiation seem to be mediated mainly by $O_2(^1\Delta_g)$. Although in a minor contribution, the participation of other active oxygen species could not be neglected particularly for *C. albicans* photoinactivated with $TMAP^{4+}$.

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