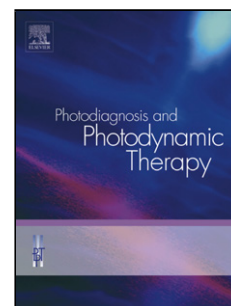# Journal Pre-proof

Mechanistic aspects in the photodynamic inactivation of *Candida albicans* sensitized by a dimethylaminopropoxy porphyrin and its equivalent with cationic intrinsic charges

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Please cite this article as: Quiroga ED, Cordero P, Mora SJ, Alvarez MG, Durantini EN, Mechanistic aspects in the photodynamic inactivation of *Candida albicans* sensitized by a dimethylaminopropoxy porphyrin and its equivalent with cationic intrinsic charges, *Photodiagnosis and Photodynamic Therapy* (2020), doi: https://doi.org/10.1016/j.pdpdt.2020.101877

Mechanistic aspects in the photodynamic inactivation of *Candida albicans* sensitized by a dimethylaminopropoxy porphyrin and its equivalent with cationic intrinsic charges

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Graphical abstract

Photodynamic inactivation mechanism and cell damage mediated by TAPP and TAPP[4+] was investigated on *Candida albicans*.
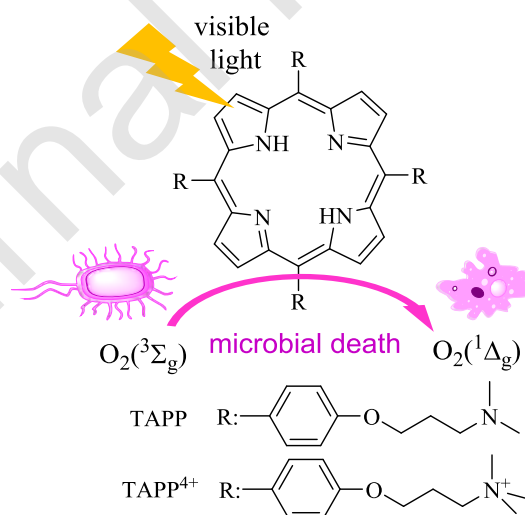
Highlights

- Photodynamic mechanism sensitized by porphyrin was investigated in *Candida albicans*.

- Singlet molecular oxygen was the main reactive species involved in cell damage.

- Morphologic photodamage was studied in yeast cells.

- Photoinactivation mainly produced alterations in the cell barriers.

Abstract

Photocytotoxic effect induced by 5,10,15,20-tetrakis[4-(3-*N,N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP) and 5,10,15,20-tetrakis[4-(3-*N,N,N*-trimethylaminepropoxy)phenyl]porphyrin (TAPP^{+4}) was examined in *Candida albicans* to obtain information on the mechanism of photodynamic action and cell damage. For this purpose, the photokilling of the yeast was investigated under anoxic conditions and cell suspensions in D$_2$O. Moreover, photoinactivation of *C. albicans* was evaluated in presence of reactive oxygen species scavengers, such as sodium azide and D-mannitol. The results indicated that singlet molecular oxygen was the main reactive species involved in cell damage. On the other hand, the binding and distribution of these porphyrins in the cells was observed by fluorescence microscopy. Morphological damage was studied by transmission electron microscopy (TEM), indicating modifications in the cell envelopment. Furthermore, deformed cells were observed after photoinactivation of *C. albicans* by toluidine blue staining. In addition, modifications in the cell envelope due to the photodynamic activity was found by scanning electron microscopy (SEM). Similar photodamage was observed with both porphyrin, which mainly produced alterations in the cell barriers that lead to the photoinactivation of *C. albicans*.

Keywords: photoinactivation; porphyrins; yeasts; photodynamic mechanism; cell damage

1. Introduction

For over a hundred years, it has been known that light and dyes can kill microorganisms. Although this therapy was overshadowed by antibiotics, the increased resistance of microorganisms to these compounds has restarted research to develop new antimicrobial strategies [1,2]. In this way, photodynamic inactivation (PDI) has shown be effective to eliminate microorganisms [3,4]. This method is originated on the addition of a photosensitizer (PS) that rapidly binds to microbial cells. Under aerobiosis, excitation of the PS with visible light of an appropriate wavelength produces reactive oxygen species (ROS), which can react with the biomolecules into de cells. Consequently, this photocytotoxic effect leads to the death of microbes.

Two mechanisms can be mainly involved in PDI after activation of the PS [5]. In the type I photoreaction, the PS excited triplet state can react with different compounds by electron or proton transfer, producing free radicals. These radicals can also interact with molecular oxygen in the ground state, $O_2(^3\Sigma_g)$, forming ROS, such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^\bullet$) and hydrogen peroxide (H_2O_2). In the type II partway, the PS excited triplet state generates singlet molecular oxygen, $O_2(^1\Delta_g)$, by energy transfer [6]. Thus, photodynamic effect can occur through type I and type II mechanisms and the ROS formed can rapidly react with several biological substrates in microbial cells. Moreover, the photodynamic activity has multiple sites of action in cells. For this reason, PDI has shown be effective against sensitive and resistant strains to conventional chemotherapeutic [7,8]. Furthermore, in PDI treatments only the components of cells that are close to the ROS production area can be directly affected. This is due to the short half-life and limited diffusion of these cytotoxic species into cells. In this sense, a lifetime of 2 µs was estimated for $O_2(^1\Delta_g)$ in water-incubated cells [6]. This value is lower than in neat water, indicating that the $O_2(^1\Delta_g)$ produced in cells is removed by interactions with cellular components by a combination of chemical reactions and physical quenching pathways. Also, a radial diffusion distance of 155 nm was

calculated for $O_2(^1\Delta_g)$ sphere of activity in cells. Therefore, cellular photodamage is produced in the adjacent area where the PS is found in cells.

In particular, the opportunistic fungus *Candida albicans* is a member of the healthy human microbiota. This yeast is able to colonize the gastrointestinal tract, female reproductive tract, oral cavity and skin [9]. Thus, *C. albicans* is an innocuous commensal that exists in congruence with other members of the microbiota. However, disorders to this delicate balance, for example due to variations in the local environment, the use of antibiotics or alterations in the immune system, can enable *C. albicans* to quickly proliferate and cause infections [10,11]. In this way, the cytoplasmic membrane of fungi is a suitable target for the action of antifungals agents [10]. In particular, the yeast cell wall is provided of two main layers that include an outer layer composed of glycoproteins and an inner layer that contains skeletal polysaccharides [12]. The outer layer predominantly consisting of *O*- and *N*-linked mannose polymers, which are covalently associated with proteins to form glycoproteins. The inner layer of the cell wall contains the skeletal polysaccharides chitin and β-1,3-glucan, which confer strength and cell shape. Therefore, the growing resistance against antifungal drugs has renewed the search for alternative treatment modalities and PDI appears to be a potential therapy [13]. Many of PSs bind to the cell envelope because the incubation times are short in the PDI [14,15]. Thus, the cell wall is expected to be one of the main areas of damage leading to the inactivation of the microorganisms [16].

In the present work, the photodynamic mechanism sensitized by 5,10,15,20-tetrakis[4-(3-*N,N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP) and 5,10,15,20-tetrakis[4-(3-*N,N,N*-trimethylaminepropoxy)phenyl]porphyrin (TAPP^{+4}, Figure 1) was investigated to eliminate *C. albicans* cells. Furthermore, the photodynamic damage induced by these porphyrins was investigated in the structure of cells. In previous studies, it was demonstrated that this intrinsically non-charged porphyrin (TAPP) and its equivalent with cationic charges (TAPP^{+4}) are effective PSs to produce ROS in solutions and photoinactive *C. albicans* cells [17,18]. Therefore, the present studies are

intended to provide insights into the photodynamic action process involved in the photokilling of *C. albicans* sensitized by these porphyrins.

2. Materials and methods

2.1. General

Absorption spectra of porphyrins were performed as previously indicated [17]. A Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector equipped with a 150W lamp was used to irradiate the *C. albicans* cells. A wavelength between 350 and 800 nm was selected by optical filters and the light was passed through a 2.5 cm glass cuvette containing water to absorb heat. [18]. The fluence rate was measured with a radiometer (Laser Mate-Q, Coherent, Santa Clara, CA) giving 90 mW/cm^2 at the treatment site. Chemicals were purchased from Aldrich (Milwaukee, WI, USA) and they were used without further purification.

2.2. Porphyrins

TAPP and TAPP^{4+} were obtained as previously reported [19]. Stock solutions (0.5 mM) of TAPP and TAPP^{4+} were prepared by weighing and dilution in of *N,N*-dimethylformamide (DMF). The concentration of each porphyrin was established by absorption spectroscopy, considering the corresponding values of the molar absorption coefficients ($\varepsilon^{421} = 1.65 \times 10^5$ L mol^{-1} cm^{-1} for TAPP and $\varepsilon^{421} = 1.64 \times 10^5$ L mol^{-1} cm^{-1} for TAPP^{4+} in DMF) [19,20].

2.3. C. albicans strain and growth conditions

The strain of *C. albicans* PC31 was previously characterized [21]. Yeast cells were aerobically grown overnight in 4 mL Sabouraud broth (Britania, Buenos Aires, Argentina) at 37 ℃ to stationary phase. Cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min). After that, they were resuspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0) to obtain a cell

suspension of $\sim 10^7$ colony forming units (CFU)/mL. Then, the cell suspension was diluted 1/10 in PBS to obtain $\sim 10^6$ CFU/mL [18].

2.4. Photoinactivation of C. albicans cells

Tests were achieved using 2 mL cell suspension ($\sim 10^6$ CFU/mL) in PBS, which were placed in Pyrex brand culture tubes (13 x 100 mm). The cultures were treated with TAPP or TAPP^{4+} that was added from the stock solution in DMF. The maximum amount of DMF used was 1% v/v, which was not toxic to *C. albicans*. Each sample was incubated 30 min in dark at 37 ºC and irradiated 30 min with visible light. *C. albicans* cells were quantified by serial dilution in PBS and counted by the spread plate method. Viable *C. albicans* cells were determined on Sabouraud agar plates after incubation for 48 h incubation at 37 ºC [21]. Studies in anoxic condition were carried out displacing the oxygen with argon in the culture flasks for 15 min before irradiation. Argon atmosphere was kept during the illumination. Sodium azide (100 mM) and D-mannitol (100 mM) were added to *C. albicans* suspensions from stock solutions 1 M and 2 M in water, respectively. After that, cells were incubated for 30 min at 37 ºC in dark previous to the treatment with the porphyrin. Photoinactivation studies in D_2O were performed using 2 mL cell suspension ($\sim 10^6$ CFU/mL) in PBS, which were centrifuged (3000 rpm for 15 min), the supernatant was discarded and the cell pellet was resuspended in 2 mL D_2O. After that, cell suspensions in D_2O were incubated with 1 μM porphyrin 30 min in dark at 37 ºC and irradiated 30 min with visible light [22]. Cell viability was determined as described above.

2.5. Fluorescence microscopy

Microscopic experiments were performed in a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) fluorescence microscope, which was equipped with a HBO 100 W mercury lamp. Images were taken with an AxioCam HRc camera and processed using AxioVision Rel. 4.3 software. Fluorescence images of porphyrins into the cells were determined with a DBP 406/23 + 530/45, DFT 435 + 570, DBP 467/30 + 618/75 filters (Carl Zeiss) [23].

2.6. Transmission electronic microscopy (TEM)

Cell suspensions were harvested by centrifugation (3000 rpm for 15 min) and resuspended in equal volumes of 4% formaldehyde and 2% glutaraldehyde for 2 h at room temperature. Samples were washed with cacodylate buffer for three times and treated with 1% osmium tetroxide in the same buffer solution for 1 h. Then, the cells were dehydrated using gradients of acetone. After that, cells were embedded in araldite epoxy resin and polymerized for 24 h at 60 ºC. Thin sections (80-100 nm thick) were obtained by cutting 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) [24]. Then, cells were analyzed with a TEM Leo 906E (Zeiss, Germany) and photographed with a MegaView III camera. Thin sections were stained with 1% toluidine blue to identify areas containing cells.

2.7. Scanning electron microscopy (SEM)

Samples were centrifugated (3000 rpm for 15 min) and the supernatants were separated. Yeast pellets were fixed in 4% formaldehyde and 2% glutaraldehyde mix in cacodylate buffer for 2 h. Cells were dehydrated with alcohol solutions of increasing concentration (30% - 100%). Samples were dropped on round glass cover slip. Them, they were dried by critical-point method (EMITECH, Model K850, East Grinstead, West Sussex, UK). Samples were sputter coating with gold (Balzers Instruments SCD 030, Balzers, Liechtenstein) to produce electrical conductivity and detected with a SEM Philips 505 (Philips, Eindhoven, NL, USA) [25].

2.8. Controls and statistical analysis

Each experiment was repeated three times and each value was determined in triplicate. Controls of *C. albicans* were carried out in presence and absence of porphyrin in the dark and in the absence of PS with irradiated cells. DMF (<1% v/v) was not toxic to yeast cells. The unpaired t-test was used to establish the significance of differences between groups. Differences between means

were tested for significance by one-way ANOVA. Results were statistically significant using a confidence level of 95% ($p < 0.05$) [20]. Data were denoted as the mean ± standard deviation of each group.

3. Results

3.1. Determination of ROS involved in the photoinactivation of C. albicans

In order to establish the photoprocess (type I and II) that predominantly occurs in the damage of yeast cells, PDI of *C. albicans* suspensions in PBS sensitized by TAPP and TAPP^{4+} were compared with those obtained in presence of an argon atmospheres, sodium azide, D_2O and D-mannitol (Figures 2 and 3). *C. albicans* cells incubated with 5 µM PS and irradiated for 30 min led to a loss of survival of 4.5 log for both agents (Figures 2 and 3, column 4). In contrast, cells incubated with these porphyrins in dark or exposed to light in absence of PS not showed a reduction in their viability (Figures 2 and 3, columns 2 and 3). The same study was done under an argon atmosphere to simulate a low oxygen pressure. Irradiation of the cells under argon without PS not affected the cell viability (Figures 2 and 3, column 5). In presence of porphyrin, cell survival was reduced by 1 log for TAPP and less than 1 log for TAPP^{4+} (Figures 2 and 3, column 6), showing the importance of oxygen in the photokilling of *C. albicans*. Moreover, azide ions was used as a quencher of $O_2(^1\Delta_g)$, it can also deactivate other compounds in its excited triplet state through an energy transfer [26]. No toxicity was detected when cells treated with 100 mM sodium azide were irradiated for 30 min (Figures 2 and 3, column 7). In addition, yeast cells were highly protected from the photodynamic activity mediated by porphyrins, reducing 1.5 log for TAPP, while photoinactivation was negligible for TAPP^{4+} (Figures 2 and 3, column 8). Furthermore, studies were performed using *C. albicans* cell suspensions in D_2O. In this medium, $O_2(^1\Delta_g)$ has a longer lifetime than in aqueous suspension. Therefore, reactions in which $O_2(^1\Delta_g)$ is involve should be favored [6]. In this study, the cell suspensions in D_2O were incubated with 1 µM PS. This concentration was selected to not produce a complete eradication of *C. albicans* and thus be able to observe the effect produced by this medium. Irradiated yeast cells in D_2O

without porphyrin did not show toxic effect respect to control (Figure 2 and 3, column 9). When cultures were treated with 1 μM porphyrin in PBS, a photoinactivation of 1.5 log was found after 30 min irradiation. Under these conditions, an increase in photokilling of *C. albicans* was detected D_2O, producing a decrease in cell survival of 1 log for TAPP and 1.5 log for TAPP^{4+} respect to the corresponding cell suspensions in PBS (Figure 2 and 3, columns 10 and 11). On the other hand, D-mannitol was used an inhibitor of type I reactions, because acts like a scavenger of radicals, such as $O_2^{\bullet-}$ and $HO^{\bullet}$ [27]. The addition of 100 mM D-mannitol in absence of porphyrin did not affect the survival of yeast cells irradiated for 30 min (Figure 2 and 3, column 12). Moreover, the photocytotoxic activity induced by both porphyrins were not affected for cells treated with 5 μM photosensitizer and 30 min irradiation in presence of D-mannitol (Figure 2 and 3, columns 4 and 13).

3.2. Fluorescence images in C. albicans cells

The binding of TAPP and TAPP^{4+} to *C. albicans* cells was explored by fluorescence microscopy. The images showed that yeast cells incubated with 5 μM PS in PBS for 30 min in the dark exhibited red fluorescence that is typical of porphyrins (Figures 4 and 5) [16,23]. Therefore, both porphyrins were strongly bound to yeast cells. For TAPP was observed that the distribution was uniform by the entire cell (Figure 4). In most cases, TAPP^{4+} was localized in certain subcellular regions more than others (Figure 5). Moreover, the fluorescence emission of these PSs was not affected by PDI treatments and similar red emission intensity was observed before and after irradiation.

3.3. Photodynamic effect on cellular structure of C. albicans cells

In order to investigate at morphological level, the photocytotoxic activity induced by TAPP and TAPP^{4+} in the cell structure of *C. albicans* were analyzed by TEM. The results are shown in Figure 6. As can be observed, control cells showed typical oval form of yeast and thick cell wall well defined [28,29]. Moreover, it can be detected an electron-dense zone in cytoplasm that corresponds

to organelles limited by a membrane. Similar remarks were detected in irradiated cells in absence of PSs or in cells incubated with porphyrins and keeping in dark. In contrast, images displayed alterations at structural level of the treated *C. albicans* cells with PS and irradiated for 30 min, which were not detected in the controls. PDI induced intracellular alteration that showed a loss of oval form with strong changes on cell wall. Photodynamic activity produced by these porphyrins presented manifest changes in the cell wall, membrane and cytoplasm respect to the controls. Moreover, control of *C. albicans* stained with toluidine blue showed normal and spherical blue cells (Figure 7). In contrast, for culture incubated with TAPP and TAPP^{4+} were found empty cells with a clear appearance and easy to identify by their irregular shapes (Figure 7, E and F).

3.4. Photodynamic effect on C. albicans cell surface

The photodynamic activity mediated by TAPP and TAPP^{4+} on the surface of *C. albicans* were analyzed by SEM (Figure 8). The untreated *C. albicans* cultures appeared ellipsoidal and pyknotic cells, characteristic of this strain of yeast [29,30]. After incubation with porphyrins in dark, the shape of the cells was not changed and the shapes were similar to untreated *C. albicans* cells. Moreover, after irradiation for 30 min of untreated cultures, the cell wall was unaffected and presented a healthy appearance. Cells incubated with porphyrins and irradiated for 30 min exhibited deformations like wrinkles and blisters, which were not detected in the untreated controls.

4. Discussion

Porphyrins substituted by cationic groups have received considerable attention due to their high effectiveness as photodynamic agents to inactivate microorganisms [3]. In this investigation, two porphyrin derivatives, TAPP and TAPP^{4+}, were used as PSs to inactivate *C. albicans*. The main difference between them is that TAPP, unlike TAPP^{4+}, does not present intrinsic positive charges in its structure. However, considering the basicity of the aliphatic amine groups (3-(*N,N*-dimethylamine)propanol, $pK_a = 9.51$) in the periphery of the macrocycle, protonation of these

substituents is expected in PBS [31]. Moreover, in both cases the aliphatic spacer provides a higher mobility of the charges facilitating the binding to yeast cells, improving their photo-inactivating capacities.

TAPP and TAPP^{4+} absorb in the visible region, showing the characteristic Soret band at 421 nm and the four Q-bands between 515 and 650 nm [17]. Furthermore, these porphyrins were able to photosensitize O$_2$($^1\Delta_g$) in DMF with quantum yields of $\Phi_\Delta \sim 0.5$ (Figure 1) [17]. In addition, these porphyrins were able to efficiently photo-oxidize the amino acid L-tryptophan (Trp) in microheterogeneous media. In addition, the formation of O$_2$$^{\bullet-}$ through a type I photoreaction process sensitized by TAPP was found in the presence of a reductant, such as NADH [20]. Previous investigations showed that porphyrins bearing cationic substituents directly attached to aromatic substituents appeared to act as photosensitizers primarily through the intermediation of O$_2$($^1\Delta_g$) in *C. albicans* [22]. However, the photodynamic effect of the porphyrins determined in solution can undergo considerable modifications in the cells, mainly depending on where the PS is localized. Therefore, depending on the structure of the PS and the experimental conditions, both O$_2$($^1\Delta_g$) and O$_2$$^{\bullet-}$ could be involved in the photodynamic activity. In particular, the photodynamic properties of TAPP and TAPP^{4+} together with their high affinity for the cells make to these compounds efficient photosensitizing agents. *In vitro* studies with *C. albicans* cultures showed that the amount of TAPP or TAPP^{4+} bound to the cells was not dependent on the incubation time between 15 and 60 min [18]. For cells treated with 5 μM PS, the binding was 1.1 nmol/10^6 cells for TAPP and 1.4 nmol/10^6 cells TAPP^{4+}. Cells treated with 1 μM porphyrin produced a photoinactivation of 99.0% (2 log decrease) after 30 min irradiation. When the *C. albicans* cells were incubated with 5 μM porphyrin a value greater than 99.996% (4.5 log decrease) of cell inactivation was observed when the cultures were irradiated for 30 min with visible light. Therefore, the most efficient conditions for inactive *C. albicans* were selected to assess the mechanism of photodynamic action. In order to establish the photoprocess (type I and II) that mainly occurs during PDI, photokilling of yeast cells were investigated under different experimental conditions. Photoinactivation was negligible when oxygen

atmosphere was replaced by argon. For both photodynamic mechanisms, the production of ROS requires aerobic conditions. Furthermore, these results indicate an insignificant contribution of a possible oxygen-independent photoinactivation pathway leading to the death of yeast cells [32]. The addition of azide ions produced a high photoprotection of *C. albicans* cells incubated with TAPP or TAPP^{4+} after 30 min irradiation. Sodium azide is known to prevent the photodynamic damage produced by $O_2(^1\Delta_g)$ in microbial cells [33,34]. Although this is an important indication of a mediation of the type II mechanism, it should be considered that azide ions can also deactivate PSs in their triplet excited state. Therefore, D$_2$O was used instead of water in order to increase the $O_2(^1\Delta_g)$ lifetime [6,35]. Irradiation of *C. albicans* cell suspensions in D$_2$O with porphyrins produced a significant increase in the reduction of yeast viability in comparison with cells in PBS. This effect also indicates the involvement of $O_2(^1\Delta_g)$ in cell death. In addition, D-mannitol was used as a free radical scavenger to investigate the contribution of type I mechanism [27,36]. Photokilling of *C. albicans* induced by TAPP or TAPP^{4+} was unaffected when D-mannitol was added to cell suspensions. Consequently, an involvement of type I pathway was not found for the photokilling of *C. albicans* induced by these porphyrins. Comparable behavior was previously observed using cationic porphyrins as PSs to inactivate *C. albicans* [22]. Therefore, this family of compound appears to act primarily through a type II photoprocess to inactivate *C. albicans* in aerobic conditions.

On the other hand, TAPP or TAPP^{4+} showed a strong affinity by *C. albicans* cells [18]. When cells were incubated with 5 μM porphyrin, the amount of TAPP recovered was 1.1 nmol/10^6 cells, while TAPP^{4+} achieved a value of 1.4 nmol/10^6 cells. Furthermore, the amount of cell-bound porphyrin was not substantially changed incubating the yeast cells for different times (15-60 min). Fluorescence microscopic images provided additional insight into the uptake of these porphyrins by *C. albicans* cells. The fluorescence quantum yields of TAPP and TAPP^{4+} showed values of $\Phi_F \sim 0.1$ in DMF (Figure 1) [17]. The red fluorescence images in yeast cells confirm that these PSs presented a high binding affinity to *C. albicans*. In most cases, strong porphyrin fluorescence was observed in the entire cell. It was previously established that 5-phenyl-10,15,20-tris(*N*-methyl-4-

pyridyl)porphyrin (TriP[4]) interact with *C. albicans* cells resulting in peripheral fluorescence patterns [16]. Also, similar patter of red emission inside *C. albicans* cells was observed for 5,10,15,20-tetrakis(4-*N,N,N*-trimethylammoniumphenyl) porphyrin (TMAP^{4+}) [23]. After 30 min irradiation, the red emission of TAPP and TAPP^{4+} was still observed indicating a low photobleaching of these porphyrins. Furthermore, a redistribution of PS can take place in cells after PDI treatments due to damage to the cell membrane and eventual increase in permeability, allowing relocation of molecules that were weakly bound to cells or remained in solution [16,23].

The photodynamic effect sensitized by TAPP and TAPP^{4+} on cellular structure of *C. albicans* cells was analyzed by TEM. The control cells presented round bodies that were characterized by a single limiting membrane, a discreetly dense matrix with a central electron-lucid core. As opposed, alterations in the structure of the *C. albicans* cells were observed for cultures incubated with these porphyrins after PDI treatment. Cells showed structural disorder within the cell cytoplasm with alterations in the cell envelope. Apparently, the cells underwent changes in a way that was mainly characterized by deformations and inflammation of the cell envelope. Moreover, photoinactivated *C. albicans* cells stained with toluidine blue showed empty cells with irregular shapes, which can be considered dead yeast. Therefore, photooxidative processes that involving $O_2(^1\Delta_g)$ are the mainly responsible for the changes produced in the cytoplasm and the components of the cell wall, conducing to alterations of the cell envelope structure. This photocytotoxic activity can lead to the inability to multiply and to produce viable *C. albicans* cells. Photodamage on the cellular surface were also observed via freeze-fracture electron microscopy images for *C. albicans* following PDI with TriP[4] [16]. 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 [28]. Comparable damage was observed in a previous study where *C. albicans* was inactivated by 5-(4-trifluorophenyl)-10,15,20-tris(4-*N,N,N*-trimethylammoniumphenyl) porphyrin and TMAP^{4+}[24]. Also, it was observed that hypocrellin B mediated PDI induced membrane ruptures and cell wall swelling of *C. albicans*, suggesting increased cell membrane permeability and impaired cell wall function [29].

Furthermore, differences on the cellular surface of *C. albicans* after PDI treatment with TAPP and TAPP[4+] were also observed by SEM. Control cells showed a smooth and homogeneous cell surface, whereas the photodynamic effect produced morphology defects, such as a highly heterogeneous, warty and rougher cell surface. In previous investigation, SEM images revealed the twisted and ruptured *C. albicans* cells after PDI treatment with hypocrellin B, suggesting that the cell envelope was severely damaged [29]. Moreover, the decrease in *C. albicans* growth after irradiation of cultures incubated with methylene blue was associated with an increase in membrane permeabilization [37]. Therefore, modifications in the cytoplasmic macromolecules and alteration in the cell barriers induced by the photodynamic action of TAPP and TAPP[4+] play a major role in *C. albicans* inactivation.

Conclusions

This investigation provides knowledge about the photodynamic mechanism of action and cell damage that occur in the PDI of *C. albicans* mediated by TAPP and TAPP[+4]. Although porphyrins with positive charges in the phenyl groups produced the photodynamic action mainly by a type II mechanism, this behavior could be different depending on where the PS is located in the cells. Unlike these, TAPP and TAPPP contain positive charges attached through a flexible spacer that allows for better interaction with cellular components, and the polarity of the microenvironment can significantly influence the mechanism of action. Therefore, to establish the photodynamic action pathway, it was determined that an oxygen atmosphere is necessary for an efficient PDI of yeast. Moreover, photoprotection was found with the addition of sodium azide as type II quencher, while an increase in photokilling was detected in cell suspensions in D_2O respect to water due to a longer lifetime of $O_2(^1\Delta_g)$. In contrast, photokilling of *C. albicans* was not affected in presence of D-mannitol, as a type I scavenger. Therefore, $O_2(^1\Delta_g)$ appears to be the main reactive species in yeast cell eradication. Furthermore, the red fluorescence emission of porphyrins into the cells confirm that these PSs have high binding affinity to *C. albicans*. Morphologic changes were found after PDI

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treatments, which showed modifications and deformation of cell envelopment. Consequently, the photodamages mediated by $O_2(^1\Delta_g)$ mainly produced modifications in the cell barriers conducing to the photoinactivation of *C. albicans* cells.

Acknowledgments

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Figure legends

Figure 1. Structure of the TAPP and TAPP^{4+} porphyrins, fluorescence quantum yields (Φ_F) and O$_2$($^1\Delta_g$) quantum yields (Φ_Δ) [17].

Figure 2. Survival of *C. albicans* ($\sim 10^6$ UFC/mL) incubated with TAPP in dark for 30 min to 37 ºC and irradiated with visible light for 30 min (90 mW/cm^2); 1) dark control; 2) irradiated control; 3) cells treated with TAPP in dark; 4) cells treated with 5 µM TAPP and irradiated; 5) irradiated cells under argon; 6) cells treated with 5 µM TAPP under argon and irradiated; 7) irradiated cells treated with 100 mM sodium azide; 8) cells treated with 100 mM sodium azide, 5 µM TAPP and irradiated; 9) irradiated cells in D$_2$O; 10) cells treated with 1 µM TAPP in PBS and irradiated; 11) cells treated with 1 µM TAPP in D$_2$O and irradiated; 12) irradiated cells treated with 100 mM D-mannitol; 13) cells treated with 100 mM D-mannitol, 5 µM TAPP and irradiated.

Figure 3. Survival of *C. albicans* ($\sim 10^6$ UFC/mL) incubated with TAPP^{4+} in dark for 30 min to 37 ºC and irradiated with visible light for 30 min (90 mW/cm^2); 1) dark control; 2) irradiated control; 3) cells treated with TAPP^{4+} in dark; 4) cells treated with 5 µM TAPP^{4+} and irradiated; 5) irradiated cells under argon; 6) cells treated with 5 µM TAPP^{4+} under argon and irradiated; 7) irradiated cells treated with 100 mM sodium azide; 8) cells treated with 100 mM sodium azide, 5 µM TAPP^{4+} and irradiated; 9) irradiated cells in D$_2$O; 10) cells treated with 1 µM TAPP^{4+} in PBS and irradiated; 11) cells treated with 1 µM TAPP^{4+} in D$_2$O and irradiated; 12) irradiated cells treated with 100 mM D-mannitol; 13) cells treated with 100 mM D-mannitol, 5 µM TAPP^{4+} and irradiated.

Figure 4. Microscopic observation of *C. albicans* incubated with 5 µM TAPP for 30 min in the dark; A) cells under a bright field; B) fluorescence; C) cells under a bright field after 30 min irradiation; D) fluorescence after 30 min irradiation (100 × microscope objective).

Figure 5. Microscopic observation of *C. albicans* incubated with 5 μM TAPP^{4+} for 30 min in the dark; A) cells under a bright field; B) fluorescence; C) cells under a bright field after 30 min irradiation; D) fluorescence after 30 min irradiation (100 × microscope objective).

Figure 6. TEM images of *C. albicans* cells treated with 5 μM porphyrin for 30 min in the dark, A) control of untreated cells; B) treated cells with TAPP in dark; C) treated cells with TAPP^{4+} in dark; D) control of untreated cells after 30 min irradiation; E) treated cells with TAPP after 30 min irradiation; F) treated cells with TAPP^{+4} after 30 min irradiation.

Figure 7. Microscopic images of *C. albicans* cells treated with 5 μM porphyrin for 30 min in the dark assessed with toluidine blue, A) control of untreated cells; B) treated cells with TAPP in dark; C) treated cells with TAPP^{4+} in dark; D) control of untreated cells after 30 min irradiation; E) treated cells with TAPP after 30 min irradiation; F) treated cells with TAPP^{+4} after 30 min irradiation.

Figure 8. SEM images of *C. albicans* cells treated with 5 μM porphyrin for 30 min in the dark, A) control of untreated cells; B) treated cells with TAPP in dark; C) treated cells with TAPP^{4+} in dark; D) control of untreated cells after 30 min irradiation; E) treated cells with TAPP after 30 min irradiation; F) treated cells with TAPP^{+4} after 30 min irradiation.

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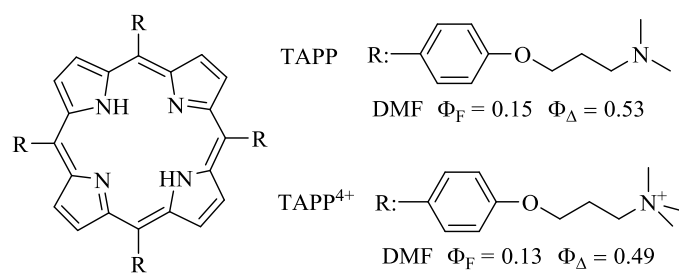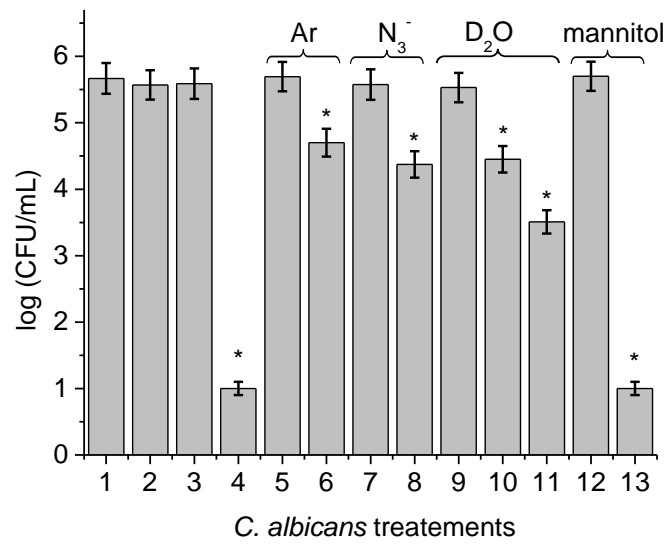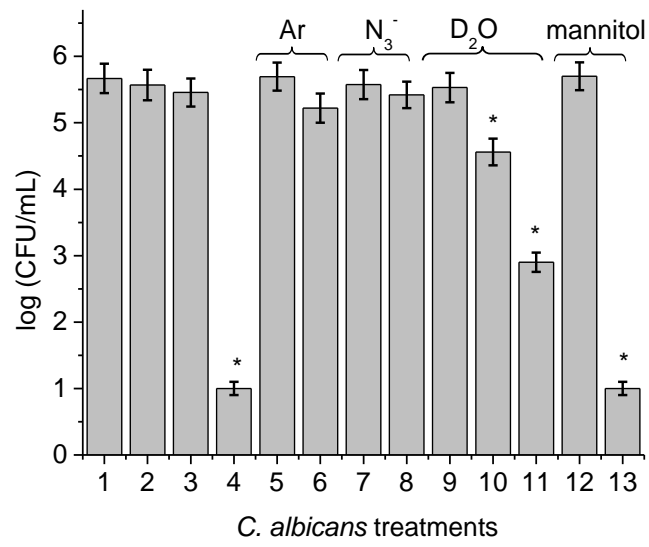
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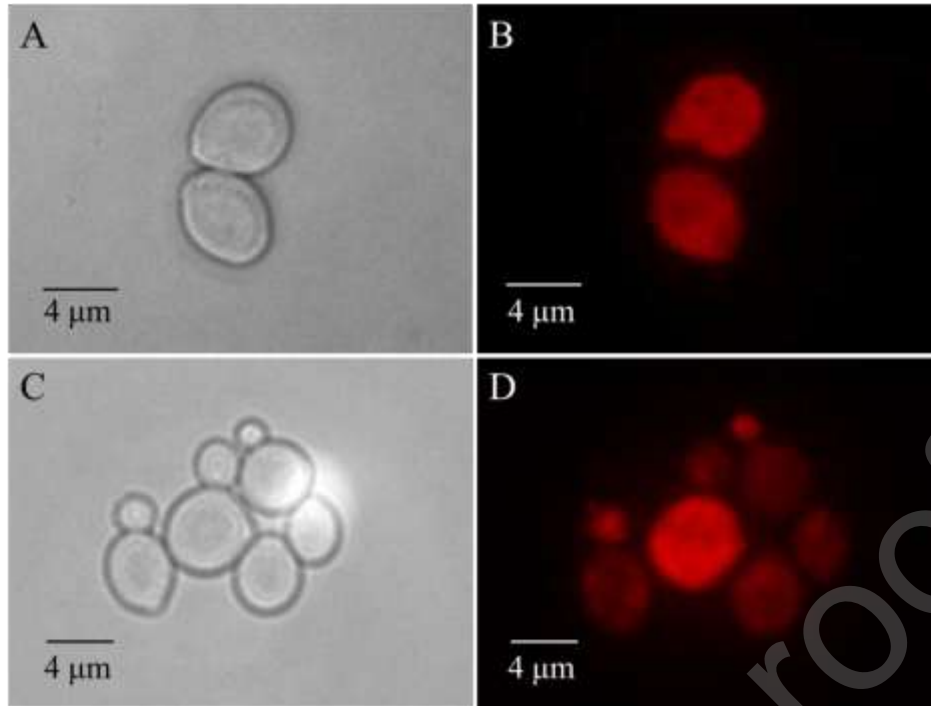
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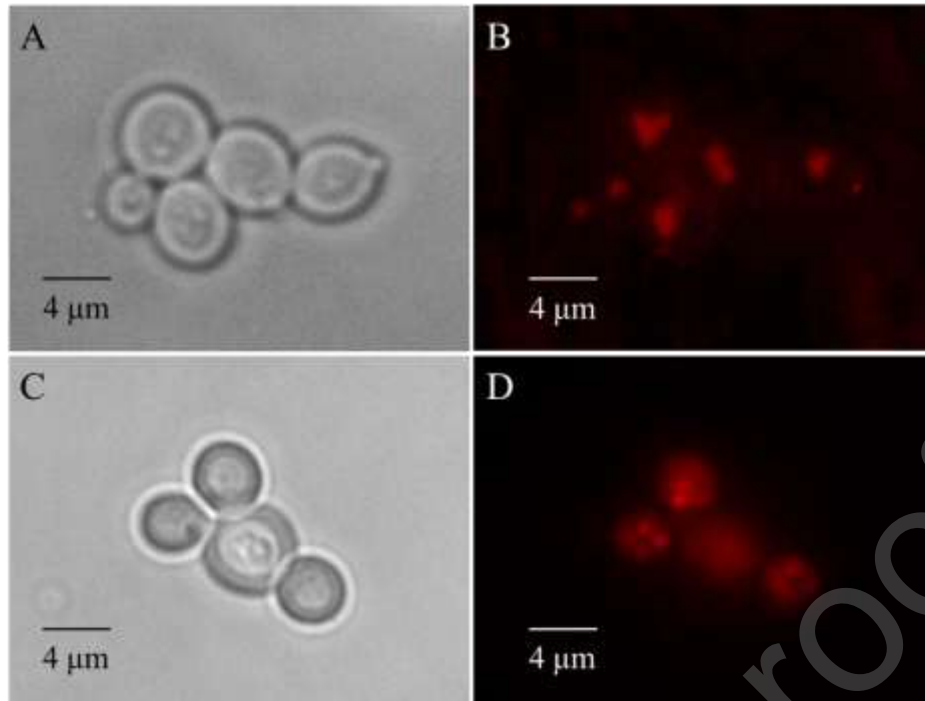
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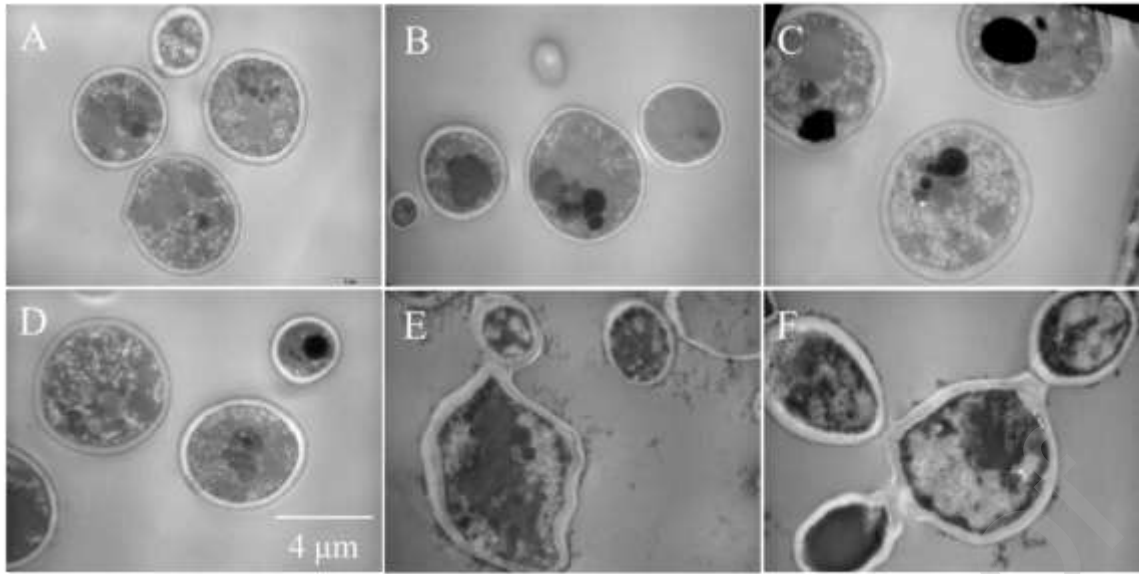
Figure 6

Figure 7

Figure 8