Available online at www.sciencedirect.com
ScienceDirect
Journal of Photochemistry and Photobiology A: Chemistry 194 (2008) 220–229
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Synthesis, spectroscopic properties and photodynamic activity of a novel Zn(II) phthalocyanine substituted by fluconazole groups

M. Paula Cormick [a], Marisa Rovera [b], Edgardo N. Durantini [a,*]

[a] *Departamento de Química, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X5804BYA Río Cuarto, Argentina*
[b] *Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X5804BYA Río Cuarto, Argentina*

Received 16 May 2007; received in revised form 1 August 2007; accepted 13 August 2007
Available online 19 August 2007

Abstract

A novel Zn(II) phthalocyanine derivative (ZnPcF) bearing four antifungal structures of fluconazole was synthesized by a two-step procedure starting from 4-nitrophthalonitrile. First, phthalonitrile-azole derivative was prepared by a nucleophilic *ipso*-nitro substitution reaction between 4-nitrophthalonitrile and fluconazole. The cyclotetramerization of phthalonitrile-azole with Zn(II) acetate in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) results in the formation of the ZnPcF as a mixtures of constitutional isomers with 27% yield. Absorption and fluorescence spectroscopic studies were analyzed in different media. The results show that ZnPcF is lowly soluble in polar solvents or in reverse micellar systems. However, addition of HCl produce an increase in the monomerization of ZnPcF in *N*,*N*-dimethylformamide (DMF)/water (10%, v/v) and in benzene/benzyl-*n*-hexadecyldimethyl ammonium chloride (BHDC, 0.1 M)/water ($W_0 = 10$). A value of 0.19 was calculated for the fluorescence quantum yield (ϕ_F) of this photosensitizer in DMF/water (10%)/HCl 1.2 mM. The photodynamic activity of ZnPcF was evaluated using 9,10-dimethylanthracene (DMA). An enhancement in the singlet molecular oxygen, $O_2(^1\Delta_g)$, production was obtained in acidified DMF/water or BHDC micellar system, which represent an appropriate system to induce monomerization of ZnPcF. Preliminary studies to evaluate the photodynamic activity of ZnPcF were tested against a typical yeast *Candida albicans*. These results indicated that ZnPcF is an interesting antifungal agent because it has antimycotic activity in dark and its efficiency increases in the presence of light due to the photodynamic inactivation caused by the photosensitizer moiety.

Keywords: Phthalocyanine; Floconazole; Photodynamic inactivation; Yeast; *Candida albicans*

1. Introduction

Phthalocyanines derivatives exhibit a high absorption coefficient ($\varepsilon > 10^5\ M^{-1}\ cm^{-1}$) in the visible region of the spectrum, mainly in the phototherapeutic window (600–800 nm) and a long lifetime of triplet excited state to produce efficiently singlet molecular oxygen, $O_2(^1\Delta_g)$. Based on these properties, one of more recent applications of phthalocyanine in medicine is in the detection and cure of tumors by photodynamic therapy [1]. In this way, photodynamic inactivation (PDI) is a new promising approach to treat microbial infections. PDI is based on the administration of a photosensitizer, which is preferentially accumulated in the cells. The subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms [2–4].

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 [5]. *Candida albicans*, which is a common inhabitant of the mouth, throat, digestive tract and skin, can become pathogenic mainly in hosts with a compromised immune system [6]. Resistance of *C. albicans* against traditional antifungal, such as fluconazole, is increasing in such patients [7–9]. These trends underscore the importance of developing novel strategies for treatment of fungal infections. Thus, in the last years several photosensitizers have been investigated for PDI applications [10–13]. However, the interest in this field and the need for new molecules with improved characteristics is always high owing to the large variety of therapeutic applications.

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

1010-6030/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2007.08.013

In this paper, a novel Zn(II) phthalocyanine derivative bearing four structures of fluconazole (ZnPcF) was synthesized. The azoles are antifungal agents that act inhibiting the activity of cytochrome $P450_{14DM}$ of the fungi. The azoles contain an imidazolic ring, which is involved in the pharmaceutic activity [14–16]. In particular, fluconazole has a hydroxylic group that allows binding this structure with a photosensitizer by alcohoxy bound. This covalent binding should not affect significantly the biological activity of the azole group. Thus, this structure present potential application as antifungal agent, not only because it could inactive microbes in dark, but also the activity could be enhanced by irradiation with visible light due to the photodynamic activity of the photosensitizer moiety.

2. Materials and methods

2.1. General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a FT-NMR Bruker Avance DPX400 multinuclear spectrometer at 400 MHz. FAB mass spectra were taken with a ZAB-SEQ Micromass equipment. The refractive indexes (η) were measured using an Atago NAR-1T (Tokyo, Japón) refractometer. Silica gel thin-layer chromatography (TLC) plates 250 μm from Aldrich (Milwaukee, WI, USA) were used. All the chemicals from Aldrich were used without further purification. Benzyl-*n*-hexadecyldimethyl ammonium chloride (BHDC) from Sigma (St. Louis, MO, USA) was recrystallized twice from ethyl acetate and dry under vacuum over P_2O_5. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Sensitizers

Zinc phthalocyanine (ZnPc) from Aldrich was used as received. According to the synthetic procedure, phthalocyanine macrocycle is obtained as a mixture of the corresponding regioisomers.

Phthalonitrile-fluconazole (PnF): A solution of 4-nitrophthalonitrile (200 mg, 1.16 mmol) and fluconazole (352 mg, 1.16 mmol) in 15 mL of *N*,*N*-dimethylformamide (DMF) was stirred for 10 min under argon atmosphere. Then, dry potassium carbonate (1.5 g, 10.8 mmol) was added and the mixture was heated at 80 °C for 3 h. The mixture was treated with water (50 mL) and extracted with dichloromethane/methanol (5%) (two portion of 50 mL each). The solvent was removed under reduced pressure. The product was purified by flash chromatography (silica gel, dichloromethane/methanol 5%) yielding 175 mg (35%) of the pure phthalonitrile PnF. TLC (silica gel) R_f (dichloromethane/methanol 5%) = 0.26. ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 4.52, 4.70 (AB, 4H), 6.15 (d, 2H, J = 7.8 Hz), 6.85 (m, 1H), 7.16 (m, 1H), 7.17 (m, 1H), 7.76 (s, 2H), 8.00 (d, 2H, J = 7.8 Hz), 8.01 (dd, 1H, J = 2.2 Hz, 8.6 Hz), 8.18 (d, 1H, J = 8.6 Hz), 8.29 (s, 2H), 8.34 (d, 1H, J = 2.2). FT-IR (KBr) cm^{-1} 3117, 3038, 2910, 2860, 2232, 1619, 1507, 1382, 1270, 1210, 1144, 1104, 960, 848. MS *m*/*z* 432 (M^+) (432.1259 calculated for $C_{21}H_{14}F_2N_8O$). Anal. calcd. C 55.33, H 3.21, N 26.12, found C 55.27, H 3.26, N 26.05.

Phthalocyanine-fluconazole (ZnPcF): A solution of phthalonitrile PnF (60 mg, 0.14 mmol) and zinc(II) acetate dihydrate (11 mg, 0.05 mmol) in 10 mL of *n*-pentanol was stirred for 10 min under argon atmosphere. Then, 30 μL of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.20 mmol) was added and the mixture was refluxed for 18 h. The reaction mixture was cooled to room temperature and precipitated with 50 mL of cyclohexane and filtered. The solid was recrystallized from methanol/water and it was washed with cyclohexane, acetone and water to yield 17 mg (27%) of ZnPcF. FT-IR (KBr) cm^{-1} 3110, 3040, 2905, 2840, 1620, 1510, 1380, 1245, 1206, 1148, 1102, 1042, 956, 845. FAB-MS *m*/*z* 1792 (M^+) (1792.4326 calculated for $C_{84}H_{56}F_8N_{32}O_4Zn$). Anal. calcd. C 56.21, H 3.14, N 24.97, found C 56.10, H 3.02, N 25.05.

2.3. Spectroscopic studies

Spectra were recorded using 1 cm path length quartz cells at 25.0 ± 0.5 °C. The fluorescence quantum yield (ϕ_F) of ZnPcF was calculated by comparison of the area below the corrected emission spectrum with that of ZnPc as a fluorescence standard, exciting at λ_{exc} = 610 nm [17]. A value of ϕ_F = 0.28 for ZnPc in DMF was calculated by the comparison with the fluorescence spectrum in pyridine using ϕ_F = 0.30 and taking into account the refractive index of the solvents (η (DMF) = 1.431; η (DMF/water 10% [HCl] = 1.2 mM) = 1.425) [18].

Studies in reverse micelles were performed using a stock solution of BHDC 0.1 M, which was prepared by weighing and dilution in benzene. The addition of water to the corresponding solution was performed using a calibrated microsyringe. The amount of water present in the system was expressed as the molar ratio between water and the BHDC present in the reverse micelle (W_0 = [H_2O]/[BHDC]). In all experiments, W_0 = 10 was used. The mixtures were sonicated to obtain perfectly clear micellar system [19].

2.4. Steady state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 35 μM) and photosensitizer in different media were irradiated in 1 cm path length quartz cells (2 mL) with monochromatic light at λ_{irr} = 670 nm for ZnPc and λ_{irr} = 678 nm for ZnPcF (sensitizer absorbance 0.3) from a 75 W high-pressure Xe lamp through a high intensity grating monochromator, Photon Technology Instrument [19]. The light intensity was determined as 1.5 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). The kinetics of DMA photooxidation were studied by following the decrease of the absorbance at λ_{max} = 378 nm. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of ln A_0/A vs. time. Photooxidation of DMA was used to determine singlet molecular oxygen, $O_2(^1\Delta_g)$, production by the photosensitizer [19,20]. ZnPc was used as

the standard, $\Phi_\Delta = 0.56$ in DMF [21] and $\Phi_\Delta = 0.62$ in benzene [22]. Measurements of the sample and reference under the same conditions afforded Φ_Δ for ZnPcF by direct comparison of the slopes in the linear region of the plots. The reaction of DMA with $^1O_2$ sensitized by the standard ZnPc was compared in DMF and in DMF/water (10%, v/v) [HCl] = 1.2 M and insignificant difference was found between both media. 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 5%.

2.5. Microorganism and growth conditions

C. albicans strain (PC31) recovered from human skin lesion was previously characterized and identified. The fungal strain was identified according to conventional procedures [23]. Primary classification of colonies from plates was based on colony characteristic (pigmentation and shape), mode of vegetative reproduction, formation of hyphae or pseudohyphae and ascopore production. Identification of the yeast isolates to species level was done using the API 20C AUX (BioMérieux, Marcy l′Etoile, France) system of carbohydrate assimilation profiles.

C. albicans strain was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth at 37 °C to stationary phase. An aliquot (1 mL) of this culture was aseptically transferred to fresh fluid Sabouraud (3 mL) medium, which produces to an absorbance of ∼0.75 at 650 nm. 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). Then the cells were diluted 1/10 in PBS, corresponding to $\sim 10^6$ colony forming units (CFU)/mL. In all the experiments, 2 mL of the cell suspensions in Pirex brand culture tubes (13 mm × 100 mm) were used and the sensitizer was added from a stock solution of ZnPcF (5×10^{-4} M) in DMF/water (10%) [HCl] = 3.3 mM. This amount of HCl does not affect the cultures because they are in PBS and the pH is not significantly changed with the addition of 40 μL. ZnPc and fluconazole were added from a stock solution (5×10^{-4} M) in DMF. Viable *C. albicans* cells were monitored and their number calculated by counting the number of colony forming units after appropriated dilution on Sabouraud agar plates. Fungal cultures grown under the same conditions with and without photosensitizers kept in the dark as well as illuminated cultures without sensitizer served as controls.

2.6. Photosensitizer binding to yeast cells

Suspensions of *C. albicans* (2 mL, $\sim 10^6$ CFU/mL) in PBS were incubated in dark at 37 °C with 10 μM of sensitizer for different times. The cultures were centrifuged (3000 rpm for 15 min) and the cell pellets were re-suspended in 2% aqueous SDS (2 mL), incubated overnight at 4 °C and sonicated for 30 min. The concentration of sensitizer in the supernatant was measured by spectrofluorimetry ($\lambda_{exc} = 668$ nm, $\lambda_{em} = 676$ nm for ZnPc; $\lambda_{exc} = 675$ nm, $\lambda_{em} = 682$ nm for ZnPcF) in solution of 2% SDS in PBS. The fluorescence values obtained from each sample were referred to the total number of cells contained in the suspension. The concentration of the photosensitizer in this sample was estimated by comparison with a calibration curve obtained with standard solutions of the sensitizer in 2% SDS ([sensitizer] ∼ 0.005–0.1 μM).

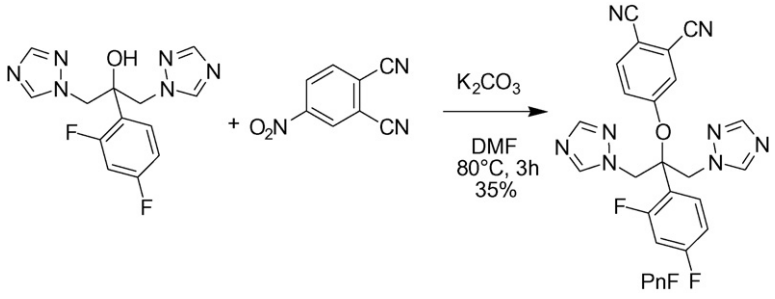
2.7. Growth delay of C. albicans cultures

Cultures of *C. albicans* cells were grown overnight as described above. A portion (1 mL) of this culture was transfer to 20 mL of fresh Sabouraud broth medium. The suspension was homogenized and aliquots of 2 mL were incubated with 10 μM of sensitizer at 37 °C. The culture grown was measured by turbidity at 550 nm using a Tuner SP-830 spectrophotometer. This λ was chosen because phthalocyanines absorbs in the range of 600–700 nm. Then the flasks were irradiated with visible light at 37 °C. The light source used was a Novamat 130 AF 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 and 800 nm was selected by optical filters. The light intensity at the treatment site was 30 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). Total fluence was 864 J/cm^2 after 8 h of irradiation. In all cases, control experiments were carried out without illumination in the absence and in the presence of sensitizer. Each experiment was repeated separately three times.

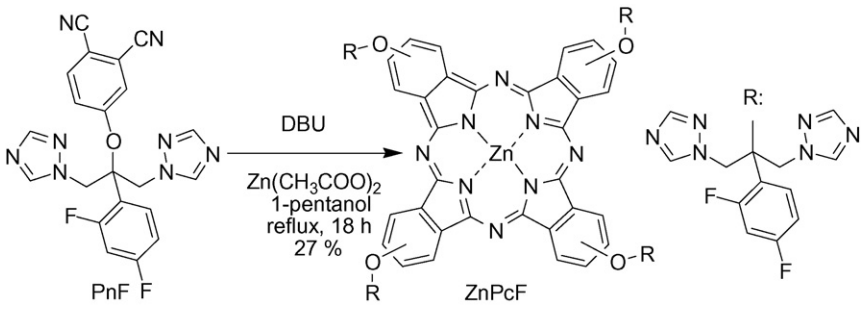
3. Results and discussion

3.1. Synthesis of Zn(II) phthalocyanine-fluconazole derivative

Zn(II) phthalocyanine substituted by four fluconazole units (ZnPcF) was synthesized by a two-step procedure [24]. First,



Scheme 1. Synthesis of phthalonitrile derivative PnF.



Scheme 2. Synthesis of phthalocyanine ZnPcF.

the phthalonitrile derivative bearing a fluconazole moiety (PnF) was prepared by a nucleophilic *ipso*-nitro substitution reaction of 4-nitrophthalonitrile with fluconazole in the presence of K_2CO_3 (Scheme 1). This dinitrile derivative PnF was isolated by flash chromatography (silica gel, dichloromethane/methanol 5%) with 35% yield. The cyclotetramerization of PnF with Zn(II) acetate in the presence of organic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was performed in *n*-pentanol (Scheme 2). After reflux for 18 h, the reaction results in the formation of the corresponding ZnPcF as mixtures of constitutional isomers with 27% yield. The product was precipitated with cyclohexane and filtered. Then, it was re-precipitated from methanol/water and the solid washed with cyclohexane, acetone and water. This procedure yields a mixture of four regioisomers with a fluconazole group at the 2- or 3-positions of each benzene ring in the ZnPcF molecule [25].

In the structure of ZnPcF, the fluconazole centers are isolated from the phthalocyanine macrocycle ring by alcohoxy bonds. This spacer provides a higher mobility of the azole moiety, which could facilitate the interaction with the heme group of the cytochrome [14–16].

3.2. Spectroscopic studies and solubilization of ZnPcF

3.2.1. Absorption spectroscopy in different media

The absorption spectrum of ZnPcF was studied in different homogeneous and microheterogeneous media. Typically, the spectra of Zn(II) phthalocyanine derivatives show two characteristic long wavelength peaks, one at ~635 nm corresponds to aggregate absorption, while the other at ~675 nm is due to absorption by the monomeric molecule [25–27]. Almost no significant absorption bands of ZnPcF are detected between 550 and 750 nm in non-hydrogen bond donor (DMF, pyridine and dimethylsulphoxide) or hydrogen bond donor (methanol and water) polar organic solvents and the sensitizer is very poorly solubilized in these media. Also, negligible solubilization was observed for ZnPcF in microheterogeneous micellar systems of cationic benzyl-*n*-hexadecyldimethyl ammonium chloride (BHDC) or anionic sodium bis(2-ethylhexyl)sulfosuccinate (AOT) surfactants. However, the addition of 10% (v/v) water/HCl 3.3 mM produces the solubilization of ZnPcF mainly as monomer in DMF, whereas in methanol the two bands corresponding to the monomer (~672 nm) and aggregated (~640 nm) are observed in Fig. 1A. Moreover, ZnPcF is

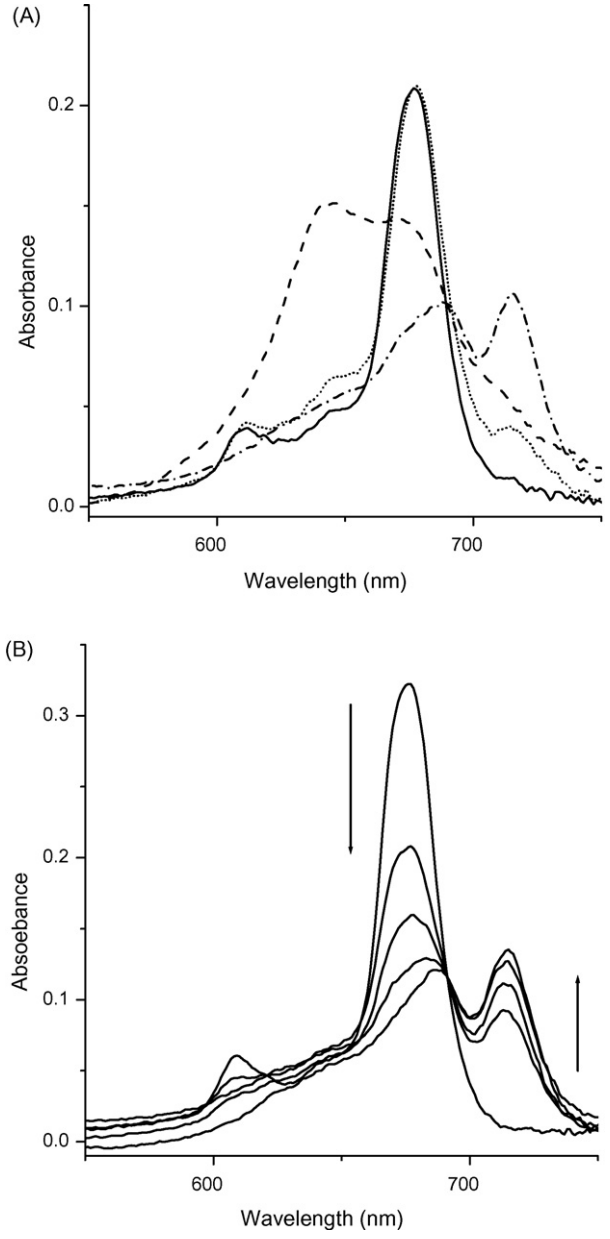
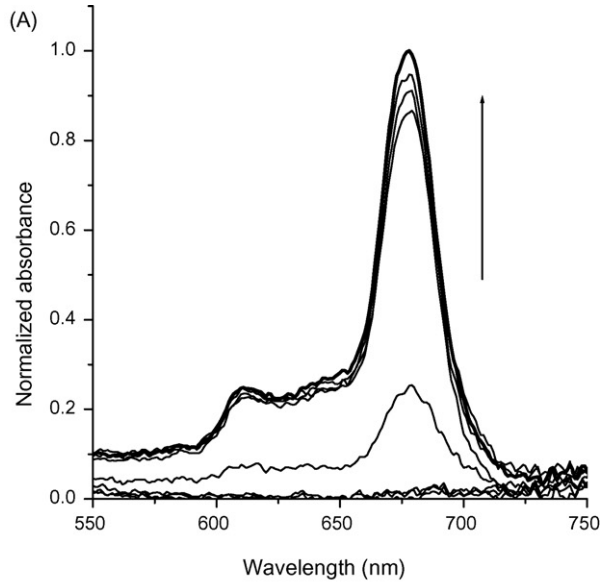
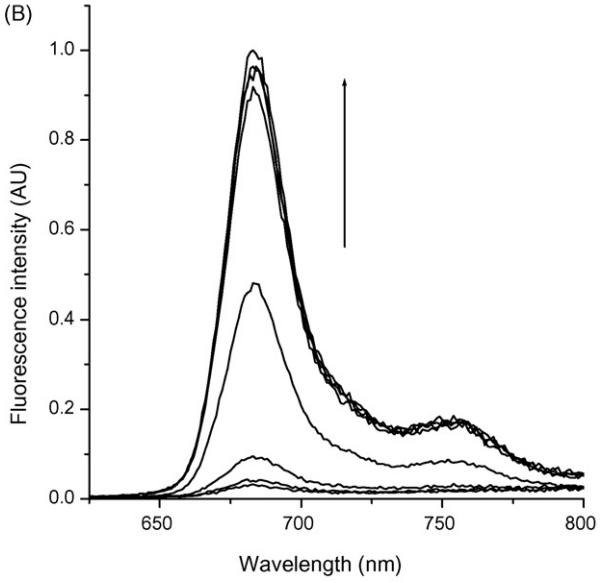
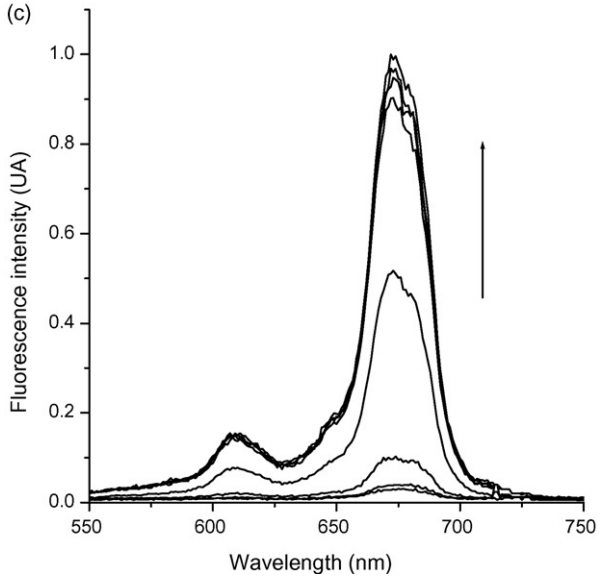


Fig. 1. (A) Absorption spectra of ZnPcF in DMF/water (10%, v/v) [HCl] = 3.3 mM (solid line), methanol/water (10%, v/v) [HCl] = 3.3 mM (dashed line), benzene/BHDC (0.1 M)/water (W_0 = 10, [HCl] = 5.0 mM) (dotted line) and DMF/water (10%, v/v) [HCl] = 1.1 M (dashed–dotted line), [ZnPcF] = 2.5 μM; (B) spectral changes of ZnPcF in DMF/water (10%, v/v) using increasing H_2SO_2 concentrations as follows: 1×10^{-3}, 0.44, 0.87, 1.67 and 2.39 M.







dissolved as monomer in cationic reverse micelles of benzene/BHDC (0.1 M)/water ($W_0 = 10$) when the aqueous pools are formed from a solution 5.0 mM of HCl (Fig. 1A) but this procedure was not effective with anionic reverse micelles of *n*-heptane/AOT (0.1 M)/water ($W_0 = 10$) (see below).

Zn(II) phthalocyanines macrocycles have four nitrogen atoms, which can participate in acid–base interaction with acid media. Monoprotonation of the external nitrogen atoms results in splitting and bathochromic shift of the Q-band [28]. First protonation shows two bands at ~680 and ~710 nm and it requires an elevated acid concentration, for example, Zn(II) phthalocyanine (ZnPc) in DMF/H_2SO_4 1.84 M [28]. In our case, it can be noted that a small band appear at 712 nm in acidified BHDC system (Fig. 1A). This peak can be assigned to the partial first protonation of ZnPcF in the reverse micellar system. However, a new band was not observed in the other organic solvent/water (10%) media contain 3.3 mM HCl. Also, monoprotonation peak was not observed with Zn(II) phthalocyanines derivatives in water/HCl 0.1 M [29]. On the other hand, when ZnPcF was studied in DMF/water (10%) at higher HCl concentration (1.1 M) a new band appears at 715 nm, evidencing the first protonation on the external nitrogen atoms (Fig. 1A).

Monoprotonation peak was also confirmed in DMF/water (10%) using H_2SO_4 as compared with previous results for ZnPc [28]. Fig. 1B shows the changes in the absorption spectra on addition of H_2SO_4. As can be observed, the protonation occurs with a clear isosbestic point at ~691 nm, as previously showed for ZnPc [28]. From the results in Fig. 1B, the formation constant for the first protonation (K_f) of ZnPcF was calculated giving a value of $K_f = 0.48 \pm 0.01$. This value is comparable to those reported in the literature for protonation of Zn(II) phthalocyanine derivatives [28]. Thus, a Zn(II) phthalocyanine derivative containing phenoxy ring substituted with *t*-butyl groups ($K_f = 0.63$) was easier protonated than unsubstituted ZnPc ($K_f = 0.23$) in acidified DMF medium [28]. Consequently, protonation take places more readily in the presence of electron donating peripheral substituents like the alcohoxy groups in ZnPcF.

3.2.2. Solubilization of ZnPcF in DMF/water

The monomerization of ZnPcF was analyzed in DMF/water (10%, v/v) at different concentrations of HCl. As it can be observed in Fig. 2A, the Q-band around 675 nm is not detected in absence of acid indicating that ZnPcF is practically insoluble as monomer in this medium. However, as acid concentration increases, the absorption band of the monomeric form enhances until the HCl concentration reaches values ~1 mM (Fig. 3). At this point, addition of a higher acid concentration (1–3.5 mM) produces only negligible changes in the spectra.

Under this condition, the monomeric Q-band of ZnPcF presents a bathochromic shift by ~10 nm when compared with that of ZnPc as previously observed for Zn(II) phthalocya-

Fig. 2. (A) Absorption, (B) fluorescence emission ($\lambda_{exc} = 610$ nm) and (C) excitation ($\lambda_{em} = 720$ nm) spectra of ZnPcF in DMF/water (10%) using increasing HCl concentrations as follows: 3.2×10^{-6}, 4.3×10^{-5}, 1.7×10^{-4}, 4.3×10^{-4}, 8.5×10^{-4}, 1.2×10^{-3}, 1.7×10^{-3}, 3.3×10^{-3} M.

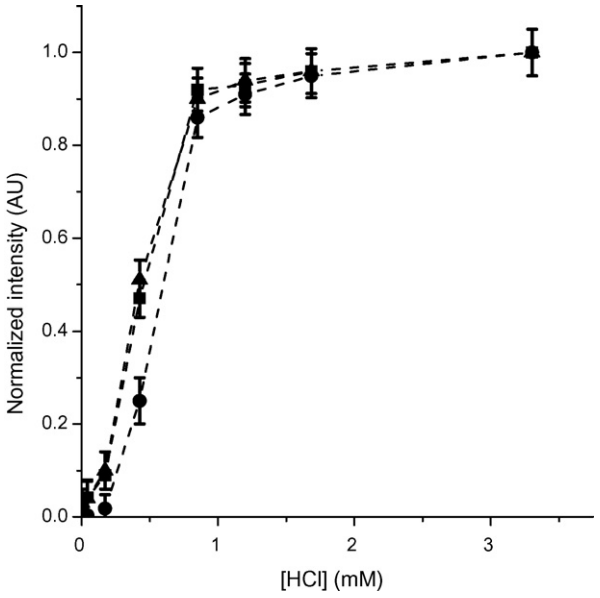


Fig. 3. Variation of normalized intensities of absorbance at λ = 678 nm (●), emission at λ = 683 nm (■) and excitation at λ = 678 nm (▲) with HCl concentrations.

nine derivates substituted by alcohoxy groups [24]. A value of $8.4 \times 10^4\ M^{-1}\ cm^{-1}$ was calculated for the molar coefficient (ε) of ZnPcF in DMF/water (10%, v/v) [HCl] = 1.2 mM.

The steady-state fluorescence emission spectrum of ZnPcF was studied in DMF/water (10%) varying HCl concentration. As showed above for absorption spectroscopy, the fluorescence intensity increases with the amount of acid in solution (Fig. 2B). Since aggregated phthalocyanines are nonemissive [27], the emission observed upon excitation at 610 nm occurs from the monomeric species. The emission spectra show two bands in the red spectral region with a more intense peak at ~683 nm, which are characteristic of similar Zn(II) phthalocyanines [24]. As can be observed in Fig. 3, only small changes in the intensity are found upon [HCl] > 1 mM. By comparison with ZnPc as a reference, a fluorescence quantum yields (ϕ_F) value of 0.19 ± 0.02 was obtained for ZnPcF in DMF/water (10%) [HCl] = 1.2 mM. A small Stokes shift (~5 nm) was observed indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. Taking into account the energy of the 0–0 electronic transitions, the energy level of the singlet excited stated (E_s) was calculated for ZnPcF giving a value of 1.80 eV. These results are in agreement with those previously reported for similar phthalocyanines in different media [29].

Also, the fluorescence excitation spectra of the ZnPcF were measured in DMF/water (10%) at different HCl concentrations (Fig. 2C), monitoring the emission at 720 nm. As can be observed in Fig. 2C, the spectra resemble the absorption spectra (Fig. 2A), indicating that the acidified DMF/water medium promote demonomerization of the ZnPcF.

The studies in homogenic media show that partial monoprotonation of the nitrogen atoms on the phthalocyanine ring require high proton concentration (>1 M). However, relatively lower acid concentrations (<3 mM) are adequate to produce the ZnPcF monomerization. Under this condition, peripheral triazole units of fluconazole can undergo protonation (for example, pK_a of 1-methyl-1,2,3-triazol is reported 3.20) [30]. Thus, the repulsion forces associated with the release of the cationic charge on the protonated heterocyclic groups helps to produce disaggregation of ZnPcF. Similar results were previously obtained for compounds with peripheral *tertiary* amino groups [31]. In this case, full monomerization was obtained already at 0.5 mM of HCl due to higher basicity of the *tertiary* amines.

3.2.3. Monomerization of ZnPcF in reverse micellar systems

The absorption spectrum of ZnPcF was analyzed in *n*-heptane/AOT (0.1 M) varying the concentration of HCl contained in the water dispersed in the reverse micelles. The effect of changing HCl concentrations keeping W_0 constant is shown in Fig. 4A. In previous studies, it was shown that AOT reverse micellar system very effectively inhibits the aggregation of cationic phthalocyanine, promoting the formation of photoactive monomeric species [32]. However, in our case the solubilization of ZnPcF does not take place in acidified AOT reverse micelles indicating that this is not an appropriated system to avoid aggregation of ZnPcF.

Similar studies were performed in benzene/BHDC (0.1 M). The results are shown in Fig. 4B. The band at ~680 nm, which can be attributed to the Q-band of the monomeric species, become more intense when the HCl concentration in the water microdrop increase. Thus, disaggregation of ZnPcF takes place when the amount of HCl dispersed increases in the micelles. This indicates that the sensitizers are mainly solubilized as monomers in BHDC system containing HCl.

3.3. Photodynamic activity in different media

9,10-Dimethylanthracene (DMA) quenches $O_2(^1\Delta_g)$ by exclusively chemical reaction, therefore, it was used to evaluate the ability of ZnPcF to produce $O_2(^1\Delta_g)$ [19]. The aerobic irradiations with monochromatic light (λ_{irr} = 670 nm for ZnPc and 678 nm for ZnPcF) of photosensitizers in DMF/water (10%) [HCl] = 1.2 mM were performed in the presence of DMA. A time-dependent decrease in the DMA concentration was observed by following a decrease in its absorbance at 378 nm (Fig. 5A). From first-order kinetic plots the values of the observed rate constant (k_{obs}) were calculate for DMA (Table 1). The quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) were calculated comparing the slope for ZnPcF with the corresponding slope obtained for the reference, ZnPc. As can be observed in Table 1, the values of Φ_Δ for ZnPcF are quite reasonable because the low solubilization of this sensitizer as monomer. While the $O_2(^1\Delta_g)$ production was negligible in these medias without HCl. Aggregation of the phthalocyanine almost invariably causes a shortening of the triplet lifetime and a drastic reduction of the overall photosensitizing efficiency [27].

Moreover, the photodynamic activity of ZnPcF was evaluated in benzene/BHDC (0.1 M)/water (W_0 = 10, [HCl] = 25 mM)

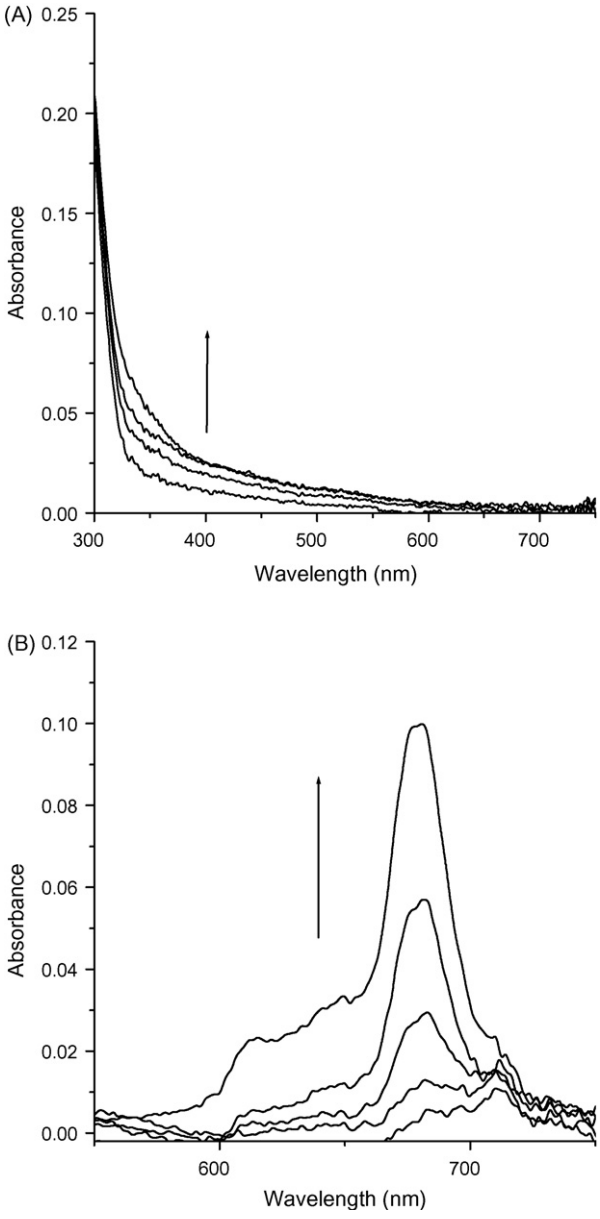


Fig. 4. Absorption spectra of ZnPcF at different HCl concentrations in the water dispersion in (A) *n*-heptane/AOT (0.1 M)/water ($W_0 = 10$, $[HCl] = 1.0 \times 10^{-2}$, 2.0×10^{-2}, 2.5×10^{-2}, 5.0×10^{-2} M) and (B) benzene/BHDC (0.1 M)/water ($W_0 = 10$, $[HCl] = 3.2 \times 10^{-6}$, 2.5×10^{-3}, 1.0×10^{-2}, 2.0×10^{-2}, 2.5×10^{-2} M).

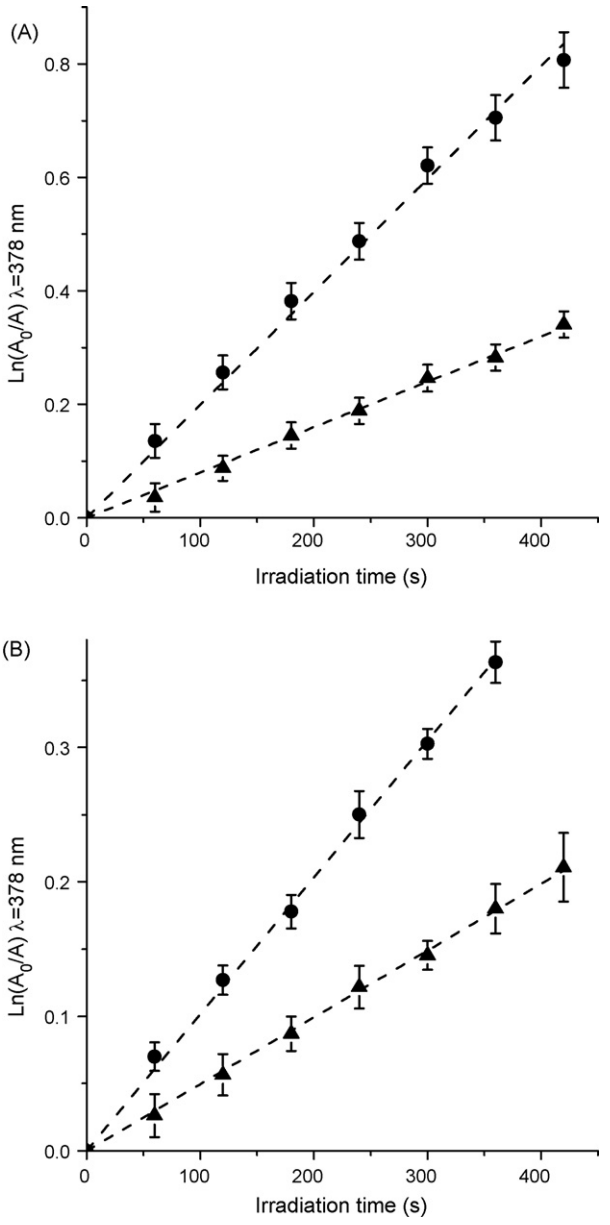


Fig. 5. First-order plots for the photooxidation of DMA (35 μM) photosensitized by ZnPcF (▲) and ZnPc (●) in (A) DMF/water (10%) [HCl] 1.2 mM and (B) benzene/BHDC (0.1 M)/water ($W_0 = 10$, $[HCl] = 25$ mM). Values represent mean ± standard deviation of three separate experiments.

(Fig. 5B). The results of DMA photodecomposition in the reverse micellar system show that $O_2(^1\Delta_g)$ production by ZnPcF reaches a value ~0.3 in the acidified BHDC system (Table 1). Therefore, this microheterogeneous system provides an appropriated biomimetic media to produce photodynamic activity.

3.4. Antimicrobial activity

ZnPcF was tested for its inhibitory activity against *C. albicans* as a model to assess the microorganism inactivation. First, the binding capacity to fungal cells of ZnPcF was evaluated after different incubation times. The *C. albicans* cultures were treated

Table 1
Kinetic parameters (k_{obs}) and quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) of phthalocyanines in different media

Phthalocyanine	Medium	k_{obs} (s^{-1})	Φ_Δ
ZnPcF	DMF/water[a]	$(0.80 \pm 0.04) \times 10^{-3}$	0.23 ± 0.02[b]
ZnPc	DMF/water[a]	$(1.98 \pm 0.07) \times 10^{-3}$	0.56[c]
ZnPcF	BHDC micelles[d]	$(0.50 \pm 0.03) \times 10^{-3}$	0.30 ± 0.02[e]
ZnPc	BHDC micelles[d]	$(1.02 \pm 0.06) \times 10^{-3}$	0.62[f]

[a] DMF/water (10%) [HCl] = 1.2 mM.
[b] Relative to ZnPc in DMF.
[c] From Ref. [21].
[d] Benzene/BHDC (0.1 M)/water ($W_0 = 10$, [HCl] = 25 mM).
[e] Relative to ZnPc in benzene.
[f] From Ref. [22].

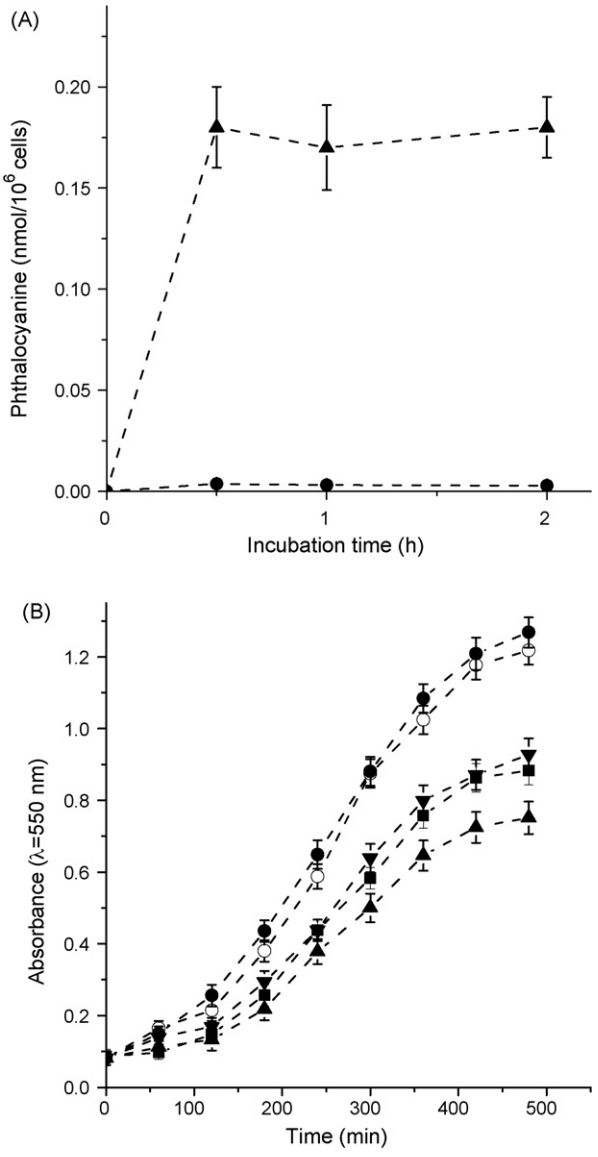


Fig. 6. (A) Amount of ZnPcF (▲) and ZnPc (●) recovered from *C. albicans* cells incubated with 10 μM of photosensitizer for different times at 37 °C in dark, (B) photosensitized growth delay curves of *C. albicans* cells treated with 10 μM of ZnPcF (▲) and irradiated with visible light (30 mW/cm^2), treated with 10 μM of ZnPcF (▼) and keeping in dark, treated with 80 μM of fluconazole and keeping in dark (■) in Saboreaoud broth at 37 °C. Control culture untreated irradiated (●), untreated in dark (◯). Values represent mean ± standard deviation of three separate experiments.

with 10 μM of phthalocyanine at 37 °C in the dark. The amounts of sensitizer associated with the cells are shown in Fig. 6A. As can be seen, ZnPcF reaches the highest value of cell-bound sensitizer at ∼30 min and prolongation of the incubation time does not cause appreciable increase in the amount of photosensitizer bound to *C. albicans* cells. In these conditions, the binding of ZnPcF to cells reaches a value of ∼0.17 nmol/10^6 cells. This value is considerably higher than that found for the cells treated with ZnPc under similar conditions. Therefore, the four fluconazole units contained in the phthalocyanine macrocycle produces an increase in the photosensitizer binding to yeast cells with respect to ZnPc.

The inactivation effect of ZnPcF was compared with fluconazole in *C. albicans* growth delay cultures. In this way, the cultures of *C. albicans* in lag phase were treated with either 10 μM of photosensitizer or 80 μM of fluconazole at 37 °C. As can be observed in Fig. 6B, growth was arrested when *C. albicans* cultures were treated with ZnPcF in dark. This behavior was similar to that found for fluconazole indicating that ZnPcF is toxic in dark probably due to the activity of the four fluconazole groups. Moreover, the cytotoxic activity of ZnPc even increases when the cultures are irradiated with visible light. The last effect can be attributed to the photodynamic action produced by the photosensitizer, which enhances the inactivation of *C. albicans* cells. On the other hand, cultures treated with unsubstituted ZnPc (10 μM) mixed with fluconazole (80 μM) and irradiated showed a similar behavior to that found for *C. albicans* treated with only with 80 μM of fluconazole. This result is probably influenced by the low binding of ZnPc to cells indicating, at least in this case, the necessity of fluconazole bound covalently to phthalocyanine macrocycle.

4. Conclusions

A novel Zn(II) phthalocyanine derivative bearing four antifungal structure of fluconazole (ZnPcF) was synthesized from phthalonitrile-azole derivative with 27% yield. Absorption and fluorescence spectroscopic studies show that ZnPcF is lowly soluble in polar solvents or in reverse micellar systems. One of the problems that affect the sensitizing ability of the phthalocyanines is the aggregation tendency due to the large π conjugate systems [27,33]. The aggregates present an efficient nonradiative energy relaxation pathway, diminishing the triplet-state population and the $O_2(^1\Delta_g)$ quantum yield. Therefore, the formation of aggregation precludes the photodynamic activity. However, for ZnPcF the addition of HCl produce an increase in the monomerization of ZnPcF in DMF/water (10%) and in benzene/BHDC (0.1 M)/water ($W_0 = 10$). This acid media ([HCl] < 3.5 mM) does not produce significant monoprotonation of the external nitrogen atoms of the macrocycle. Microheterogeneous systems such as reverse micelles are frequently used as an interesting model to mimic the water pockets that are often found in various bioaggregates such as proteins, enzymes and membranes [34]. Thus, water-soluble and water-insoluble compounds can be dissolved simultaneously in reverse micelles, which simulate a biomimetic microenvironment [35]. The present studies show that acidified cationic reverse micelles can efficiently avoid aggregation of ZnPcF, enhancing its photodynamic activity. The value of ϕ_F obtained in DMF/water (10%, v/v, [HCl] = 1.2 mM) indicates that it is appropriated for quantification and detection of the sensitizer in biological media [24]. The photodynamic studies show that the values of $O_2(^1\Delta_g)$ generation are also indicative that acidified DMF or BHDC micellar media produce monomerization of ZnPcF. However, the values of Φ_Δ can significantly change in a different medium, diminishing when the sensitizer is partially aggregated. Also, the biological microenvironment of the sensitizer can induce important modifications in the photophysics of the photosensitizer established in solution [36]. In consequence, there are limitations to predict photodynamic

efficiencies of sensitizers in biological systems on the basis of photophysical investigations in solution. Preliminary studies to evaluate the photodynamic activity of these phthalocyanines were tested against a typical yeast *C. albicans*. In the biological medium, the amount of sensitizer that remains in solution is mainly aggregated; however, the photodynamic inactivation of microorganism is produced by photosensitizers that are bound to *C. albicans* cells, which is probably dissolved as monomer in the cellular microenvironment and from this location the photodynamic effect takes place inducing the cellular photodamages. Thus, the present results indicated that ZnPcF is an interesting antifungal agent because it has antimycotic activity in dark and its efficiency increases in the presence of light due to the photodynamic inactivation caused by the photosensitizer moiety.

Acknowledgements

Authors thank Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina, SECYT Universidad Nacional de Río Cuarto and Agencia Nacional de Promoción Científica y Técnológica (ANPCYT) of Argentina for financial support. E.N.D. is a Scientific Members of CONICET. M.P.C. thanks Agencia Córdoba Ciencia-CONICET for a doctoral fellowship.

References

[1] K. Langa, J. Mosinger, D.M. Wagnerová, Photophysical properties of porphyrinoid sensitizers non-covalently bound to host molecules; models for photodynamic therapy, Coord. Chem. Rev. 248 (2004) 321–350.
[2] M.R. Hamblin, T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem. Photobiol. Sci. 3 (2004) 436–450.
[3] G. Jori, S.B. Brown, Photosensitized inactivation of microorganisms, Photochem. Photobiol. Sci. 5 (2004) 403–405.
[4] E.N. Durantini, Photodynamic inactivation of bacteria, Curr. Bioactive Compd. 2 (2006) 127–142.
[5] P.G. Calzavara-Pinton, M. Venturini, R. Sala, A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin, J. Photochem. Photobiol. B: Biol. 78 (2005) 1–6.
[6] G. Ganber, A overview of fungal infections, Drugs 61 (2001) 1–12.
[7] J.H. Rex, M.G. Rinaldi, M.A. Pfaller, Resistance of *Candida* species to fluconazole, Antimicrob. Agents Chemother. 39 (1995) 1–8.
[8] G.H. Goldman, M.E. da Silva Ferreira, E. dos Reis Marques, M. Savoldi, D. Perlin, S. Park, P.C. Godoy Martinez, M.H.S. Goldman, A.L. Colombo, Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil, Diagn. Micr. Infec. Dis. 50 (2004) 25–32.
[9] M.A. Ribeiro, C.P. Rodrigues, Up-regulation of ERG11 gene among fluconazole-resistant *Candida albicans* generated in vitro: is there any clinical implication? Diagn. Micr. Infec. Dis. 57 (2007) 71–75.
[10] J.M. Bliss, C.E. Bigelow, T.H. Foster, C.G. Haidaris, Susceptibility of *Candida* species to photodynamic effects of photofrin, Antimicrob. Agents Chemother. 48 (2000) 2000–2006.
[11] L. Polo, A. Segalla, G. Bertoloni, G. Jori, K. Schaffner, E. Reddi, Polylysine-porphycene conjugates as efficient photosensitizers for the inactivation of microbial pathogens, J. Photochem. Photobiol. B: Biol. 59 (2000) 152–158.
[12] B. Cosimelli, G. Roncucci, D. Dei, L. Fantetti, F. Ferroni, M. Rici, D. Spinelli, Synthesis and antimycotic activity of new unsymmetrical substituted zinc phthalocyanines, Tetrahedron 59 (2003) 10025–10030.
[13] S.A.G. Lambrechts, M.C.G. Aalders, J. Van Marle, Mechanistic study of the photodynamic inactivation of *Candida albicans* by a cationic porphyrin, Antimicrob. Agents Chemother. 49 (2005) 2026–2034.
[14] Y. Yoshida, Y. Aoyama, Stereoselective interaction of an azole antifungal agent with its target, lanosterol 14 alpha-demethylase (cytochrome P-45014DM): a model study with stereoisomers of triadimenol and purified cytochrome P-45014DM from yeast, Chirality 2 (1990) 10–15.
[15] H. Ji, W. Zhang, Y. Zhou, M. Zhang, J. Zhu, Y. Song, J. Lu, A three-dimensional model of lanosterol 14-alpha-demethylase of *Candida albicans* and its interaction with azole antifungals, J. Med. Chem. 43 (2000) 2493–2505.
[16] T. Fukuoka, D.A. Johnston, C.A. Winslow, M.J. de Groot, C. Burt, C.A. Hitchcock, S.G. Filler, Genetic basic for differential activities of fluconazole and voriconazole against *Candida krusei*, Antimicrob. Agents Chemother. 47 (2003) 1213–1219.
[17] J.N. Demas, G.A. Crosby, The measurement of photoluminescence quantum yields, J. Phys. Chem. 75 (1971) 991–1024.
[18] P.G. Seybold, M. Gouterman, Porphyrins XIII: fluorescence spectra and quantum yields, J. Mol. Spectroscopy 31 (1969) 1–13.
[19] M.E. Milanesio, M.G. Alvarez, E.I. Yslas, C.D. Borsarelli, J.J. Silber, V. Rivarola, E.N. Durantini, Photodynamic studies of metallo 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin: photochemical characterization and biological consequences in a human carcinoma cell line, Photochem. Photobiol. 74 (2001) 14–21.
[20] R.W. Redmond, J.N. Gamlin, A compilation of singlet yields from biologically relevant molecules, Photochem. Photobiol. 70 (1999) 391–475.
[21] U. Michelsen, H. Kliesch, G. Schnurpfeil, A.K. Sobbi, D. Wöhrle, Unsymmetrically substituted benzonaphthoporphyrazines: a new class of cationic photosensitizers for the photodynamic therapy of cancer, Photochem. Photobiol. 64 (1996) 694–701.
[22] A. Ogunsipe, D. Maree, T. Nyokong, Solvent effect on the photochemical and fluorescence properties of zinc phthalocyanine derivatives, J. Mol. Struct. 650 (2003) 131–140.
[23] W.F. Harrigan, M.E. McCance, Laboratory Methods in Food and Dairy Microbiology, Academic Press, London, 1986.
[24] E.I. Yslas, V. Rivarola, E.N. Durantini, Synthesis and photodynamic activity of zinc(II) phthalocyanine derivatives bearing methoxy and trifluoromethylbenzyloxy substituents in homogeneous and biological media, Bioorg. Med. Chem. 13 (2005) 39–46.
[25] E.A. Dupouy, D. Lazzeri, E.N. Durantini, Photodynamic activity of cationic and non-charged Zn(II) tetrapyridinoporphyrazine derivatives: biological consequences in human erythrocytes and *Escherichia coli*, Photochem. Photobiol. Sci. 3 (2004) 992–998.
[26] S. Wei, J. Zhou, D. Huang, X. Wang, B. Zhang, J. Shen, Syntesis and typeI/type II photosensitizing properties of a novel amphiphilic zinc phthalocyanine, Dyes Pigm. 71 (2006) 61–67.
[27] M. Kostka, P. Zimcik, M. Miletin, P. Klemera, K. Kopecky, Z. Musil, Comparison of aggregation properties and photodynamic activity of phthalocyanines and azaphthalocyanines, J. Photochem. Photobiol. A:Chem. 178 (2006) 16–25.
[28] A. Ogunsipe, T. Nyokong, Effects of substituents and solvents on the photochemical properties of zinc phthalocyanine complexes and their protonated derivatives, J. Mol. Struct. 689 (2004) 89–97.
[29] C. Marti, S. Nonell, M. Nicolau, T. Torres, Photophysical properties of neutral and cationic tetrapyridinoporphyrazines, Photochem. Photobiol. 71 (2000) 53–59.
[30] E. Diez-Barra, M.C. Parde, Reactivity of tetracyanoethylene oxide towar heteroaromatic compounds. Synthesis and structure of heterocyclic dicyanomethylides, J. Org. Chem. 47 (1982) 4409–4412.
[31] P. Zimcik, M. Miletin, Z. Musil, K. Kopecky, L. Kubza, D. Brault, Cationic azaphthalocyanines bearing aliphatic *tertiary* amino substituents—synthesis, singlet oxygen production and spectroscopic studies, J. Photochem. Photobiol. A: Chem. 183 (2006) 59–69.
[32] Z. Chen, X. Li, T. Ngai, C. Wu, D.K.P. Ng, Monomerization of cationic phthalocyanine in AOT reversed micelles, Langmuir 17 (2001) 7957–7959.

[33] C.A. Hunter, J.K.M. Sanders, The nature of π–π interactions, J. Am. Chem. Soc. 112 (1990) 5525–5534.
[34] I. Scalise, E.N. Durantini, Photodynamic effect of metallo 5-(4-carboxyphenyl)-10,15,20-tris(4-methylphenyl) porphyrins in biomimetic AOT reverse micelles containing urease, J. Photochem. Photobiol. A: Chem. 162 (2004) 105–113.
[35] J.J. Silber, A. Biasutti, E. Abuin, E. Lissi, Interactions of small molecules with reverse micelles, Adv. Colloids Interf. Sci. 82 (1999) 189–252.
[36] B.M. Aveline, R.W. Redmond, Can cellular phototoxicity be accurately predicted on the basic of sensitizer photophysics? Photochem. Photobiol. 69 (1999) 306–316.