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

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Abstract. A novel Zn(II) phthalocyanine derivative bearing four antifungal structure of fluconazole (ZnPcF) 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 mixtures of constitutional isomers with 23% 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 a appropriate system to induce monomerization of ZnPcF.

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, $O_2(^1\Delta_g)$ [1]. Based in these properties, one of more recent applications of phthalocyanine in medicine is in the detection and cure of tumors by photodynamic therapy [2]. A new promising approach to treat microbial infections is called photodynamic inactivation (PDI). This is based in the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. The subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms [3]. Thus, phthalocyanines were proposed for PDI of microorganisms in an attempt to overcome the problem of microbial strains resistant [4-6].

In this paper, a novel Zn(II) phthalocyanine derivative bearing four structure 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 [7-9]. 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 enhance by irradiation with visible light due to the photodynamic activity of the photosensitizer moiety.

One of the problems that affect the sensitizing ability of the phthalocyanines is the aggregation tendency due to the large π conjugate systems [1]. 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. 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 [10,11]. Thus, water-soluble and water-insoluble compounds can be dissolved simultaneously in reverse micelles, which simulate a biomimetic microenvironment [12]. The present studies show that acidified cationic reverse micelles can efficiently avoid aggregation of ZnPcF, enhancing its photodynamic activity.

Synthesis of Zn(II) phthalocyanine derivative

Zn(II) phthalocyanine subtituded by fluconazole (ZnPcF) was synthesized by a two-step procedure [11,13]. First, the phthalonitrile derivative bearing a fluconazole moiety was prepared by a nucleophilic *ipso*-nitro substitution reaction of 4-nitrophthalonitrile with fluconazole in the presence of K$_2$CO$_3$ (Scheme 1). This dinitrile derivative was isolated by flash chromatography (silica gel, dichloromethane/methanol 5%) with 12% yield [MS m/z 432 (M$^+$) (432,1259 calculated for C$_{21}$H$_{14}$F$_2$N$_8$O); elemental analysis: calcd. C 55.33, H 3.21, N 26.12 found C 55.27, H 3.26, N 26.05]. The cyclotetramerization of dinitrile 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 23% yield [FT-IR (KBr) cm^{-1} 3040, 2905, 2840, 1635, 1560, 1245, 1040, FAB-MS m/z 1792 (M$^+$) (1792,4326 calculated for C$_{84}$H$_{56}$F$_8$N$_{32}$O$_4$Zn); elemental analysis: calcd. C 56.21, H 3.14, N 24.97 found C 56.10, H 3.02, N 25.05]. The product was precipitated with cyclohexane and filtered. Them, 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 [14].

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 can facilitate the interaction with the heme group of the cytochrome [7-9].
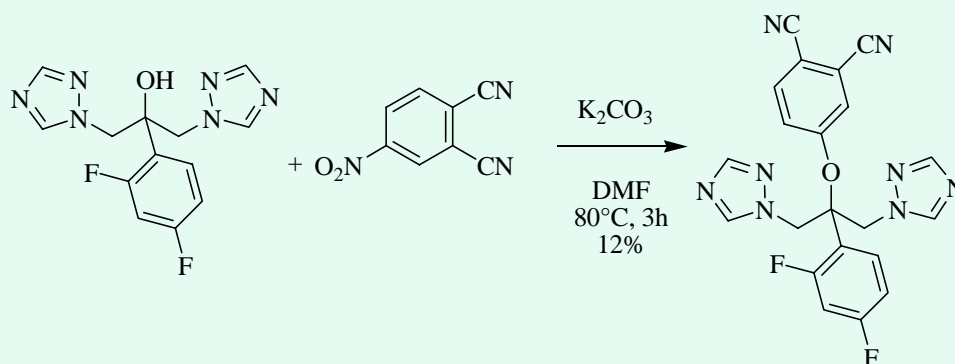
Scheme 1. Synthesis of phthalonitrile derivative
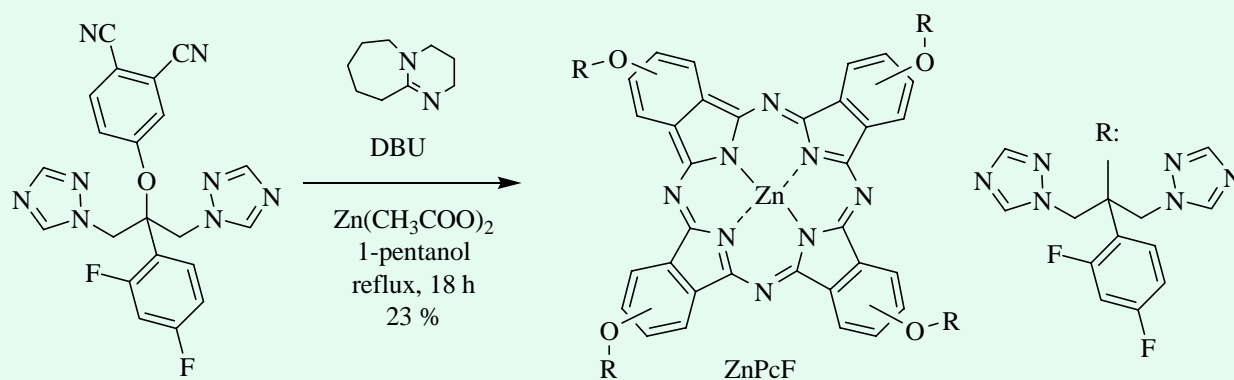
Scheme 2. Synthesis of ZnPcF

Spectroscopic studies and solubilization of ZnPcF

Absorption spectroscopy in different media. The absorption spectrum of ZnPcF was studied in different homogeneous and microheterogeneous media. The results are gathered in Figure 1A. 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 [14,15]. Almost no significant absorption bands of ZnPcF are detected between 550-750 nm in non-hydrogen bond donor or hydrogen bond donor polar organic solvents and the sensitizer is very poorly solibilized in these media. Also, negligible solubilization was observed 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 N,N-dimethylformamide (DMF), whereas in methanol the two bands corresponding to the monomer (~640 nm) and aggregated (~672 nm) are observed in Figure 1A. Also, ZnPcF is dissolved as monomer in

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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 but this procedure was not effective with anionic reverse micelles of AOT (0.1 M) (see below).

Zn(II) phthalocyanines 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 [16]. First protonation shows two bands at ~680 and ~710 nm and it requires an elevated acid concentration, per example Zn(II) phthalocyanine (ZnPc) in DMF/H_2SO_4 1.84 M. In our case, it can be note that a small band appear at 712 nm in acidified BHDC system. This peak can be assigned to the partial first protonation of ZnPcF. 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 [17]. However, 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 (Figure 1 A). Also, it was established in DMF/water (10%) using H_2SO_4 as compared with previous results for ZnPc [16]. Figure 1 B shows the absorption spectra on addition of H_2SO_4. The protonation occurs with isosbestic point at ~691 nm, as previously showed for ZnPc [16].
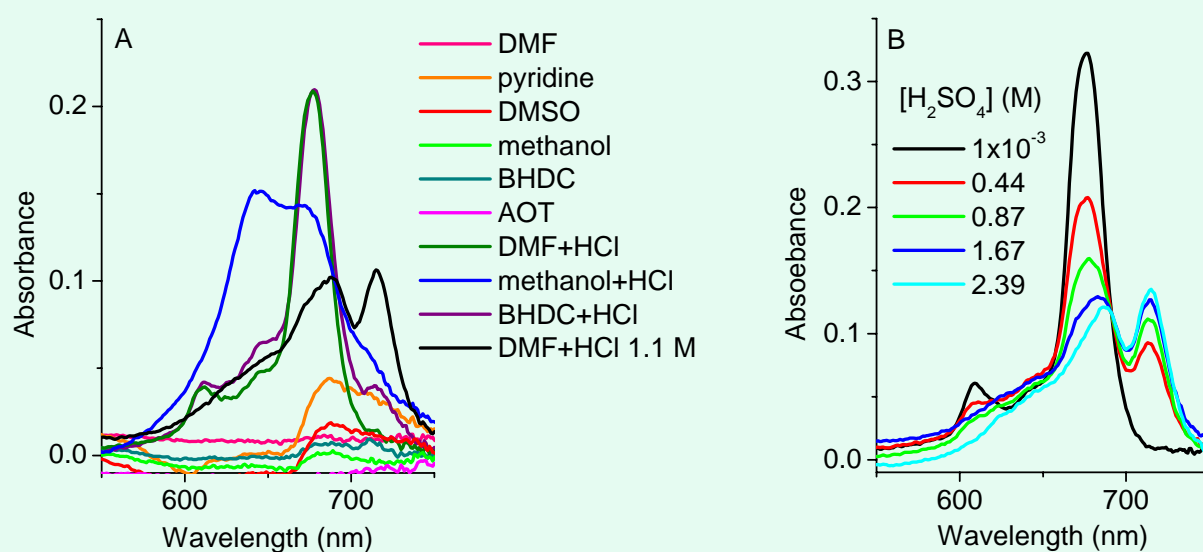

Figure 1. Absorption spectra of ZnPcF in different media (A) N,N-dimethylformamide (DMF), pyridine, dimethylsulphoxide (DMSO), methanol, benzene/BHDC (0.1 M)/water ($W_0=10$) and *n*-heptane/AOT (0.1 M)/water ($W_0=10$). Organic solvent/water mixtures were 10% v/v water/HCl 3.3 mM. Reverse micellar systems at $W_0=10$ were prepared using water/HCl 5.0 mM. [ZnPcF]=2.5μM; (B) spectral changes of ZnPcF in DMF/water (10% v/v) at different H_2SO_2 concentrations.

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 Figure 2A, the Q-band around 675 nm is not detected in absence of acid indicating that ZnPcF is practically insoluble as monomer. However, as acid concentration increases, the absorption band of the monomeric form enhances until the HCl concentration reaches values ~1 mM (Figure 2D). At this point, addition of a higher acid concentration produce only negligible changes en the spectra.

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) phthalocyanine derivates substituted by alcohoxy groups [13]. A value of 8.4×10^4 M^{-1} cm^{-1} was calculated for the molar coefficient (ε) of ZnPcF in DMF/water (10% v/v)/HCl 1.2 mM.
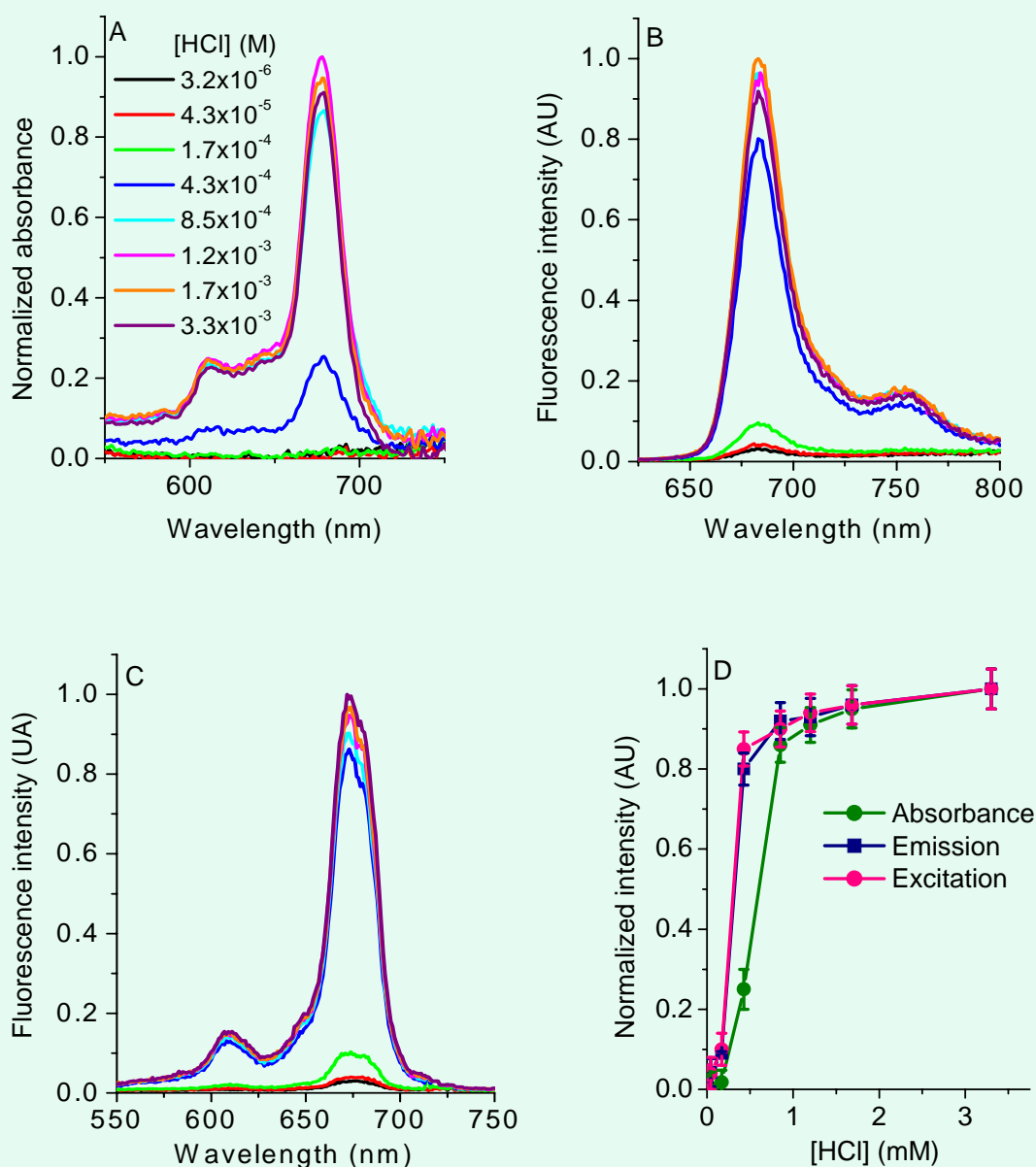
Figure 2. (A) Absorption, (B) fluorescence emission (λ_{exc}=610 nm) and (C) excitation (λ_{em}=720 nm) spectra of ZnPcF in DMF/water (10%) at different HCl concentrations. (D) Variation of intensities (absorbance λ=678 nm, emission λ=683 nm and excitation λ=678 nm) with HCl concentration.

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 (Figure 2B). Since aggregated phthalocyanines are nonemissive [1], 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 for similar Zn(II) phthalocyanines [6]. As can be observed in Figure 2D, 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) [6]. 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 in 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 values of 1.80 eV. These results are in agreement with those previously reported for similar phthalocyanines in different media [17].

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

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 Figure 1A. As can be observed, solubilization of the sensitizer 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 show in Figure 3B. The band at ~680 nm, which can be attributed to the Q-band of the monomeric species, become more intense when the HCl concentration increase. Thus, deaggregation 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.
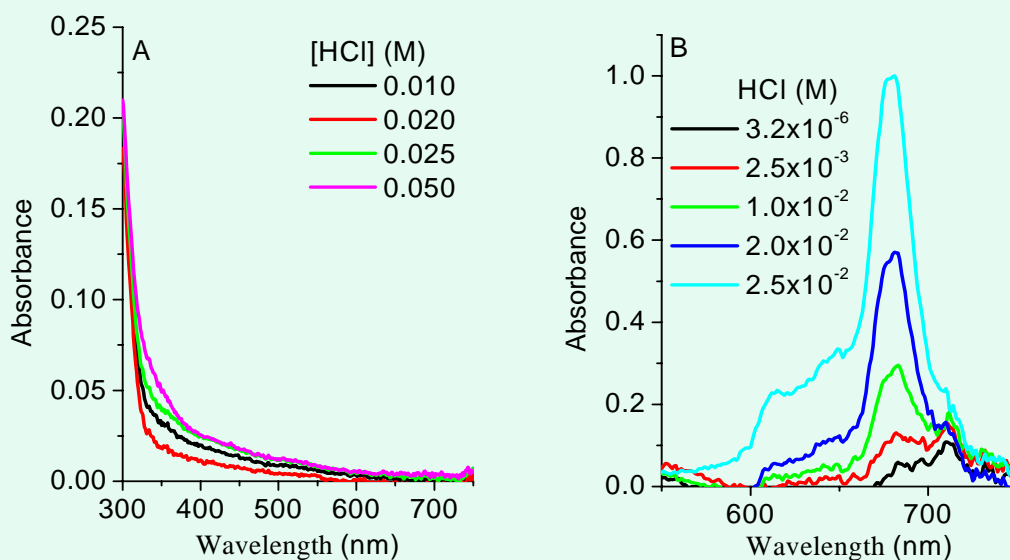
Figure 3. Absorption spectra of ZnPcF in (A) *n*-heptane/AOT (0.1 M)/water (W_0=10) and (B) benzene/BHDC (0.1 M)/water (W_0=10) at different HCl concentration in the water dispersion.

Photodynamic activity

Photooxidation of 9,10-dimethylanthracene (DMA). 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 9,10-dimethylanthracene (DMA). This substrate quenches $O_2(^1\Delta_g)$ by exclusively chemical reaction [10]. Therefore, it was used in this work to evaluate the ability of the sensitizers to produce $O_2(^1\Delta_g)$. A time-dependent decrease in the DMA concentration was observed by following a decrease in its absorbance. From first-order kinetic plots the values of the observed rate constant (k_{obs}^{DMA}) 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. On the other hand, it reaches a value ~0.2 in the acidified BHDC system. This microheterogeneous system provides an appropriated biomimetic media to produce photodynamic activity.
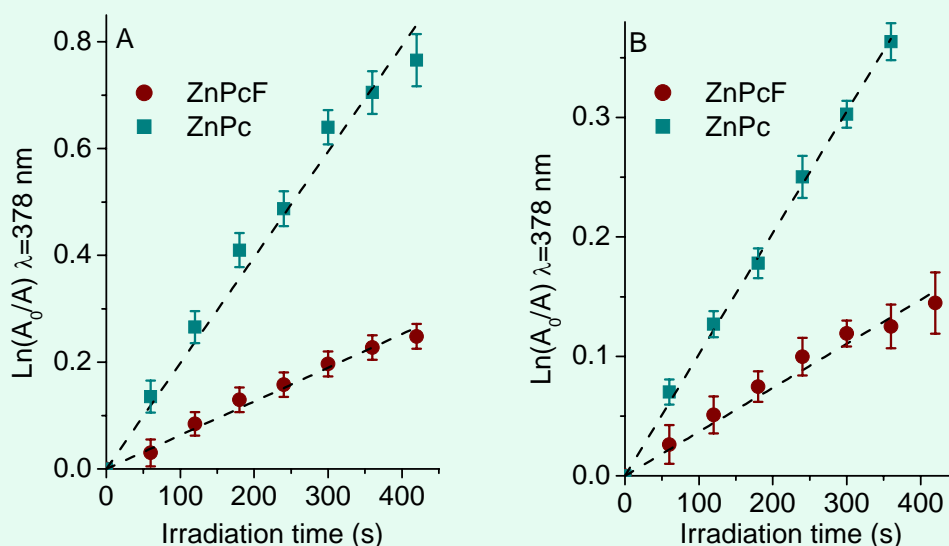
Figure 3. First-order plots for the photooxidation of DMA (35 μM) photosensitized by ZnPcF and ZnPc in (A) DMF/water (10%)/HCl 1.2 mM and (B) benzene/BHDC (0.1 M)/water (W_0=10, HCl 25 mM). Values represent mean ± standard deviation of three separate experiments.

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]	$(6.3\pm0.2)\times10^{-4}$	0.14 ± 0.02
ZnPc	DMF/water[a]	$(2.5\pm0.1)\times10^{-3}$	0.56[c]
ZnPcF	BHDC micelles[b]	$(3.8\pm0.2)\times10^{-4}$	0.19 ± 0.02
ZnPc	BHDC micelles[b]	$(1.0\pm0.1)\times10^{-3}$	0.50[d]

[a]DMF/water (10%)/HCl 1.2 mM, [b]benzene/BHDC (0.1 M)/water (W_0=10, HCl 25 mM), [c]ref. [18], [d]ref. [19].

Conclusions

A novel Zn(II) phthalocyanine derivative bearing four antifungal structure of fluconazole (ZnPcF) was synthesized from phthalonitrile-azole derivative with 23% yield. Absorption and fluorescence spectroscopic studies 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 DMF/water (10%) and in benzene/BHDC (0.1 M)/water (W_0=10). This acid media does not produce significant monoprotonation of the of the external nitrogen atoms of the macrocycle. 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 [6]. 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 porphyrin established in solution [20]. In consequence, there are limitations to predict photodynamic efficiencies of sensitizers in biological systems on the basic of photophysical investigations in solution.

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