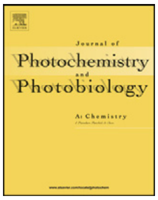
Journal of Photochemistry and Photobiology A: Chemistry 246 (2012) 67–74

Contents lists available at SciVerse ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem



Spectroscopic and time-resolved fluorescence emission properties of a cationic and an anionic porphyrin in biomimetic media and *Candida albicans* cells

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ARTICLE INFO

Article history:
Received 3 December 2011
Received in revised form 8 June 2012
Accepted 23 June 2012
Available online 2 August 2012

Keywords:
Porphyrin
Fluorescence
Micelles
DNA
Candida albicans

ABSTRACT

Spectroscopic and time-resolved fluorescence emission techniques were used to provide information for the interaction of 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl) porphyrin ($TMAP^{4+}$) and 5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrin ($TPPS^{4-}$) with different biomimetic media and with *Candida albicans* cells. In *n*-heptane/sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/water and benzene/benzyl-*n*-hexadecyldimethylammonium chloride (BHDC)/water reverse micelles interactions were dependent on the micellar interface and the amount of water dispersed in the microemulsion. It was also observed that the DNA binding of cationic porphyrin $TMAP^{4+}$ led to two lifetimes. *In vitro* investigations showed that $TMAP^{4+}$ is bound to *C. albicans*. Fluorescence lifetime measurements and fluorescence microscopic images provided additional insight into the effects of porphyrin uptake by cells. The results reveal a double localization of $TMAP^{4+}$ inside of *C. albicans* cells. Thus, a redistribution of $TMAP^{4+}$ was observed in unwashed cells, probably due to a relocalisation of molecules that were weakly bound to the cells or remained in solution. However, this effect was not found with molecules tightly bound in the cells, after one washing step.

1. Introduction

In the last years, positively charged porphyrins have attracted considerable attention because of their remarkable ability as phototherapeutic agents. In particular, cationic porphyrin derivatives have been proposed for the treatment and control of microorganisms by photodynamic inactivation (PDI) [1]. This methodology is mainly based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. Subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms [2,3]. Also, porphyrins containing cationic groups are able to interact with DNA bases, inducing DNA lesions upon photoactivation [4,5]. In general, three binding models have been described for the interaction of cationic porphyrins with DNA, which involve intercalation, outside groove binding and outside binding with porphyrins self-stacking [6–9]. The DNA complexes involving cationic porphyrins are presumably stabilized by electrostatic interaction between the positively charged substituents on the macrocycle periphery and the negatively charged phosphate oxygen atoms of DNA.

In biological processes, the solubilization of photosensitizers plays an important role. In this sense, reverse micelles have been frequently used as an interesting model to mimic the water pockets often found in various bioaggregates such as proteins, enzymes and membranes [10,11]. Water-soluble and water-insoluble compounds can be dissolved simultaneously in reverse micelles. In these microheterogeneous systems, a solute can be located in a variety of microenvironments, namely the organic surrounded solvent, the water pool or at the micellar interface.

In previous studies, cationic porphyrin derivatives have been investigated for PDI applications in the treatment and control of yeast [12–15]. In particular, 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) has shown to be an effective photosensitizer to eradicate *Candida albicans* [14]. The PDI induced by $TMAP^{4+}$ was compared with that produced by 5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrin ($TPPS^{4-}$), which was used as an anionic photosensitizer model. *In vitro* studies showed that *C. albicans* cellular suspensions in PBS were efficiently photo inactivated by $TMAP^{4+}$, whereas a negligible effect was found for $TPPS^{4-}$.

In the present work, we examined fluorescent spectroscopic properties of $TMAP^{4+}$ and $TPPS^{4-}$ in reverse micelles biomimetic systems, in calf thymus DNA solutions and in *C. albicans* cells suspensions. The fluorescence lifetimes (τ) of these porphyrins were measured previously in solution and in microheterogeneous systems [16,17]. However, extrapolation from measurements made in homogeneous solution to biological media is difficult to be done due to the influence of the microenvironment where the

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http://dx.doi.org/10.1016/j.jphotochem.2012.06.024

photosensitizer can be localized. Also, the porphyrin can be distributed heterogeneously in different cellular compartments. The singlet molecular oxygen generated by the photosensitizer in a given intracellular location produces its initial effects in a specially confined site of action [18]. Therefore, the cellular localization of photosensitizer molecules is determinant in PDI efficiency. In general, porphyrins which localize at intracellular sites are more effective photosensitizers than those which are not bound to cells. The results of fluorescence decay times obtained for these porphyrins in *C. albicans* cells were complemented with those of fluorescence microscopy, which represents an useful procedure to observe the localization of photosensitizer in cells.

2. Materials and methods

2.1. General

All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) from Sigma (St. Louis, MO, USA) was dried under vacuum. Benzyl-*n*-hexadecyldimethylammonium chloride (BHDC) from Sigma was recrystallized twice from ethyl acetate and dry under vacuum over P_2O_5. Calf thymus double-stranded DNA from Sigma was used as received. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from a Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Porphyrins

5,10,15,20-Tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl) porphyrin *p*-tosylate ($TMAP^{4+}$) and 5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrin ($TPPS^{4-}$) sodium salt were purchased from Aldrich. A porphyrin stock solution (~0.5 mM) was prepared by dissolution in 1 mL of water. The sensitizers concentrations were checked by spectroscopy, taking into account the value of molar extinction coefficients (ε); $TMAP^{4+}$ $\varepsilon = 178{,}144\ M^{-1}\ cm^{-1}$ at 412 nm and $TPPS^{4-}$ $\varepsilon = 163{,}000\ M^{-1}\ cm^{-1}$ at 413 nm in water [19].

2.3. Spectroscopic studies

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. The emission spectra were recorded exciting the samples at $\lambda_{exc} = 515$ nm. The measurements were performed at $25.0 \pm 0.5\ ^\circ C$ using 1 cm path length quartz cells. Fluorescence decays were recorded with a time-correlated single photon counting system (Edinburgh Instruments OB 900, Livingston, UK) equipped with a PicoQuant (Berlin, Germany) subnanosecond pulsed LED PLS370 with emission centered at 380 nm. Fluctuations in the pulse and intensity were corrected by making an alternative collection of scattering and sample emissions. In all cases, the fluorescence decay time were fitted with an exponential function, optimizing Chi-square, residuals and standard deviation parameters.

2.4. Studies in reverse micelles

Measurements in reverse micelles were performed using a stock solution of AOT and BHDC 0.1 M, which was prepared by weighing and dilution in *n*-heptane and benzene, respectively. 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 surfactant present in the reverse micelle ($W_0 = [H_2O]/[\text{surfactant}]$). The mixtures were sonicated for about 10 s to obtain perfectly clear micellar system. *n*-Heptane and benzene were chosen as organic solvents to form AOT and BHDC reverse micelles, respectively, because both combinations can disperse water to high W_0 [20].

2.5. Studies in calf thymus DNA

Stock solution of DNA was prepared by weighing and dilution in water. The concentration of calf thymus double-stranded DNA stock solution (2.7 mM), calculated in base pairs, was determined spectrophotometrically using molar extinction coefficient $\varepsilon_{260} = 1.31 \times 10^4\ M^{-1}\ cm^{-1}$ [4]. Solutions were prepared by adding concentrated stock solution of DNA directly to a cuvette containing porphyrin solution (2 mL, ~2 μM).

2.6. Microorganism and growth conditions

Strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified [14]. Cultures of *C. albicans* was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (4 mL) at 37 °C to stationary phase. Cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0), corresponding to ~10^7 colony forming units (CFU)/mL. The cells were appropriately diluted to obtain ~10^6 CFU/mL in PBS. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and the porphyrin was added from a stock solution ~0.5 mM in water. Cellular suspensions of *C. albicans* (2 mL, ~10^6 CFU/mL) in PBS were incubated with 5 μM porphyrin in the dark for 30 min at 37 °C. To obtain one washing step, the cells were centrifuged (3000 rpm for 15 min) and the cell pellets were re-suspended in 2 mL PBS. After that, the cultures were exposed to visible light for different time intervals. The visible light source used to irradiate *C. albicans* cells was a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. A wavelength range between 350 and 800 nm was selected by optical filters. The light intensity at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Each experiment was repeated separately three times.

2.7. Fluorescence microscopy

Microscopic observations and photographs were performed using a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) fluorescence microscope equipped with a HBO 100 W mercury lamp. Images were captured using an AxioCam HRc camera and subsequently processed using AxioVision Rel. 4.3 software. Fluorescence images of $TMAP^{4+}$ in *C. albicans* cells were visualized using a DBP 406/23 + 530/45, DFT 435 + 570, DBP 467/30 + 618/75 filter (Carl Zeiss).

3. Results and discussion

3.1. Spectroscopic studies in homogenic solvents and micellar system

Absorption spectra of $TMAP^{4+}$ and $TPPS^{4-}$ were compared in solvents used to form reverse micellar systems, as shown in Figs. 1 and 2. In water, spectra of these porphyrins show the typical *Soret* and *Q*-bands characteristic of free-base porphyrin derivatives. The relative intensities of the *Q*-bands for these porphyrins show an *etio*-type spectrum ($\varepsilon_{VI} > \varepsilon_{III} > \varepsilon_{II} > \varepsilon_{I}$). Also, sharp *Soret* absorption bands were obtained indicating that these porphyrins are mainly

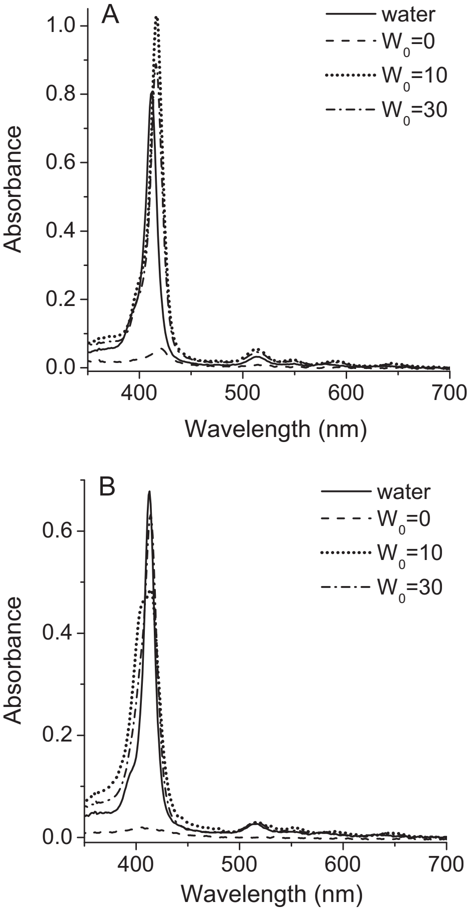

Fig. 1. Absorption spectra of (A) $TMAP^{4+}$ (4.5 μM) and (B) $TPPS^{4-}$ (4.2 μM) in water and *n*-heptane/AOT (0.1 M) reverse micelles at $W_0 = 0$, $W_0 = 10$ and $W_0 = 30$.

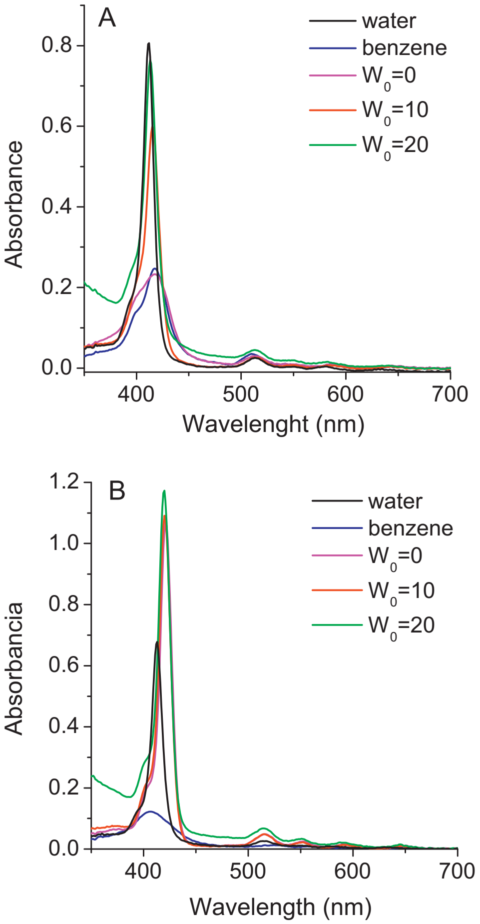

Fig. 2. Absorption spectra of (A) $TMAP^{4+}$ (4.5 μM) and (B) $TPPS^{4-}$ (4.2 μM) in water, benzene and benzene/BHDC (0.1 M) reverse micelles at $W_0 = 0$, $W_0 = 10$ and $W_0 = 20$.

not aggregated in water. In contrast, both photosensitizers are not soluble in *n*-heptane, while a low intensity and broadening of *Soret* bands were observed in benzene (Fig. 2). In the aromatic solvent, aggregation of $TMAP^{4+}$ and $TPPS^{4-}$ take places as it is typical for many porphyrin derivatives [21,22].

The solubilization and interaction of $TMAP^{4+}$ and $TPPS^{4-}$ were spectroscopically analyzed in AOT and BHDC reverse micelles. Both porphyrins were aggregated in *n*-heptane/AOT (0.1 M) at $W_0 = 0$ (Fig. 1). This behavior may be due to the high ionic strength in the micellar interface, which is produced by the AOT charged heads and their counterions in absence of a water pool [20]. However, BHDC micelles at $W_0 = 0$ were able to solubilize $TPPS^{4-}$ as monomer but not $TMAP^{4+}$, which appears to remain aggregated in benzene (Fig. 2). Furthermore, both porphyrins were unaggregated in *n*-heptane/AOT (0.1 M)/water at $W_0 = 10$ and 30, indicating that the presence of water is necessary to dissolve these porphyrin as monomer in AOT systems. Upon solubilization in AOT micelles at $W_0 = 10$ and 30, the *Soret* absorption bands of $TMAP^{4+}$ showed a slight red shift of ~4 nm respect to that in water (Fig. 1A). Since these molecules have opposite charges of the surfactant forming the micelle, they interact mainly with the surfactant headgroups and therefore the position of the *Soret* band is not changed by addition of water. In contrast, the absorption spectrum of $TPPS^{4-}$ in *n*-heptane/AOT for $W_0 = 10$ showed that the *Soret* band is broad (Fig. 1B). The spectra of $TPPS^{4-}$ in AOT resembled that in water with increasing W_0 from 10 to 30 (Fig. 1B). This effect may be due to electrostatic repulsions of the anionic porphyrin with the AOT headgroups. Thus, in these systems $TPPS^{4-}$ is mainly dissolved in the aqueous core. This observation was opposite to that found in cationic benzene/BHDC (0.1 M) micelles, where the bathochromic shift was found for $TPPS^{4-}$ (Fig. 2B), while the spectra of $TMAP^{4+}$ were similar to that in pure water with increasing W_0 (Fig. 2A). In these media, the positive charges of BHDC headgroups repel the cationic porphyrin $TMAP^{4+}$ into water.

Steady-state fluorescence emission spectra of these porphyrins were compared in the same media as absorption studies. Fluorescence spectra showed in Figs. 3 and 4 were obtained dissolving (A) $TMAP^{4+}$ (4.5 μM) and (B) $TPPS^{4-}$ (4.2 μM) in the different media without normalizing the absorbance at the excitation wavelength. In water, spectra showed two bands in the red spectral region (Fig. 3). These bands have been assigned to Q(0–0) and Q(0–1) transitions. Fluorescence quantum yields (Φ_F) of 0.07 for $TMAP^{4+}$ and 0.08 for $TPPS^{4-}$ were previously reported in water [16,17]. These values are appropriated for detection and quantification of the sensitizer in the biological media [14]. A very low emission of fluorescence was found for $TMAP^{4+}$ in benzene (Fig. 4A) evidencing that this porphyrin is poorly soluble as monomer in this medium. As expected, no fluorescence emission was observed for $TPPS^{4-}$ in benzene (Fig. 4B) due to the low solubility. On the other hand, in these microheterogenic media, $TMAP^{4+}$ and $TPPS^{4-}$ porphyrins showed fluorescence emission spectra bathochromically shifted with respect to those in water. Both porphyrins exhibited small fluorescence Stokes shifts (~5) in micellar systems, according to the rigid planar structure of the tetrapyrrolic macrocycle [17].

The lifetimes of the singlet excited state (τ) of $TMAP^{4+}$ and $TPPS^{4-}$ in different media are shown in Tables 1 and 2, respectively. In water, the fluorescence observed at either 655 or 714 nm decayed monoexponentially and the τ values are in agreement

Table 1
Fluorescence emission maxima (λ_{max}^{em}) and lifetimes (τ) of TMAP^{4+} in different media.

Media	λ_{max}^{em} (nm)	τ_1 (ns)	% species$_1$	τ_2 (ns)	% species$_2$	χ^2
Water	643	10.54 ± 0.02	100	–	–	1.04
PBS	643	10.43 ± 0.02	100	–	–	1.04
Benzene	650	6.33 ± 0.08	64.4	2.54 ± 0.06	35.6	1.07
Benzene	711	6.25 ± 0.10	24.6	2.47 ± 0.09	75.4	1.08
DMSO	647	12.51 ± 0.03	100	–	–	1.01
DMF	648	11.60 ± 0.03	100	–	–	1.04
AOT ($W_0 = 10$)[a]	649	11.99 ± 0.03	100	–	–	1.07
AOT ($W_0 = 30$)[a]	650	11.61 ± 0.03	100	–	–	1.05
BHDC ($W_0 = 0$)[b]	652	6.86 ± 0.06	61.8	1.82 ± 0.03	38.2	1.30
BHDC ($W_0 = 10$)[b]	648	11.96 ± 0.03	98.2	0.74 ± 0.07	1.8	1.04
BHDC ($W_0 = 20$)[b]	648	11.08 ± 0.04	97.2	0.32 ± 0.05	2.8	1.02
DNA[c]	652	12.24 ± 0.11	90.0	3.11 ± 0.16	10.0	1.03

[a] *n*-Heptane/AOT (0.1 M)/water.
[b] Benzene/BHDC (0.1 M)/water.
[c] DNA = 11 μM. Where none is shown, the results are fitted to a single exponential.

with information given in the literature ($\tau = 9.3$ for TMAP^{4+} and 10.4 for TPPS^{4-}) [16,17]. In contrast, two contributions were found for both porphyrins in benzene, possibly due to the formation of aggregates in this medium. Percentages of the contributions were dependent of the emission wavelength (Tables 1 and 2), indicating the presence of aggregates. In micellar system, the main interaction of TMAP^{4+} and TPPS^{4-} is basically of electrostatic character. Thus, TMAP^{4+} is effectively bound to the AOT anionic micelles head group. In this case, the τ value was increased due to this binding as compared with water. Moreover, it can be observed in Table 1 that these values in AOT were not affected by the amount of waters dispersed in the micelles. This effect was also observed for TPPS^{4-} with BHDC micelles (Table 2). Otherwise, TMAP^{4+} is physically repelled from the surface to the water core in BHDC cationic micellar system. A short-lived component ($\tau < 1$ ns) with a low contribution was detected for TMAP^{4+} in benzene/BHDC with $W_0 > 0$, probably due to a very low population of aggregate molecules localized in an environment sensing higher benzene concentration than in the micelle core [23]. Furthermore, a decrease in the longer τ value was observed from $W_0 = 10$ to $W_0 = 20$ due to the increase in water content. Similar behavior was found for TPPS^{4-} with AOT micelles (Table 2). These results indicate that the photophysical properties of these porphyrins are dependent on the electrostatic properties of the media. This fact could lead to a model of interaction of porphyrins with biomimetic systems.

3.2. *Spectroscopic studies in the presence of DNA*

The absorption and emission spectra of TMAP^{4+} and TPPS^{4-} in water were analyzed in the presence of DNA. As can be observed in Fig. 5, practically no change in the absorption and emission spectra of TPPS^{4-} was observed, while changes in the band position and intensity were found for TMAP^{4+}. It was assumed that spectral perturbations upon addition of DNA are due to association of the cationic porphyrin with the DNA matrix. This interaction between TMAP^{4+} and DNA is characterized by a red shift of the *Soret* maximum of ~7 nm and by a large hypochromicity (45%). The large hypochromicity suggests that porphyrin π electrons are perturbed by the association with DNA. Otherwise, the addition of DNA in the concentration range studied (0–11 μM) did not perturb the absorption (Fig. 5A) or steady-state fluorescence (Fig. 5B) spectra of TPPS^{4-}, which indicates a negligible interaction with nucleotides possibly by electrostatic repulsion with negative charges of phosphate groups. It was previously reported a high value of apparent binding constant (K_{DNA}) of TMAP^{4+}-DNA (7.5×10^5 M^{-1}) [24]. The K_{DNA} for TMAP^{4+} is comparable with those previously reported for other tetracationic porphyrin derivatives with charges directly attached to the tetrapyrrolic macrocycle. For example, values of 1.3×10^6 and 4.35×10^5 M^{-1} have been calculated for TMPyP [4,25]. The τ values of TMAP^{4+} and TPPS^{4-} in the presence of 11 μM DNA are reported in Tables 1 and 2, respectively. The obtained results for TMAP^{4+} showed that τ values were affected by addition of DNA, similar to that observed in absorption and emission spectra. Also, τ value for TPPS^{4-} was very similar to that in pure water in agreement with the absence of interactions between the anionic porphyrin and DNA. The analysis of τ found for the complex of TMAP^{4+} with DNA showed two lifetimes of 12.24 and 3.11 ns, which could be explained considering two different populations of TMAP^{4+} associated with DNA. It is known that DNA can exhibit different types of interaction with cationic porphyrins [26]. A similar behavior was previously found for TMPyP and the shorter time was assigned to a DNA complex corresponding to the

Table 2
Fluorescence emission maxima (λ_{max}^{em}) and lifetimes (τ) of TPPS^{4-} in different media.

Media	λ_{max}^{em} (nm)	τ_1 (ns)	% species	τ_2 (ns)	% species	χ^2
Water	642	10.87 ± 0.02	100	–	–	1.01
PBS	642	11.30 ± 0.02	100	–	–	1.08
Benzene	659	4.09 ± 0.14	52.3[a]	1.18 ± 0.05	47.7[a]	1.10
Benzene	733	4.12 ± 0.15	6.3	1.23 ± 0.07	93.7	1.02
DMSO	651	12.18 ± 0.02	100	–	–	1.02
DMF	651	11.73 ± 0.03	100	–	–	1.07
AOT ($W_0 = 10$)[a]	655	11.75 ± 0.03	100	–	–	1.01
AOT ($W_0 = 30$)[a]	647	11.18 ± 0.03	100	–	–	1.06
BHDC ($W_0 = 0$)[b]	653	11.91 ± 0.03	100	–	–	1.01
BHDC ($W_0 = 10$)[b]	648	11.27 ± 0.04	100	–	–	1.10
BHDC ($W_0 = 20$)[b]	651	12.01 ± 0.05	100	–	–	1.03
DNA[c]	643	10.92 ± 0.02	100	–	–	1.06

[a] *n*-Heptane/AOT (0.1 M)/water.
[b] Benzene/BHDC (0.1 M)/water.
[c] DNA = 11 μM. Where none is shown, the results are fitted to a single exponential.

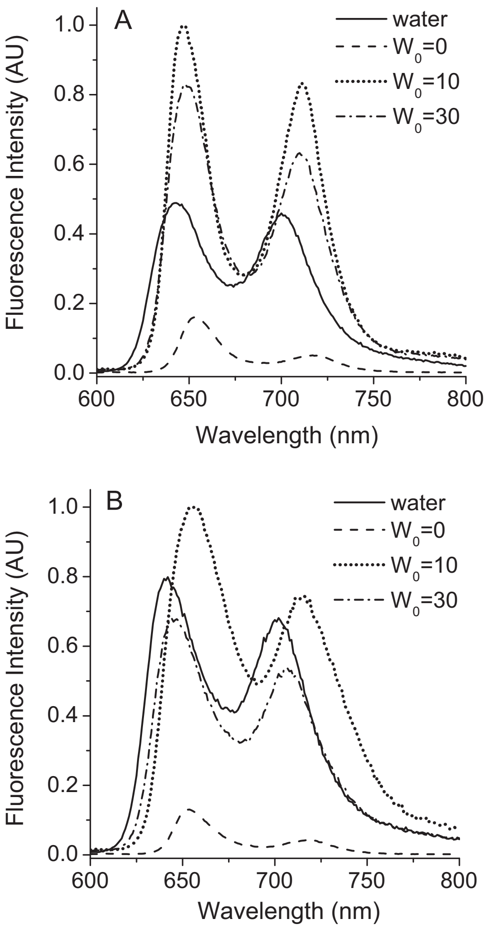

Fig. 3. Fluorescence emission spectra of (A) $TMAP^{4+}$ (4.5 μM) and (B) $TPPS^{4-}$ (4.2 μM) in water and *n*-heptane/AOT (0.1 M) reverse micelles at $W_0 = 0$, $W_0 = 10$ and $W_0 = 30$ ($\lambda_{exc} = 515$ nm).

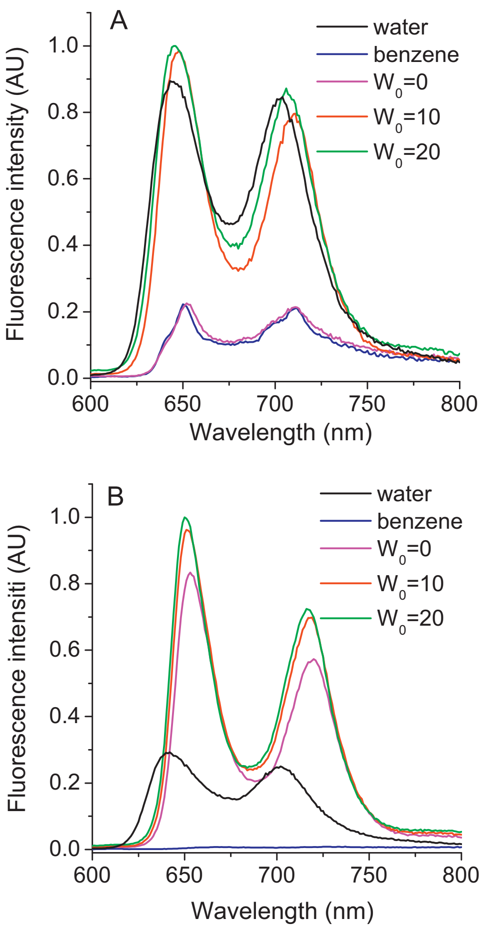

Fig. 4. Fluorescence emission spectra of (A) $TMAP^{4+}$ (4.5 μM) and (B) $TPPS^{4-}$ (4.2 μM) in water, benzene and benzene/BHDC (0.1 M) reverse micelles at $W_0 = 0$, $W_0 = 10$ and $W_0 = 20$ ($\lambda_{exc} = 515$ nm).

intercalation, while the longest time constant was related with the lifetime found for external binding [26].

3.3. Spectroscopic studies in cell suspensions of C. albicans

Absorption spectroscopic results (Fig. 6A) indicated an interaction between $TMAP^{4+}$ and yeast cells. Also, cellular suspensions in PBS showed fluorescence emission spectrum for $TMAP^{4+}$ with maxima at ~646 and 710 nm (Fig. 6B), which are bathochromically shifted ~4 nm respect to the porphyrin in PBS. As can be observed in Fig. 6, the shape and intensity of the bands of $TMAP^{4+}$ performed in cellular suspensions closely match the corresponding spectra of this photosensitizer in presence of DNA, possibly favored by the cellular microenvironment where the sensitizer is localized. In contrast, negligible changes were detected in the spectra of $TPPS^{4-}$ in cellular suspension, indicating that no significant interaction take places between this anionic porphyrin and *C. albicans* cells.

Time-resolved fluorescence measurements provided additional insight into the effects of porphyrin uptake by cells. In contrast to the results observed in water, the signals obtained for $TMAP^{4+}$ in *C. albicans* required two exponential terms (Table 3). Moreover, the fluorescence decay of $TPPS^{4-}$ in presence of cells was better fitted by biexponential decay, although the contribution of shorter lifetime component is about 1% of the relative amplitudes (Table 4). The longer lifetime is typical of $TPPS^{4-}$ in a polar environment (Table 2). Therefore, these results indicated that almost all of the $TPPS^{4-}$ molecules are in the same aqueous medium. Otherwise, the fluorescence lifetime data for $TMAP^{4+}$ with *C. albicans* yielded two lifetimes of 10.50 and 1.83 ns, with 94.1 and 5.9 relative amplitudes, respectively. Similar behavior was previously observed for photosensitizers that interact with microbial cells [27,28].

On the other hand, the subcellular localization of $TMAP^{4+}$ in *C. albicans* cells was investigated by fluorescence microscopy (Fig. 7). Images show that cells incubated with 5 μM $TMAP^{4+}$ in PBS for 30 min in the dark exhibited red fluorescence typical of porphyrin derivatives. However, no red fluorescent image inside of *C. albicans* cells was detected for cells treated with 5 μM $TPPS^{4-}$ (results not shown). The capacity of these porphyrins to bind to *C. albicans* cells was previously compared in cellular suspension of ~10^6 cells/mL in PBS incubated with 5 μM porphyrin at 37 °C in the dark [14]. After 5 min incubation, the binding of $TMAP^{4+}$ tended to a saturation value of ~1.4 nmol/10^6 cells, whereas $TPPS^{4-}$ was poorly uptake by yeast cells. Thus, the cationic porphyrin was higher bound to cells in comparison with the anionic porphyrin $TPPS^{4-}$. These results are in agreement with images shown in Fig. 7 and they suggest that these cationic porphyrins have particularly high binding affinity for *C. albicans* cells.

After one washing step of the cells, porphyrin molecules that remain in PBS and those that are weakly bound to the cells are removed. Thus, under this condition only the emission of $TMAP^{4+}$ molecules bound to the cells was observed and the contribution of the shorter lifetime increased to 18.1% with respect to that in unwashed cells (Table 3). Also, this distribution of species practically did not change after 15 min irradiation with visible light. However, this was not the tendency observed for cells treated

Table 3
Fluorescence emission maxima (λ_{max}^{em}) and lifetimes (τ) of TMAP^{4+} in *C. albicans* cells.

Irr. time (min)[a]	λ_{max}^{em} (nm)	τ_1 (ns)	% species	τ_2 (ns)	% species	χ^2
0[b]	648	10.50 ± 0.04	94.1	1.83 ± 0.08	5.9	1.10
15[b]	648	10.03 ± 0.07	81.1	1.71 ± 0.04	18.9	1.15
30[b]	648	9.80 ± 0.09	78.1	1.52 ± 0.04	21.9	1.09
0[c]	648	10.01 ± 0.06	81.9	1.48 ± 0.05	18.1	1.18
15[c]	648	9.49 ± 0.05	83.2	1.04 ± 0.16	16.8	1.30

[a] Irradiation time of cell suspensions in PBS with visible light (90 mW/cm^2).
[b] Cell suspensions without washing.
[c] Cell suspensions with a washing step.

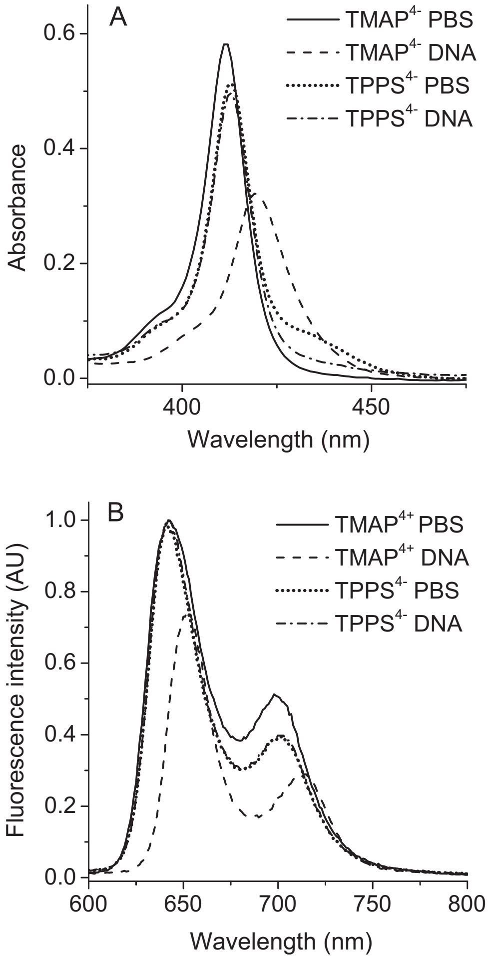

Fig. 5. (A) Absorption spectra of TMAP^{4+} and TPPS^{4-} and (B) fluorescence emission spectra of TMAP^{4+} (λ_{exc} = 515 nm) in PBS and in the presence of 11 μM DNA.

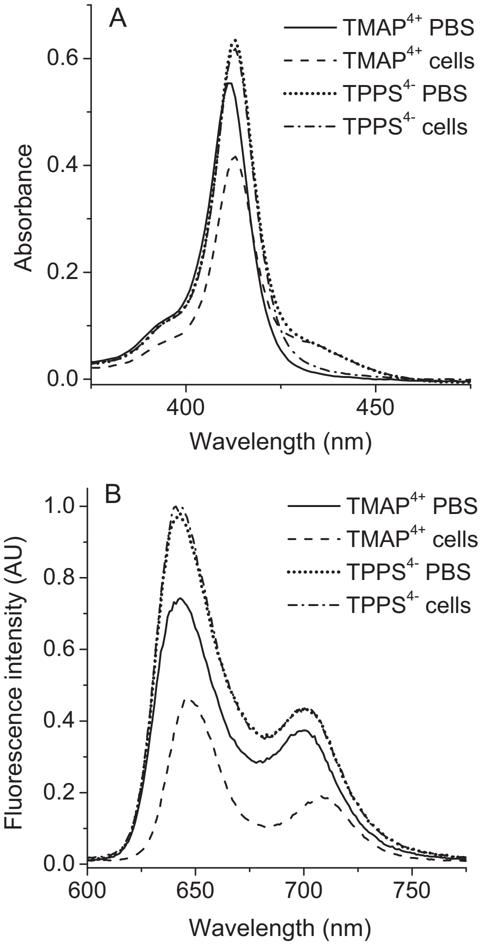

Fig. 6. (A) Absorption spectra and (B) fluorescence emission spectra (λ_{exc} = 515 nm) of TMAP^{4+} and TPPS^{4-} in PBS and in presence of *C. albicans* (1 × 10^6 cells/mL).

with TMAP^{4+} without washing. In the last case, the contribution of shorter lifetime increased with the irradiation times. It was previously found that TMAP^{4+} is highly bound to *C. albicans* cells [14]. However, a small fraction of porphyrin molecules remains in the PBS, producing emission outside the cells. Thus, both species may be contributing to the longer lifetime observed. Also, it was found that TMAP^{4+} exhibited a high photoinactivation (>99.9997%) of *C. albicans* cells after 30 min of irradiation [14]. Thus, when cells were irradiated the photodynamic activity can induce membrane damages, which could allow a redistribution of the molecules that remain in the PBS, producing an increase in the contribution of the shorter lifetime.

Table 4
Fluorescence emission maxima (λ_{max}^{em}) and lifetimes (τ) of TTPS^{4-} in *C. albicans* cells.

Irr. time (min)[a]	λ_{max}^{em} (nm)	τ_1 (ns)	% species	τ_2 (ns)	% species	χ^2
0[b]	644	11.44 ± 0.03	99.0	0.54 ± 0.14	1.0	1.17
15[b]	644	11.20 ± 0.02	98.8	0.31 ± 0.10	1.2	1.05
30[b]	644	11.80 ± 0.02	98.9	0.45 ± 0.15	1.1	1.01

[a] Irradiation time of cell suspensions in PBS with visible light (90 mW/cm^2).
[b] Cell suspensions without washing.

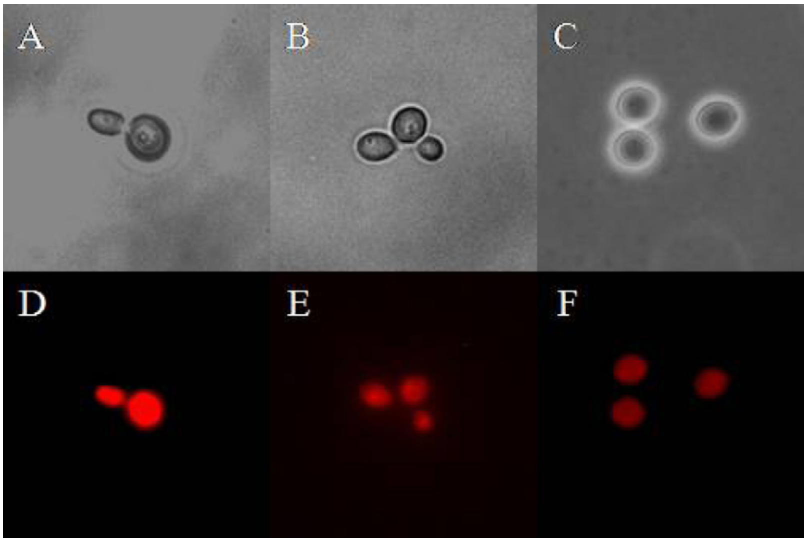

Fig. 7. Microscopic observation of the *C. albicans* cells under bright field (A–C) and fluorescence (D–F) conditions. Cells (10^6 CFU/mL) were incubated with 5 μM $TMAP^{4+}$ for 30 min at 37 °C in dark and exposed to visible light for different times 0 min (A and D), 15 min (B and E) and 30 min (C and F) (100× microscope objective).

4. Conclusions

This study provides information on the spectroscopic and time-resolved fluorescence emission properties of a cationic ($TMAP^{4+}$) and an anionic ($TPPS^{4-}$) porphyrin derivatives in reverse micellar systems, calf thymus DNA solution and *C. albicans* cells. Both porphyrins interact with *n*-heptane/AOT and benzene/BHDC reverse micelles. When the charge on the macrocycle is equal to the charge of the head of the surfactant, porphyrin is repelled into the water and the τ values tend to those in pure water with increasing W_0. However, when the charges are opposite the porphyrin interacts strongly with the micellar interface. The electrostatic interaction of $TMAP^{4+}$ with anionic micelles or DNA increase the tendency of cationic porphyrin binding to anionic domains. This effect can improve the photodynamic action in the location site. Also, $TMAP^{4+}$ is strongly bound to *C. albicans* cells. When $TMAP^{4+}$ is in the presence of *C. albicans* the molecules experience a distribution of microenvironments. The results reveal a double localization of $TMAP^{4+}$ inside of *C. albicans* cells. After photodynamic treatment, a redistribution of $TMAP^{4+}$ was observed in unwashed cells. The formation of reactive oxygen species sensitized by $TMAP^{4+}$ inside de cells can lead to damage and loss in the functionality of the cell membrane. This effect is accompanied by a relocation of $TMAP^{4+}$, mainly involving molecules that remained weakly bound to the cells or in PBS solution.

Acknowledgements

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

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