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Photodynamic inactivation of *Candida albicans* using bridged polysilsesquioxane films doped with porphyrin

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ABSTRACT

Novel photoactive bridged polysilsesquioxane films were prepared by doped with a porphyrin derivative. The films were formed by acid-catalyzed polycondensation reaction of a precursor of a bridged silsesquioxane, based on the reaction product of (glycidoxypropyl)trimethoxysilane with *n*-dodecylamine in the presence of 5-(4-carboxyphenyl)-10,15,20-tris(4-methylphenyl)porphyrin, followed by solvent evaporation. This procedure allowed obtaining flexible thin films. Absorption and fluorescence spectroscopic analysis showed the characteristic bands of the porphyrin in the visible region indicating that the photosensitizer is mainly embedded as monomer in the films. Photodynamic properties of the polymeric films were studied in solution containing photooxidizable substrates. Singlet molecular oxygen, $O_2(^1\Delta_g)$, production was observed by the reaction with 9,10-dimethylanthracene and 9,10-anthracenediyl-bis(methylene)dimalonic acid in different media. Also, these films photosensitized the decomposition of L-tryptophan. In vitro investigations showed that these films produce photodynamic inactivation of *Candida albicans* cells in aqueous suspensions and on their surfaces. These films exhibit a photosensitizing activity causing a ~2.5 log (99.7%) decrease of cellular survival after 60 min of irradiation with visible light. Also, the photocytotoxicity of the surfaces was tested under condition of microbial growth. Yeast cells exposed to the film and illuminated showed growth delay compared with controls. Studies of photodynamic action mechanism showed that the photoinactivation increased in D_2O, while cells were protected in the presence of azide ion. In contrast, the addition of mannitol produced a negligible effect on the cellular phototoxicity. These results provide evidence that $O_2(^1\Delta_g)$ produced by the polymeric film doped with porphyrin can successfully inactivate *C. albicans* in cell suspensions and deposited on the film surface.

1. Introduction

The increasing diffusion of infectious diseases represents a major challenge for human health worldwide, especially as a consequence of the continuous emergence of antifungal-resistant yeast.[1] The ubiquitous commensal member of the human microflora, *Candida albicans*, is the most important fungal opportunistic pathogen, which can cause various diseases from superficial mucosal infections to life-threatening systemic disorders.[2,3] Superficial skin mycosis caused by *Candida* species is one of the most frequent diseases in humans and animals. Conventional treatments are prolonged and the appearance of drug resistant strains is more frequent in high risk groups.[4,5] For these reasons, effective alternatives are required to treat fungal superficial infections. A promising modality for the treatment of candidiasis is photodynamic inactivation (PDI) of microorganisms. PDI combines a photosensitizer, light and oxygen to produce cytotoxic reactive oxygen species (ROS), which specifically generates a cascade of biochemical events that produce cell damages inactivating the microorganisms.[6,7]

In PDI process, the photosensitizer excited state can react with molecules from its direct environment by electron or hydrogen transfer, leading to the production of radicals (type I reaction), or it can transfer its energy to oxygen, generating the highly reactive singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II reaction).[8] 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. 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.

Several photosensitizers have been investigated for PDI applications in the inactivation of yeast.[9,10] Most of PDI studies have been carried out adding the photosensitizer to cell suspensions. In this procedure, after treatment traces of the photosensitizer can remain in the medium, leading to an undesired remnant photodynamic effect. An alternative to avoid this inconvenient is represented by photosensitizers immobilized on polymeric supports.[11,12] Also, this

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procedure could allowing the re-utilization of the photodynamic polymer. Most studies have been performed in bacterial cultures.[13–17] However, investigations of PDI using photoactive polymeric films are very scarce on yeast. A bioadhesive patch-based delivery of 5-aminolevulinic acid (ALA) to the nail was proposed for photodynamic therapy of onychomycosis.[18] Also, a mucoadhesive patch containing toluidine blue O (TBO) was reported as a potential delivery system for use in photodynamic antimicrobial of oropharyngeal candidiasis.[19] Other surfaces used involve electrochemically generated porphyrin polymeric films on optically transparent indium tin oxide electrodes.[20] The photoantimicrobial action of these films was used to inactivate *C. albicans* cells. In addition, the inactivation of *Saccharomyces cerevisiae* was evaluated on the surface of a photoactive polymer comprising covalently bound anthraquinone moieties.[21]

In this work, bridged polysilsesquioxane plastic films doped with 5-(4-carboxyphenyl)-10,15,20-tris(4-methylphenyl)porphyrin (P-acid), SSO-P, were evaluated as photodynamic surfaces both in solution containing photooxidizable substrates and in *C. albicans* cells.

2. Materials and methods

2.1. General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan) and on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc, Edison, NJ, USA), respectively. A solid sample holder placed at 45° was employed for fluorescence spectra of the films; all measurements were performed in a front surface geometry at room temperature employing samples with the same thickness. Spectra in solution were recorded using 1 cm path length quartz cuvettes at room temperature. Fourier-transformed infrared (FTIR) spectra were recorded with a Nicolet 6700 device (Thermo Scientific, Corp., Madison, WI USA) in the absorbance mode, in the range 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. FTIR spectra were obtained by ATR employing 32 scans. Differential scanning calorimetry (DSC) scans, were performed with a DSC-50 Shimadzu (Tokyo, Japan) equipment at 10 °C/min. Surface hydrophobicity was evaluated by initial static contact angle measurements of a water drop deposited over the film surface. Photographs were taken with a digital camera after stabilization (about 5 min). Results were the mean values of eight independent measurements.

Irradiation experiments with *C. albicans* cells were performed using a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. A wavelength range between 350–800 nm was selected by optical filters. Irradiation of the cultures was performed as previously described.[22] The light fluence rate 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. Dodecylamine (DA) was provided by Fluka (Buchs, Switzerland) and glycidoxypropyltrimethoxysilane (GPTMS) from Sigma (St. Louis, MO, USA) were used without further purification. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Tetrahydorfuran (THF) was refluxed in KOH and distilled over 4 Å molecular sieves. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Synthesis of bridged polysilsesquioxane films doped with porphyrin

5-(4-Carboxyphenyl)-10,15,20-tris(4-methylphenyl)porphyrin (P-acid) was synthesized as previously described.[23] The synthesis of the films based on bridged silsesquioxane was reported before.[24] The precursor of the silsesquioxane was synthesized employing stoichiometric amounts of DA and GPTMS. The reaction was carried out in 0.4 M THF solution at 58 °C for 48 h under nitrogen atmosphere, attaining complete conversion. The hydrolysis and condensation was performed at room temperature, employing 0.1 M solutions in THF adding an appropriate amount of water and catalyst (formic acid) in order to obtain the molar ratio Si/$HCOOH/H_2O$ = 1/0.1/3. Solutions (25 mL) were cast in polyacetal recipients of 5 cm diameter with an initial height of liquid of close to 5 mm and place in an oven at 30 °C for 24 h. Hydrolysis and condensation reactions took place together with solvent evaporation. Films doped with porphyrin were synthesis as follow: after adding the reactants for hydrolysis and condensation, the corresponding amount of porphyrin was incorporated from a 4×10^{-4} M P-acid stock solution in THF. The porphyrin concentration was checked by spectroscopy, taking into account the value of molar extinction coefficients (ε); P-acid $\varepsilon = 4.05 \times 10^5$ M^{-1} cm^{-1} at 424 nm in THF.[25] The mixture was placed in the polyacetal recipients until complete solvent evaporation. Three kinds of films were synthesized, film A: undoped (SSO); film B: doped with 8.8×10^{-6} M P-acid (SSO-P) and film C: doped with 1.8×10^{-5} M P-acid. These concentrations were calculated with all the reactants before hydrolysis and polycondensation of the films take place.

2.3. Steady state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 35 μM) in *N*,*N*-dimethylformamide (DMF), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA, 35 μM) in water or L-tryptophan (Trp, 25 μM) in DMF were irradiated in 1 cm path length quartz cells (2 mL) containing the films (surface 0.7×25 mm) in different media. The samples bearing DMA and ABDA were irradiated as described above but using a wavelength range between 455 and 800 nm (GG455 cutoff filter). The kinetics of DMA and ABDA photooxidation were studied following the decrease of the absorbance (A) at λ_{max} = 378 nm, while the fluorescence intensity (F) decrease at λ = 350 nm was used for Trp. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of Ln A_0/A or Ln F_0/F vs time. All the experiment were performed at 25.0 ± 0.5 °C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 10%.

2.4. Microorganisms and growth conditions

The strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified.[26] Yeast was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (3 mL) at 37 °C to stationary phase. An aliquot of this culture (1 mL) was dissolved in 3 mL Sabouraud broth. Then, cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH 7.0), corresponding to $\sim 10^7$ colony forming units (CFU)/mL.

2.5. Quantification of cells and control cultures

The *C. albicans* cells were appropriately diluted to obtain $\sim 10^6$ CFU/mL in PBS. After the irradiation period, cellular suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar plates after ~48 h incubation at 37 °C.

In all cases, control experiments were carried out in the absence of SSO-P film with cellular suspensions irradiated and in the dark, in presence of SSO-P film in the dark and in presence of SSO film

irradiated and in the dark. Each experiment was repeated separately three times.

2.6. Photosensitized inactivation of C. albicans in PBS suspension

The films were positioned in a Pyrex brand culture tubes (13 × 100 mm) containing the cellular suspensions of microorganisms (2 mL, ~10^6 CFU/mL) in PBS. After that, the cultures were exposed to visible light for different time intervals. Cellular suspensions were serially diluted with PBS and the number of colonies formed was counted as described above. In the experiments with P-acid, the sensitizer was added from a stock solution 0.5 mM in DMF as previously reported.[27] The cells were treated with 5 μM P-acid for 30 min in dark at 37 °C and irradiated with visible light.

2.7. Photosensitized inactivation of C. albicans on the film surface

For the antifungal drop-test each film was placed into a sterilized Petri dish. Then, a droplet (100 μL) of the diluted saline solution containing *C. albicans* (~10^6 CFU/mL) was placed on the surface of the film. After exposing the films to light irradiation, each film was placed in a Pyrex brand culture tubes (13 × 100 mm) containing 4 mL of PBS. Then, the cellular suspensions were serially diluted with PBS and the number of colonies formed was counted as described above.

2.8. Growth curves of *C. albicans* cultures

Cultures of *C. albicans* cells were grown overnight as described above. A portion (1 mL) of this culture was transferred to 20 mL of fresh Sabouraud broth medium. The suspension was homogenized and aliquots of 2 mL were incubated with the films at 37 °C. The culture grown was measured by turbidity at 660 nm every 60 min using a Turner SP-830 spectrophotometer (Dubuque, IA, USA). Then the flasks were irradiated with visible light at 37 °C, as described above.

2.9. Statistical analysis

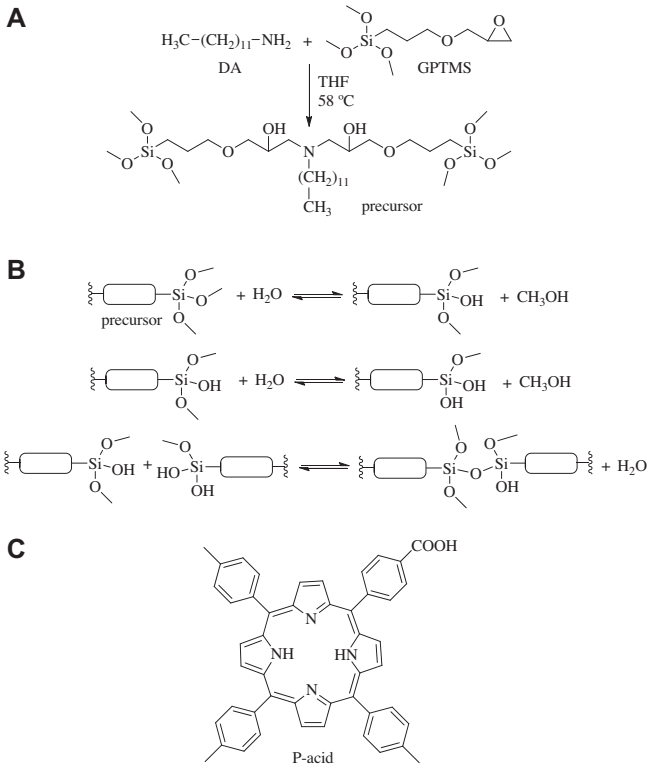
All data were presented as the mean ± standard deviation of each group. Variation between groups was evaluated using the Students *t*-test, with a confidence level of 95% (p <0.05) considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of the films

First, terminal Si(OCH_3) groups of the precursor were converted into Si(OH) groups in the presence of an acid catalyst (HCOOH) and water. These groups react among themselves or with Si(OCH_3) groups, leading to a cross-linked polymer through the formation of Si–O–Si bonds.[24] The bridged silsesquioxane undergoes a nanostructuration process during which organic bridges become self-assembled through dodecyldodecyl interactions. Molecular structures and schematic representation of the synthesis are presented on Scheme 1. Films obtained without porphyrin (SSO) were transparent with a slight yellow color (Fig. 1A). A low content of acid was used to diminish the polycondensation rate. This procedure allowed obtaining organized bridge groups in the matrix producing flexible thin films.

Films doped with porphyrin (SSO-P) were synthesized adding the corresponding amount of a P-acid stock solution in THF after



Scheme 1. (A) Synthesis of the precursor, (B) hydrolysis and polycondensation of silane groups and (C) P-acid porphyrin structure.

Figure 1. Photograph of the flexible bridged polysilsesquioxane plastic films undoped SSO (A) and doped SSO-P with 550 μL (B) and 1100 μL (C) of P-acid 4×10^{-4} M stock solution in THF.

adding the reactants for hydrolysis and condensation. The concentrations of P-acid calculated with all the reactants were 8.8×10^{-6} M and 1.8×10^{-5} M, while the final concentrations in the films were 2.6×10^{-4} w/w and 5.2×10^{-4} w/w, respectively. Films were obtained as a flexible and light pink material with a thickness around 400 μm (Fig. 1).

In order to analyze these materials as potential photoactive films, several experiments were performed with the aim to establish their chemical and thermal resistance. First, no changes were found in the FTIR spectrum of the SSO-P film with respect to that of SSO indicating that this amount of porphyrin did not produce significant alteration in the unmodified film[24]A low refraction

index of 1.51 was observed for the films. The hydrophobicity of the films was measured by the water contact angle. A value of 103 ± 5° was obtained for the undoped films. A comparable value was obtained for the doped films. Those values are similar to the contact angle reported for Teflon.[28,29] The thermal stability of the films was analyzed by DSC. The matrix displayed little weight loss from room temperature to 250 °C. A small weight loss was observed under analysis; this might be attributed to the volatilization of water or ethanol due to the condensation of Si–OH or Si–OCH_3 groups or to the loss of solvent. A second scan did not show neither transitions nor loss of mass.

Chemical resistance of the films was studied under different media such as water, buffer pH 7, ethanol and DMF. The films were immersed in solvents at ambient temperature 20 ± 2 °C for 7 days. The results show that the polymers are stable under the different chemical environments; a minor amount of swelling, less than 4% was observed in the aqueous media, nor loss of water is observed in ethanol and DMF. FTIR and UV–vis absorption spectra (see below) of the films remain equal after a week in the solvents. Finally the UV–Vis spectra of the solutions in contact with the films were analyzed after one week. No presence of porphyrin in solution was detected by this technique. Moreover, the extraction with chloroform did not show the presence of porphyrin. Therefore, these results indicate that the porphyrin is strongly trapped in the matrix possible by the formation of covalent bonds between the carboxylic group of the porphyrin and the silane groups of the precursor.

3.2. Spectroscopic studies

The absorption spectra of SSO-P film, SSO film and P-acid in THF are shown in Figure 2A. The SSO-P film showed the typical *Soret* and *Q*-bands, characteristic of a free-base porphyrin.[30] In the films, the *Soret* and *Q* bands show similar electronic transitions to those observed for the corresponding porphyrin in solution. For SSO-P film, no broader and shifted was observed in comparison with that of monomeric P-acid in solution. These facts indicate that the porphyrin was dissolved in the film mainly as monomer. Also, SSO-P showed an absorption band in the UV region (~316 nm) due to the SSO material. The spectrum of SOO-P is essentially a linear combination of the spectra of the corresponding P-acid and SSO film, with only minor differences in wavelength maxima and band shapes (Figure 2A). Thus the absorption spectra are consistent with only a weak interaction between the polymer and the porphyrin in the ground state and the tetrapyrrolic macrocycle retains its individual identities.

The steady-state fluorescence emission spectrum of the SSO-P film is shown in Figure 2B. The two bands are characteristic for free-base porphyrins in solution and they have been assigned to *Q*(0–0) and *Q*(0–1) transitions.[25] From the absorption and fluorescence spectra, a small Stokes' shift of 5 nm was calculated for SSO-P film indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. That suggests that only a minor geometric relaxation occurs in the first excited state. Also, the fluorescence excitation spectrum of SSO-P film coincides with the corresponding absorption spectra (Fig. 2A inset). These results indicate that the spectroscopic properties of the units of P-acid remain unalterable when the porphyrin is incorporated in the solid media and this porphyrin can be dispersed in the matrix without aggregation.

3.3. Photodynamic properties

The $O_2(^1\Delta_g)$ photosensitized by SSO-P was identified by the change in the absorption spectrum of chemical probes, DMA and ABDA, in different media.[31] Under aerobic conditions, the $O_2(^1\Delta_g)$ generated converts both substrates into their corresponding endoperoxide forms, leading to the reduction in the intensity of the peaks in its absorption spectra. Typical results are shown in Figure 3 for ABDA in water photosensitized by SSO-P film. From first-order kinetic plots of the substrate absorption at 378 nm with time the values of the observed rate constant (k_{obs}) were calculated. As can be observed in Table 1, the value of k_{obs} for ABDA in water was lower than that for DMA in DMF. This behavior can be influenced by a different decomposition rate of the substrates and mainly by the decrease in the $O_2(^1\Delta_g)$ lifetime in water.[32]

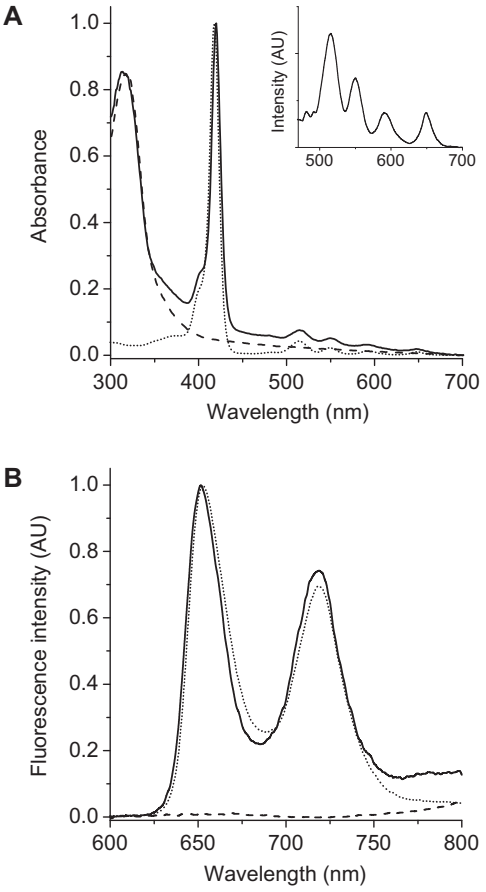


Figure 2. Absorption (A) and fluorescence emission (B) spectra of SSO (dashed line), SSO-P (solid line) films and P-acid (dotted line) in THF, λ_{exc} = 420 nm. Insert (A): fluorescence excitation spectra of SSO-P film, λ_{em} = 722 nm.

Also, the photooxidations of DMA and ABDA sensitized by SSO-P film were compared with those induced by 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) in DMF and water, respectively. It is know that $TMAP^{4+}$ represents an active photosensitizer to produce $O_2(^1\Delta_g)$ in these solvents.[33] Under these conditions, higher values of k_{obs} for both DMA and ABDA were obtained for the reaction sensitized in solution by this cationic porphyrin. Values of k_{obs} were measured using an absorbance 0.22 at 515 nm to avoid dependence on the porphyrin concentration in the films. As can be observed in Table 1, the k_{obs} for the films are ca. two orders of magnitude lower than for the reference cationic porphyrin. This result is expected since in the polymeric films the $O_2(^1\Delta_g)$ is only formed on the surface, while using $TMAP^{4+}$ the photodynamic effect takes place in the solution bulk. The diffusion length of $O_2(^1\Delta g)$ in the ethylene-acrylic acid copolymer containing an anthraquinone derivative was calculated to be approximately 100 nm.[16] Consequently, it is expected that only $O_2(1\Delta g)$ produced within this distance from the irradiated surface of the film are likely to be detected by the substrate. Also, it is possible

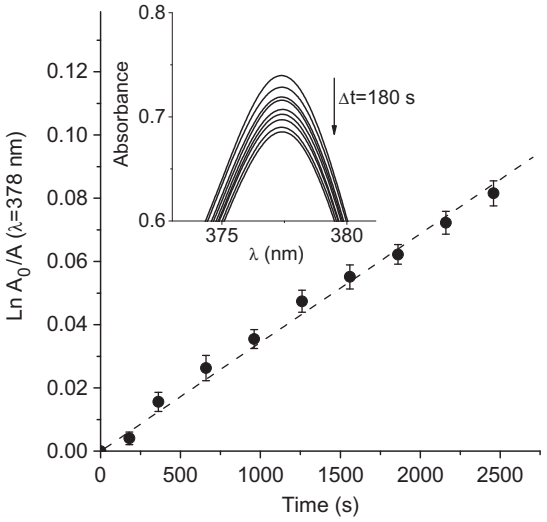


Figure 3. First-order plots for the photooxidation of ABDA (35 μM) in water, photosensitized by SSO-P film (A^{515} = 0.50), irradiated with light at λ = 455–800 nm. Values represent mean ± standard deviation of three separate experiments. Insert: absorption spectra changes of ABDA after different irradiation times (Δt = 180 s).

Table 1
Kinetic parameters for the photooxidation of substrates photosensitized by the SSO-P film and $TMAP^{4+}$ in different media.

Substrate	Solvent	Light (nm)	k_{obs}[a].(s^{-1})	k_{obs}[b].(s^{-1})
DMA	DMF	455–800	$(6.3 \pm 0.3) \times 10^{-4}$	$(4.5 \pm 0.3) \times 10^{-2}$
ABDA	water	455–800	$(1.5 \pm 0.2) \times 10^{-5}$	$(6.5 \pm 0.4) \times 10^{-2}$
Trp	DMF	300–800	$(4.3 \pm 0.2) \times 10^{-5}$	$(2.5 \pm 0.2) \times 10^{-3}$

[a] SSO-P film.
[b] $TMAP^{4+}$, absorbance 0.22 at 515 nm.

that limited availability of dissolved ground-state oxygen in the film may be a further limiting factor in $O_2(1\Delta g)$ production in the film.

On the other hand, the amino acid Trp was used as a substrate model for the compounds of biological interest that can be potential targets of porphyrin photodynamic action. In general, this substrate can be efficiently photooxidized by both type I and type II reaction mechanisms.[34] In presence of SSO-P or $TMAP^{4+}$ as sensitizers, the photoprocess follows first-order kinetics with respect to Trp concentration in DMF (result not shown). The *k*obs values are summarized in Table 1. Similar to that found previously with DMA, Trp was more rapidly photooxidized in the presence $TMAP^{4+}$. Despite this, the results indicate that SSO-P film is also a photoactive surface to decompose Trp.

3.4. Photoinactivation of *C. albicans* cells

The antifungal activity photoinduced by the polysilsesquioxane film doped with porphyrin was evaluated against *C. albicans* cells. This yeast represents a significant human and animal pathogen, which has the ability to colonize and cause disease within a diverse range of mammalian host sites.[35] Figure 4 shows typical microscopic images of SSO film, SSO-P film and SSO-P film bearing *C. albicans* cells on the surface.

First, PDI of *C. albicans* produced by the immobilized porphyrin films was evaluated in PBS cellular suspensions. Thus, the polymeric surface ($0.7 \times 2.5 = 1.75\ cm^2$) was placed inside of the cell suspension in culture tube containing $\sim 10^6$ CFU/mL and irradiated with visible light for different times. Typical results of cellular survival of *C. albicans* cells are shown in Figure 5A. Control experiments showed that the viability of *C. albicans* was unaffected by illumination alone or by dark incubation with the films. Also, the cell viability was not changed by irradiation of the cultures in the presence of SSO undoped polysilsesquioxane film. This indicates that the cell mortality obtained after irradiation of the cultures containing the SSO-P film is due to the photosensitization effect of the immobilized porphyrin, produced by visible light. After treatment, spectroscopic analyses showed that the cultures were not contaminated with porphyrin.

As can be observed in Figure 5A, the viability of *C. albicans* cells in presence of SSO-P and irradiated with visible light was depended on the light exposure. No difference in cell survival was observed using SSO-P films with different absorption between 0.2 and 0.5 at 515 nm. The SSO-P film produced a photosensitizing activity causing a ~2.5 log decrease of *C. albicans* survival after 60 min of irradiation. These results represent a value greater than 99.7% of cellular inactivation. Also, the photodynamic action of SSO-P film produces 1.4 log (96%) decrease in the cell viability of yeast, when the cultures are irradiated for 30 min. Both differences were statistically significant (Fig. 5A). Similar results were obtained re-utilizing the SSO-P film twice.

In previous investigation, it was demonstrated that using cationic porphyrins in solution, such as $TMAP^{4+}$, was possible to obtain an inactivation of >99.99%.[26] However, 99.7% could be considered appropriated in the present study considering that the photosensitizer was incorporated in a film and the phototherapeutic agent was not bound to the cells.

Also, the PDI for the free form of the photosensitizer was evaluated in *C. albicans* cells treated with 5 M P-acid. A small photoinactivation effect of 0.5 log decrease was observed after 60 min irradiated with visible light. This is an expected result for a non-cationic porphyrin derivative used to inactivate *C. albicans* or Gram-negative bacteria.[26,27]

Taking into account the result in PBS suspensions, the PDI of *C. albicans* was evaluated depositing a drop with the cells on the polysilsesquioxane films. This antifungal drop-test can be used to inactivate *C. albicans* cells growing on surfaces. Thus, 100 L of PBS containing $\sim 1 \times 10^5$ cells was located on SSO-P film and the plates were irradiated with visible light for 30 min. After this treatment, a reduction to 3×10^4 cells was found, which represented a 95% decrease of cell survival. In contrast, cells viability was unaffected in control experiments, showing that the combination of light and SSO-P film was appropriated to photoinactivate *C. albicans* on a surface.

On the other hand, photodynamic experiments were also performed under condition of microbial growth to ensure that PDI of cells is still possible when the cultures are not under starvation conditions or the potential damaging effects of PBS washing. The photocytotoxic activity on growth of *C. albicans* cultures photosensitized by SSO-P film was achieved in Sabouraud medium. The film was placed into fresh cultures of cells reaching the log phase and the flasks were irradiated with visible light at 37 °C. The effects on growth of *C. albicans* cells after different irradiation times are shown in Figure 5B. After irradiation in the same conditions of the experiments with cells, porphyrin was not detected by UV–visible spectra of the solution in contact with the film (PBS or Sabouraud medium).Yeast cells exposed to SSO-P film in the dark or not treated with the films and illuminated showed no growth delay compared with controls. Therefore, the data illustrate that the observed growth delay is due to the photoinactivation effect of the SSO-P film on the cells. Growth was suppressed when *C. albicans* cultures were treated with the SSO-P film and illuminated. The lag phase in the typical yeast growth curve was considerably enlarged due to the photodynamic activity of the antifungal films. After PDI treatment, the *C. albicans* cells appeared to be slowly growing as measured by turbidity at 660 nm. The slower growth

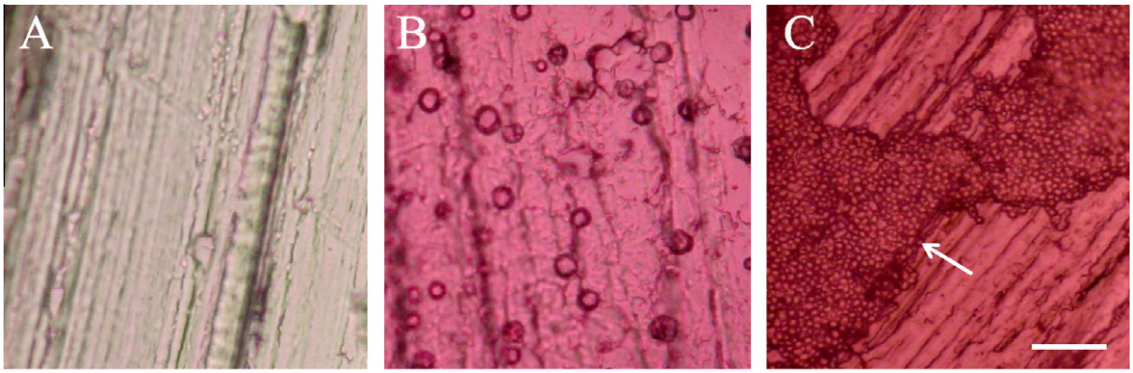


Figure 4. Microscope photograph of (A) SSO, (B) SSO-P and (C) SSO-P containing *C. albicans* cells (arrow); 100× microscope objective (scale bar 50 μm).

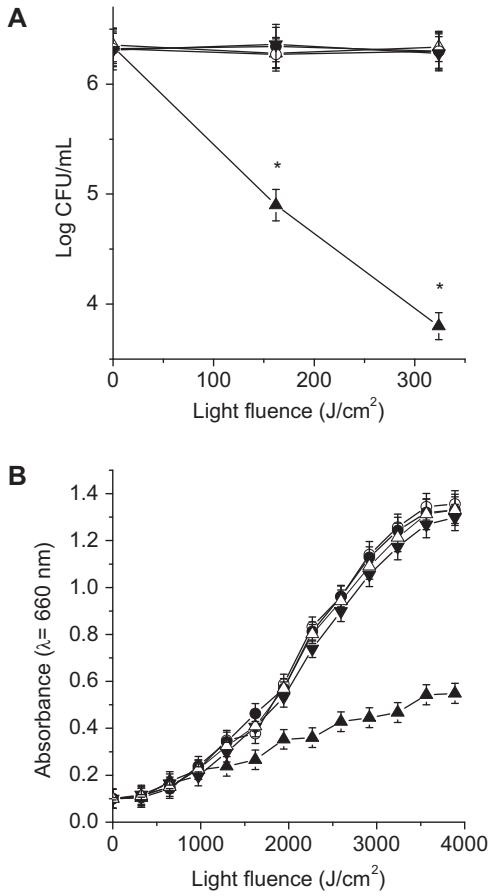


Figure 5. (A) Survival curves and (B) growth delay curves of *C. albicans* cells photosensitized by SSO-P film (▲) after different irradiation periods with visible light (90 mW/cm^2). Control cultures irradiated (●), in dark (○), in the presence of SSO film (▼) irradiated and in the presence of SSO-P film (△) in the dark. Values represent mean ± standard deviation of three separate experiments. $^*p$ <0.05, compared with untreated yeast.

of *C. albicans* cells indicates that this culture can be efficiently eradicated by the antifungal action of the SSO-P film.

It was reported that cellulose laurate esters films containing 0.52% protoporphyrin IX showed photobactericidal activity on nutrient agar when irradiated for 24 h with visible light of 1.7 mW/cm^2 fluence rate.[13] Also, pyridinium porphyrinic chloroacetyl cellulose ester chlorides films containing higher than 0.20% inactivated the two bacterial strains whereas films with lower porphyrin content were found more active against *Staphylococcus aureus* than against *Escherichia coli*.[14] On the other hand, experiments were designed to test the photodynamic effect of photosensitizers incorporated into chitosan membranes.[14] The conjugate of chitosan and the zinc(II) phthalocyanine tetrasulfonic acid reinforced with a nylon net proved to have an effective photokilling effect with *E. coli* at a level of 10^5 cells/mL. The in vitro penetration of ALA was evaluated across human nail and into neonate porcine hoof when released from a bioadhesive patch containing 50 mg cm^{-2} ALA.[18] Patch application for 24 h allowed an ALA concentration of 2.8 mM to be achieved on the ventral side of excised human nail. Incubation of *C. albicans* and *Trichophyton interdigitale* with ALA concentrations of 10.0 mM for 30 min and 6 h, respectively, caused reductions in viability of 87% and 42%, respectively, following irradiation with red light. Reports on a mucoadhesive patch containing toluidine blue O was proposed as a potential delivery system for oropharyngeal candidiasis.[19] When releasing directly into an aqueous sink, patches containing 50 and 100 mg TBO cm^{-2} both generated receiver compartment concentrations exceeding the concentration (2.0–5.0 mg mL^{-1}) required to produce high levels of kill (>90%) of both planktonic and biofilm-grown *C. albicans* upon illumination.

In previous investigations, electrochemically generated polymeric films bearing porphyrin units on optically transparent indium tin oxide electrodes were studied as potential photodynamic surfaces to inactivate *C. albicans* cells.[20] These films exhibited a photosensitizing activity causing a ~2 log decrease of *C. albicans* cellular survival after 60 min of irradiation with visible light. The main difference of the present SSO-P films is its versatility as plastic flexible material at room temperature, easily obtainable and it can mold the shape of the surfaces. Also, SSO-P films keep a higher antifungal activity in the delay in the growth curve of *C. albicans*.

3.5. Mechanistic insight of the *C. albicans* photoinactivation

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.[36] 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. 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. In order to elucidate the photodynamic mechanism involved

in the photosensitized inactivation of *C. albicans* cells by SSO-P film, the effect of D_2O and two suppressors, sodium azide and mannitol, were investigated.

First, PDI of *C. albicans* was performed in D_2O to evaluate the $O_2(^1\Delta_g)$-mediated cytotoxicity. This solvent was used to increase the lifetime of $O_2(^1\Delta_g)$.[32] Cell viability of *C. albicans* was not affected in D_2O under irradiation without polymeric surface (Fig. 6, line 5). However, irradiation of *C. albicans* cells containing SSO-film in D_2O produced a higher photoinactivation than that observed in PBS (Fig. 6, line 6).

Also, the photoinactivation of *C. albicans* was studied in presence of 100 mM azide ion. Sodium azide is a know quencher of singlet oxygen $O_2(^1\Delta_g)$ preventing type II photoprocess.[37] This concentration of sodium azide was not toxic under irradiation without SSO-P film (Fig. 6, line 7). 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. 6, line 8). Under this condition, no more than a 0.5 log decrease was observed in the survival of the yeast treated with SSO-P film after 60 min irradiation. Therefore, the azide ion quenched the photocytotoxic species, producing a protective effect on *C. albicans*.

Otherwise, 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.[38] The addition of 100 mM mannitol was not cytotoxic for irradiated cells without SSO-P film (Fig. 6, line 9). In presence of SSO-P film, PDI of *C. albicans* was quite similar in the presence or absence of mannitol after 60 min of irradiation (Fig. 6, line 10).

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.[38–41] In contrast, the phototoxicity efficacy was not significantly affected when mannitol was added to *C. albicans* cells photosensitized by SSO-P film. This suggests that the involvement of $O_2(^1\Delta_g)$ is the main reactive oxygen species involved in the photodamage of *C. albicans* cells. This is also the case of free cationic porphyrins that are bound to the cells, such as $TMAP^{4+}$. Recently, studies of the photodynamic mechanism of action on *C. albicans* cells indicated that this cationic porphyrin appears to act as photosensitizers mainly via the intermediacy of $O_2(^1\Delta_g)$.[42]

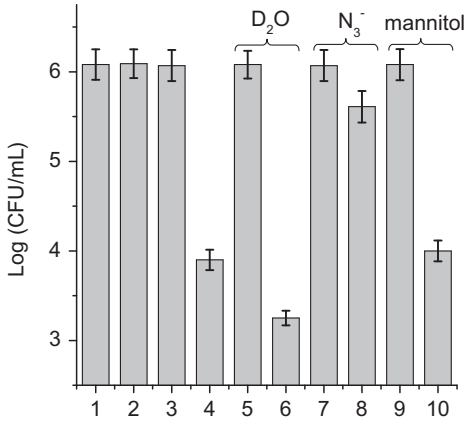


Figure 6. Survival of *C. albicans* cells ($\sim 10^6$ CFU/mL) under different experimental conditions keeping in dark or exposed to visible light for 60 min (90 mW/cm^2); (1) control culture in dark; (2) control culture irradiated; (3) control culture treated with SSO-P and keeping in dark; (4) culture treated with SSO-P and irradiated; (5) control culture in D_2O and irradiated; (6) culture treated with SSO-P in D_2O and irradiated; (7) control culture containing 100 mM azide and irradiated; (8) culture treated with SSO-P containing 100 mM azide and irradiated; (9) control culture containing 100 mM mannitol and irradiated; (10) culture treated with SSO-P containing 100 mM mannitol and irradiated. Values represent mean ± standard deviation of three separate experiments.

4. Conclusions

In the present work, a bridged polysilsesquioxane film doped with porphyrin was formed by polymerization in presence of P-acid. These plastic films represent an appropriated surface to obtain mechanically stable surfaces. Irradiation of these surfaces with visible light induces efficient photooxidation of substrates in solutions, indicating an efficient photodynamic action of the polymeric film.

In vitro investigations showed that these SSO-P films produce photosensitized inactivation of *C. albicans* in aqueous suspensions. After 60 min of irradiation, the films exhibit a photosensitizing activity causing a ~2.5 log decrease of *C. albicans* cellular survival. The photodynamic effect induced by the photoactive films can be used to prevent the growth of *C. albicans* cells in an appropriated culture medium. Also, the films can photoinactivate yeast cells contained in a drop on the surface. In this sense, the SSO-P film could also be effective in killing other microorganisms that are common causes of medical device.

To elucidate the oxidative processes that occur during the killing of yeast, first, the effect of the D_2O was analyzed on cell photoinactivation. 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. Thus, in the present in vitro experiments, the killing of *C. albicans* cells by SSO-P film and visible light irradiation seem to be mediated mainly by $O_2(^1\Delta_g)$.

The results indicate that a bridged polysilsesquioxane films doped with porphyrin are interesting photodynamic surfaces to inactivate *C. albicans*. The main advantage of heterogenic eradication of microorganisms using antifungal surfaces is that they can be easily and quickly removed from the media after cell inactivation, avoiding permanent photodynamic effects. Therefore, practical applications of SSO-P films could involve the elimination of *C. albicans* growing in a liquid media, such as a biological fluid contaminated with yeast. Also, since the films can be easily manipulated with the shape of the surface to be covered, they could be used to form permanent antifungal surfaces activated by visible light. For example, to control fungal proliferation and maintain aseptic conditions on surfaces involved in the healthcare. Therefore, the photoinactivation activity of these plastic films showed promising applications as antifungal surfaces for controlling *C. albicans*.

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