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Photophysicals and photochemicals studies of zinc(II) phthalocyanine in long time circulation micelles for Photodynamic Therapy use

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Abstract

Long time circulation systems, such as polymeric micelles, represent a growing area in biomedical research. These microparticles can be used in many biological systems to provide appropriate drug levels with a specific biodistribution. Long time circulation micelles (LTCM) were routinely prepared using PEG-5000-DSPE (polyethyleneglycol-5000-distearoil-phosphatidyl-ethanolamine) and zinc(II) phthalocyanine (ZnPc) as a photosensitizer and fluorescent probe. This compound belongs to a second generation of photoactive agents, mainly used in photodynamic therapy (PDT) of neoplasic tissues. Their high selectivity for tumoral target tissues as well as high phototoxicity based on singlet oxygen generation renders the utilization of these compounds feasible as an alternative therapy for cancer treatment. LTCM were characterized by classical spectroscopic techniques. Absorbance measurements indicated that the drug was s completely loaded into LTCM ($\varepsilon = 2.41 \times 10^5\ cm^{-1}$). This was also verified by steady state and time-resolved fluorescence measurements. The lifetime profiles of ZnPc decay curves were fitted according to biexponential function ($\tau_1 = 3.9$ ns and $\tau_2 = 15.5$ ns) indicating different locations for ZnPc into LTCM. The time-resolved spectroscopy measurements for ZnPc triplet excited state lifetimes (τ_T) were calculated from the kinetic analysis of transient decays at the absorption maximum (480 nm), by using laser flash photolysis technique. All the spectroscopy measurements performed allowed us to conclude that, ZnPc in LTCM is a promising drug delivery system (DDS) for PDT.

Keywords: PEG-5000-DSPE; ZnPc; Polymeric micelles; Long time circulation micelles

1. Introduction

Sustained delivery systems have been emerging as a main goal to provide a modificated release of drugs. These systems avoid plasmatic fluctuations, lower side effects, facilitate dosage administration and reduce the required frequency of administration increasing patient compliance (Madan, 1985). Among them, long time circulation micelles (LTCM) have been studied in the last decade and clearly shown the capacity to be utilized to maintain adequate therapeutic levels of drugs in the body (Kataoka et al., 1993; Kwon and Kataoka, 1995; Liu et al., 2001). Therefore we believe that this drug delivery system (DDS) could be an important administration pathway for PDT drugs acting directly on neoplasic tissue.

PDT, first developed for cancer treatment, is being actively exploited in many other clinical applications such as the treatment of age-related macular degeneration, of hardening arteries, sun induced precancerous skin lesions, and wound infections (Dougherty et al., 1998; Sharman et al.,

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1999; Bonnett, 2000). In most of the applications studied, the first step in the treatment involves the administration of a photosensitive drug (know as a photosensitizer), which has a high affinity to malignant tissues and other non-normal cells. In a second step, the biological systems are irradiated with appropriated low doses of light (in special at the maximum absorption wavelength of the photoactive compound and frequently isolated from laser system), leading to the production of many reactive oxygen species such as H_2O_2, $^1O_2$, $^\bullet O_2$, $^\bullet$OH (EROS) and peroxinitrites. Singlet oxygen is reported to be the predominant cytotoxic agent when the photosensitizers are excited by light of appropriate wavelength and power. Among the various photosensitizer drugs investigated, phthalocyanines have been found to be highly promising (Ben Hur and Rosenthal, 1985; Ali and Van Lier, 1999; Tedesco et al., 2003).

Zinc(II) phthalocyanine (ZnPc) (Fig. 1), a second generation photosensitizer much utilized in PDT (Spikes et al., 1985; Reddi et al., 1990; Gantchev et al., 2003), was used in our experiments due its high selectivity for tumoral targets and enhanced cytotoxic efficiency due to singlet oxygen photogeneration (Ben Hur and Rosenthal, 1985). In addition, the hydrophobic character of ZnPc facilitates the incorporation of this photosensitizer in many drug delivery systems especially in LTCM. The main structure of the phthalocyanine dye is presented in Fig 1.

One of the main advantages of polyethyleneglycol (PEG) conjugated micelles in comparison to conventional micelles are the smaller value of the critical micellar concentration (CMC) of LTCM. The CMC of the PEG conjugated micelles is around 10^{-7} M, while in conventional micelles is about 10^{-3} to 10^{-4} M. This renders PEG conjugated micelles a very stable system in diluted solution with a slow dissociation process, which permits incorporation and retention of drugs for longer time resulting in higher drug concentration in the target tissues (Kataoka et al., 1993). Besides, polymeric micelles have reduced diameters with a narrow size distribution; these properties constitute an additional advantage for their distribution control.

The complex lipid/polymeric derivative, PEG-5000-DSPE (Diasteroil phosphatidil-ethanolamine-polyethyleneglycol/MW 5000 Da), may form micelle systems with a long circulation time. The structure of the complex micelle/polymer formed, reduces the recognition of these particles by the Reticular Endothelial System (RES), leading

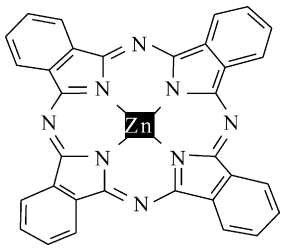

Fig. 1. Zinc(II) phthalocyanine (ZnPc).

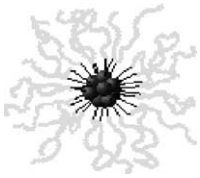

Fig. 2. Polymeric micelle (PEG-5000-DSPE) (Duncan, 2001).

to a better drug biodistribution (Fig. 2). Many researches and clinical trials worldwide have proven the efficiency of LTCM as a DDS (Weissig et al., 1998; Torchilin, 1999; Torchilin, 2001; Fu et al., 2002).

In the present work, ZnPc as a classical photosensitizer for PDT incorporated in LTCM of PEG-5000-DSPE was prepared and characterized by steady state and time-resolved spectroscopics techniques. All the results clearly indicated that ZnPc was incorporated in LTCM and most of its spectroscopic properties already studied in homogeneous media electing ZnPc as a promising photosensitizer for cancer therapy. Most of these properties are also present in this new formulation rendering their utilization in clinical trials of PDT feasible.

2. Materials and methods

2.1. Materials

PEG-5000-DSPE was purchased from Avanti Polar Lipids. ZnPc and sodium dodecyl sulfate (SDS) were from Aldrich. Solvents and commercial reagents are of HPLC grade, purchased from Merck, and utilized as obtained.

2.2. Methods

2.2.1. Preparation of LTCM

LTCM containing ZnPc were prepared from PEG-5000-DSPE lipid according to the methodology previously described by Weissig et al. (1998). Briefly, 10 mg of PEG-5000-DSPE were solubilized in chloroform and ZnPc (5 μM in ethanol) was added. After removal of the organic solvent on a rotary evaporator, micelles formed were ressuspensed in phosphate buffered saline (PBS) at pH 7.4 and submitted to ultra-sonication process for 5 min, using a bath sonicator, yielding a clear blue solution. The final concentration of the incorporated drug was determined by absorption spectroscopy using the ZnPc molar extinction coefficient (ε_{673} = $2.41 \times 10^5\ M^{-1}\ cm^{-1}$). Freshly prepared stock solutions of Zn/LTCM were used for all spectroscopy studies.

2.2.2. Spectroscopic and photophysical studies

2.2.2.1. Absorption spectroscopy and fluorescence emission. Absorption measurements of ZnPc in micellar media and in ethanol were made on a Perkin-Elmer Lambda 20 setup whose background was corrected using matched quartz cuvettes with scanning over the wavelength range from 300 to

800 nm. Fluorescence emission spectra were performed on a Hitachi F-4500 spectrofluorimeter and a Fluorog 3 SPEX from Ivon-Jobin, working, at 610 nm of excitation, and a maximum emission setup at 682 nm. The system was equipped with stirring and temperature control of the sample.

2.2.3. Time-resolved fluorescence measurement

A time-correlated single-photon counting technique (TCSPC) was used to determine the fluorescence lifetimes of 5 μM of ZnPc in micelar media and in ethanol. Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was an Edinburgh Analytical Instrument FL-900 lifetime spectrometer, operating with a hydrogen-filled nanosecond flash lamp at 40 kHz pulse frequency. The emission wavelength was selected by a monochromator (Edinburgh Instruments), and emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. Software provided by Edinburgh Instruments was used to analyse the decay curves; the exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as the reduced chi-square (χ^2).

The excitation and emission wavelengths used were setup at 610 and 682 nm, respectively. All measurements were made in the presence of oxygen, at 25 °C.

2.2.4. Determination of the fluorescence quantum yield

The fluorescence quantum yield (ϕ_F) of ZnPc was measured using the ratio method described by Eaton (1988) and Demas and Crosby (1971), utilizing ZnPc in ethanol as a standard (ϕ_F = 0.28). Optical densities were set below 0.1 a.u. in LTCM at the wavelength of maximum absorbance (674 nm) and corrected fluorescence emission spectra recorded on a Fluorog 3 spectrofluorimeter between 650 and 800 nm, following the technique described by Eaton (1988), using Eq. (1):

$$\phi_u = \left[\left(\frac{A_s F_u n^2}{A_u F_s n_o^2}\right)\right]\phi_s \quad (1)$$

where u is unknown, s is standard, ϕ_F is fluorescence quantum yield, A is absorbance, F is fluorescence area and n is refractive index.

2.2.5. Determination of photobleaching quantum yield

Photobleaching quantum yield of ZnPc (ϕ_{PB}) in LTCM was determined following the procedure described by Aveline et al. (1998). Potassium ferroxalate actinometer was used to evaluate the total amount of energy used in the photobleaching experiments. The photobleaching quantum yield (ϕ_{PB}) is defined as the number of molecules undergoing bleaching (η), divided by the number of photons absorbed by the sample. η was calculated by the decrease in the absorption maximum divided by the molar extinction coefficient as shown in Eqs. (2) and (3):

$$\eta = \frac{\Delta A \times \text{volume} \times 6.02 \times 1023}{\varepsilon} \quad (2)$$

$$\phi = \frac{\eta}{I_s \times t \times (1 - 10^{-\text{Abs}})} \quad (3)$$

where I_s is the number of photons per second emitted by the light source, t is the time in seconds and $1 - 10^{-\text{Abs}}$ is the amount of light absorbed by the sample.

I_s can then be calculated by using the equation:

$$I_s = \frac{n_{Fe}}{\phi_{Fe} \times t \times (1 - 10^{-\text{Abs}})} \quad (4)$$

$$n_{Fe} = \frac{6.02 \times 10^{23} \times \text{Abs}\,(510\,\text{nm}) \times V1 \times V3}{\varepsilon \times V2} \quad (5)$$

where $\varepsilon = 1.11 \times 10^4$, ϕ_{Fe} is the quantum yield of iron formation (ϕ_{Fe} = 1.22 at 355 nm), t is time and Abs is the absorbance of the $K_3Fe(C_2O_4)_3$ solution at the excitation wavelength.

All experiments were performed in the dark. The classical procedure for actinometer was previously described by Aveline et al. (1998).

2.2.6. Laser flash photolysis

2.2.6.1. Analysis of transient absorption spectra and ZnPc triplet excited state lifetimes. Triplet state transient species and lifetime measurements were made using a laser flash photolysis spectrometer, which allowed the simultaneous capture of the transient absorption spectrum (λ = 300–800 nm) and the transient kinetics at a single wavelength. The system is reported in detail elsewhere by Oldham and Phillips (2000), Tedesco et al. (2003). Briefly, the system uses the third harmonic (355 nm) of a Nd-YAG laser, Continuum, model SURELITE I-10. The pulse length was 8 ns, and the beam diameter of the incident spot on the sample was 6 mm, with a repetition rate of 10 Hz. The pulse energy was typically 50 mJ per pulse as measured with a Field Master power meter with a L-30V head. A 400 W tungsten–halogen lamp was used as the probe light. The lamp beam is collimated (1 mm in diameter) through the sample in the cell holder, which is held in a cuvette 1 cm in size. Growth–decay kinetic was measured at a single wavelength using a monochromator (M300 from Bentham) and a photomultiplier (Hamamatsu, model R928P). Transient decays were averaged using a Tetronix TDS 340A digital oscilloscope. The stored digitized kinetic decays were transferred to a personal computer for analysis in software supplied by Edinburgh Instruments.

2.2.7. Physical chemistry characterization

2.2.7.1. Laser light scattering (LLS). LLS studies were performed with a Zetasizer 3000-DTS 5300, Malvern Instruments, with angle scattering 90° and particle size measurements of 2 nm to 3 μm. For each experiment three sequences of 10 measurements each were performed. LTCM were analyzed in the presence and in the absence of the ZnPc photosensitizer.

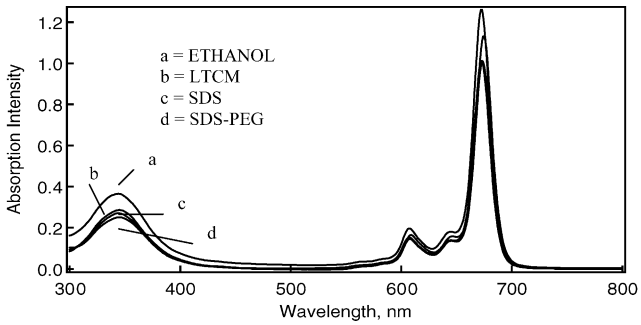

Fig. 3. Absorption spectra of ZnPc (5 μM) in SDS (a); SDS-PEG-5000 (b); LTCM (c); and in ethanol (d).

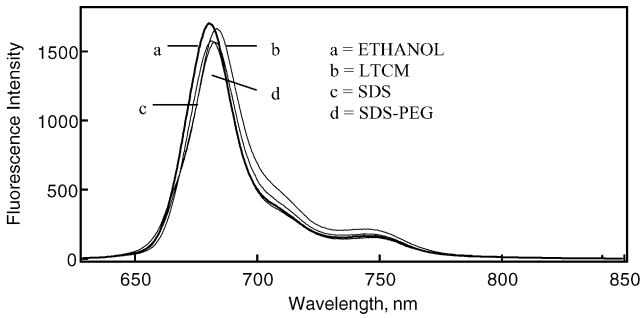

Fig. 4. Fluorescence emission spectra of ZnPc (5 μM) in SDS (a); SDS-PEG-5000 (b); LTCM (c); and in ethanol (d).

3. Results and discussions

3.1. Absorption and fluorescence spectroscopy

The absorption spectra of ZnPc (5 μM) in the micellar medias of SDS, SDS-PEG-5000, LTCM and in ethanol are illustrated in Fig. 3. In all the medias, ZnPc exhibits a strong absorbance in the red region, with a maximum wavelength at 674 nm (670 nm in ethanol). Furthermore, it presents a group of Q bands in the region from 600 to 710 nm and a Soret band at 340 nm. The Q bands represent the vibration levels referring to first and second excited electronically states. The Soret band is a group of ten electronic transitions with different spins and dipole moments sizes (Eichwurzel et al., 2000).

In all the studied systems (LTCM, SDS-PEG-5000 and SDS micelles) ZnPc was not dimer aggregation states form. This can be observed clearly in Fig. 3 behind of spectral profile Q and Soret bands (monomeric aggregation) in the micellar medias. The red shifts observed are in agreement with the process drug of incorporation in the micellar media. This behavior as frequently observed and previously described (Viswanathan and Natarajan, 1996; Ringsdorf et al., 1993; Balasubramaniam and Natarajan, 1997) for many porphyrins and phthalocyanines dyes incorporated in microheterogeneous medias.

Thus, if ZnPc had dimer aggregation states form it could be observed changes drug spectral profile, which this cannot be observed.

Based on Nunes et al. (2004), and other work developed in the group with ZnPc in different drug delivery systems, we believed that aggregation process is not present in the range of concentration used in the present work. The monomeric state of ZnPc in organic media (all spectroscopy analysis) clearly indicates that our results are in agreement with the dye behavior. ZnPc is one of the highest hydrophobic phthalocyanine derivatives with critical spectroscopic changes when aggregation process takes place.

One of the best manners to investigate the behavior of microheterogeneous or biological systems consists of the study of their photophysical properties using fluorescent compounds as probes. The fluorescence emission of the photosensitizer is sensitive in its environment yielding much information (Cuccovia et al., 1982).

The fluorescence emission spectra shown in Fig. 4 indicate the positions of the maximum emission at 682, 682 and 680 nm of the classical ZnPc emission maximum, after light excitation at 610 nm in SDS, SDS-PEG-5000 and LTCM, respectively. In ethanol, ZnPc emission maximum was at 685 nm.

Fluorescence band shifts for red in the studied medias can be related with the connection site of the micellar media and the organic media (ethanol). Some research related with photosensitizers shifts attached the albumin had been studied (Morgan et al., 1980; Lamola et al., 1981; Reddi et al., 1981; Genov et al., 1983).

According to Fig. 4, the incorporation of ZnPc in ethanol, SDS, SDS-PEG-5000 and LTCM resulted in a blue shift of the fluorescence emission maximum position. The fluorescence emission maximum position was shifted from 685 to 682, 682 and 680 nm, respectively. Of the same way, bathochromic shifts in the absorption and in the fluorescence emission maximum were going observed for monomerization of ZnPc and/or solubilization of the drug in an apolar environment (Kessel, 1982). This blue shift of the fluorescence maximum position indicates the incorporation of the drug into hydrophobic microenvironments.

Otherside, this can indicate that ZnPc molecules are located in less hydrophobic environment than the organic (ethanol), and therefore can perceive the water presence in the micellar environment and however are locating near to surfactant–polymer interface (SDS-PEG-5000), and near lipid–polymer interface (LTCM). Nevertheless, it occurs a red shift for the band located in the region of 750 nm due homogeneous environment (ethanol) changes for the micellar environments of SDS, SDS-PEG-5000 and LTCM.

Besides, this red shift of the fluorescence maximum position indicates the incorporation of the drug into hydrophobic microenvironments.

3.2. Measurements of time-resolved fluorescence

Fluorescence lifetime measurements were carried out using a time-resolved spectrofluorimeter FL900CD (Edinburgh

Instruments) with a H_2 lamp operating at 40 KHz frequencies. All measurement was done collecting around 3000 counts. Fluorescence lifetimes were estimated by fitting the decays data analyzed by an interactive deconvolution procedure based on Marquardt-algorithm using software supplied by Edinburgh. The reduced chi-square test (χ^2) and the deviation function criteria were used to test the quality of the fit during the analysis procedure. Also the global analysis software exhibits the number of population present. In organic phase ZnPc presents monoexponential decay (Table 1), clearly indicating one population of the drug. In microheterogeneous media, studies for ZnPc and many other photosensitizers, in micelles and liposomal media present biexponential decay, clearly indicating two populations. The results of fluorescence quenching for ZnPc in organic and liposomal media (Nunes et al., 2004) prove the presence of two different populations of ZnPc, located in the inner and outer position in a double bilayer of liposome. Considering that in both micellar medias studied, conventional micelle and LTCM presented the same hydrophobic region as liposome system, we believe that the ZnPc in these medias are located in two different positions in these heterogeneous systems, as observed for photosensitizers dyes in liposomes. The second life time observed in micellar media (Table 1) longer (around 19.9 ns) is the some order of magnitude as found to liposomes, belong to the population located in the inner region of the micelles alkyl chain. Other hydrophobic probes as pyrene and other photosensitizers dyes also present the same biexponential decay adjustment with two different populations lifetimes.

Singlet excited state lifetime was determined through the decay curves profiles of ZnPc in the micelar medium and in ethanol according to Fig. 5A and B.

In LTCM, smaller lifetimes were observed (τ_1 = 3.9 ns and τ_2 = 15.5 ns, with population percentages of 91 and 9%, respectively) (Table 1), probably related to ZnPc location near a polar media, since hydrophobic probes located next to polar regions emit shorter fluorescence lifetimes than those located in apolar regions (Siemiarczuk and Ware, 1990). ZnPc molecules are hydrophobics and therefore they locate in apolar environments. Previous studies in liposomal media (Nunes et al., 2004) clearly indicate that the hydrophobic ZnPc in microheterogeneous media present two different population, one locate in the inner double bilayer phase (more apolar) and other in the double layer region, close to the polar surface. The position near to the surface presents more polar behavior leading to the reduction of the fluorescence lifetime. Thus, the localization of ZnPc in region with higher polarity leads to the reduction of singlet excited state lifetimes (τ_s), as observed by Nunes et al. (2004). In LTCM, the presence PEG chain attach to the micelle increase of the polarity, of the media leading to two different populations of the dye in the excited state (two lifetimes).

Table 1
Lifetimes of the ZnPc and population distribution (%) in the micelar medias and in ethanol

Media	τ_1 (ns)	τ_2 (ns)	τ_1 (%)	τ_2 (%)
SDS	4.3 ± 0.1	19.9 ± 0.1	94	6
SDS-PEG-5000	4.1 ± 0.1	19.6 ± 0.1	93	7
LTCM	3.9 ± 0.1	15.5 ± 0.1	91	9
Ethanol	4.1 ± 0.1	–	100	–

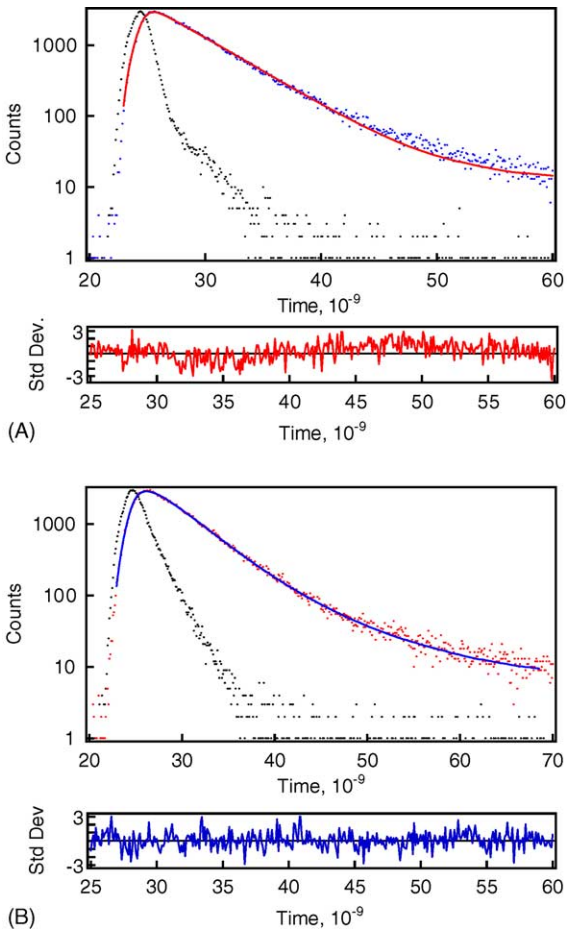

Fig. 5. Fluorescence decay curve of ZnPc (5 μM) in (A) ethanol; (B) LTCM.

ZnPc in ethanol has a best fit to monoexponential function with a decay time of 4.1 ns. However, the biexponential decay observed in the medias studied can be related to the heterogeneity of the micelar system (Valduga et al., 1988).

3.3. Determination of the fluorescence quantum yield

The relationship between emitted and absorbed photons results in the fluorescence quantum yield (ϕ_F), by which values near unity indicate that the fluorophores are more efficient as fluorescence probes for diagnostic use than as photosensitizers for PDT.

In the micellar medias of SDS, SDS-PEG-5000, LTCM, and in ethanol were obtained fluorescence quantum yields (ϕ_F) values about 0.15, 0.14, 0.23 and 0.28, respectively. It can be observed that in LTCM the ϕ_F (0.23) increased in relation to others micelar medias. This value found for ZnPc in LTCM showed a small increase in relation to the others medias studied. However this value is still low enough to

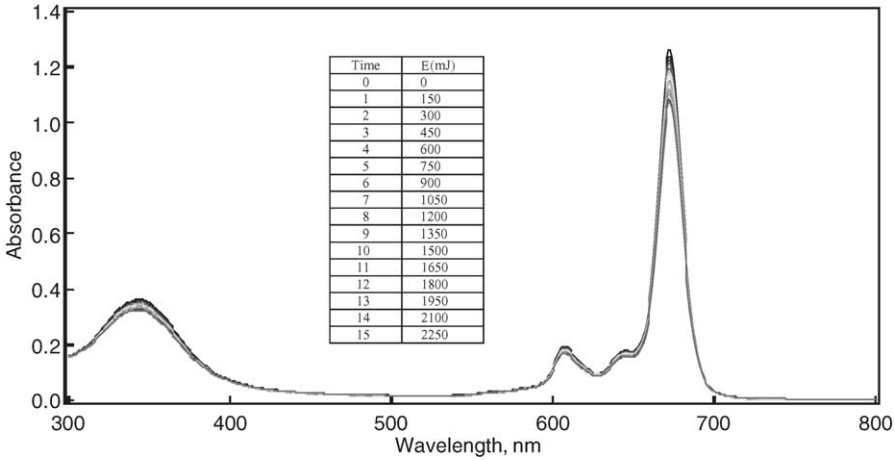

Fig. 6. Photobleaching spectrum of the ZnPc in LTCM with insertion of the quantity of energy (*E*) added to the system.

allow this class of phthalocyanine to be used as photodiagnostic and photosensitizer in neoplastic diseases. This data could be directly related to the photodynamic efficiency of the photosensitizer (Foote, 1991).

3.4. Determination of the photobleaching quantum yield

Photobleaching quantum yield of ZnPc in LTCM was calculated as ±0.055. This small value indicates a better stability of the photosensitizer in comparison to the photobleaching process. Several publications have reported that lower values of the photobleaching quantum yield are associated with a lower aggregation of the dye (Aveline et al., 1998; Fu et al., 2002) This behavior leads to a better photodynamic action since aggregation reduces the photophysical activity of the molecules. Fig. 6 shows the photobleaching spectrum of ZnPc in LTCM.

The determination of the photobleaching quantum yield (ϕ_{PB}) provide information of the energy maximum that can be added to system, avoiding photodegradation of the dye, reducing your photodynamic action. The photobleaching quantum yield (ϕ_{PB}) was determined only for LTCM, where a very small value of this constant (0.055) was obtained, indicating that in this system the photosensitizer shows a better stability after receiving high doses of energy during photoactivation. Photobleaching is a term used to indicate that the optical density of the photosensitizer utilized for photosensitization activation decreases during exposure by light. The lower value found for the photobleaching also allowed us to evaluate the degree of aggregation of the dye in the DDS used. ZnPc in LTCM seems to be less aggregated than in other DDS (such as liposomes), where a lower (ϕ_{PB}) was found due to secondary aggregation (Aveline et al., 1998).

3.5. Laser flash photolysis

3.5.1. Analysis of the ZnPc triplet excited states

Triplet excited state decay of ZnPc was calculated at the maximum absorption wavelength (480 nm) in function of time (Fig. 7). The decay profiles obtained in micelar medias presented biexponential decay kinetic, using an adjusted computer program.

Transient spectra of ZnPc in the micelar systems (Fig. 7) presented one transient specie with two different lifetimes in the order of μs. Triplet excited state of ZnPc presented absorption in the wavelength range from 380 to 650 nm, with

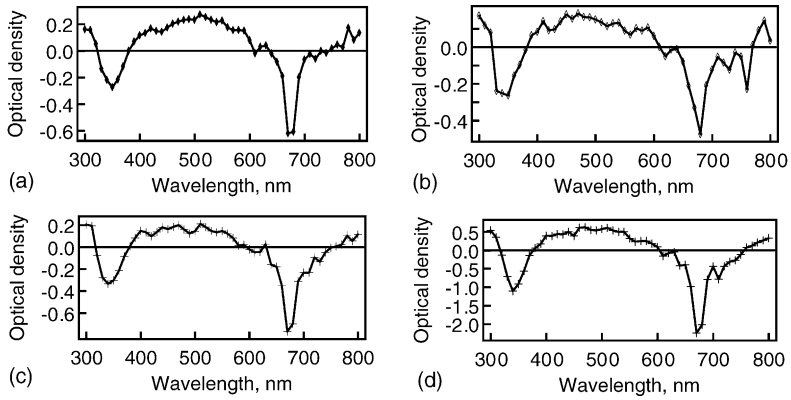

Fig. 7. Transient absorption spectra of ZnPc obtained by laser pulse photolysis in SDS (a); SDS-PEG-5000 (b); LTCM (c); and in ethanol.

maximum transient absorption at 480 nm. Beyond the triplet absorption, in the excitation process the ground state photobleaching of the Q and Soret bands of the ZnPc can be observed.

ZnPc in ethanol has a best fit to monoexponential function with lifetime of 0.17 μs (100%). However, it forms simple specie with one lifetime.

Lifetime values of ZnPc in LTCM were τ_{T1} = 4.5 μs (87.9%) and τ_{T2} = 2.2 μs (12.1%). The presence of two different lifetimes indicates the distribution of the photosensitizer in distinct populations with different locations in LTCM. The main population (about 88%) presented nearly a double triplet lifetime. This is considered to be an advantage in relation to monomeric state in ethanol, because two populations in two different locations in the LTCM enable the transfer of energy to the molecular oxygen present in the media leading to an efficient production of singlet oxygen. The decay curves adjustments were performed according to the chi-square function and in all the cases these values were 1.0 ± 0.2, which are entirely acceptable.

Photodynamic efficiency of the photosensitizer is critically dependent on the quantum yield and the triplet excited state lifetime (Foote, 1991). Therefore, the bigger the triplet excited state lifetime, the better is the probability that drug energy can be transferred to molecular oxygen and consequently to produce singlet oxygen.

3.6. Physical chemistry characterization

3.6.1. Laser light scattering (LLS)

LLS analysis determined the average diameter values of the LTCM in the presence and in the absence of ZnPc, which were 163 and 40 nm, respectively (Fig. 8). The size distribution of the LTCM is quite narrow, with a monomodal profile and uniform distribution of the particles. The small dimensions in the nanometer range confirm that this system is appropriate for drugs delivery in PDT.

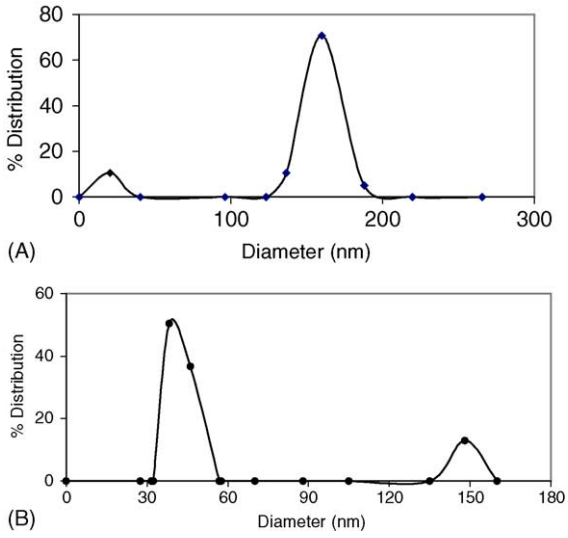

Fig. 8. Size distribution of LTCM in the presence (A) and the absence (B) of ZnPc.

The physical chemistry characterization of the LTCM was made by LLS (Fig. 8). The size distributions are monomodals. The average diameter obtained for the LTCM in the presence of the ZnPc, was larger than in its absence (163 and 40 nm, respectively). In Fig. 8A, in the first observed peak, ZnPc was not completely incorporated in LTCM and in Fig. 8B, the observed peak in 150 nm can correspond the some structures formed through aggregation process of the micelles. However, after the incorporation of ZnPc in LTCM, the average diameter obtained was 160 nm. These values confirm the incorporation of the drug in this system.

Since ZnPc maintains most of its photophysical and photodynamic properties unaltered, the results presented here clearly indicate a good potentiality for the utilization of ZnPc/LTCM in PDT.

4. Conclusions

Following incorporation of ZnPc into either one of the three systems studied (SDS, SDS-PEG-5000 and LTCM) no significant spectroscopic changes of the photosensitizer properties are observed. ZnPc loaded in LTCM presented suitable conditions for its utilization in PDT and photodiagnostic procedures, including an enhanced fluorescence quantum yield, and lifetimes of the triplet excited state lasting longer than those obtained in conventional micelles and in ethanol solution (monomeric state). Furthermore, this system provides a better stability of the incorporated drug, has a narrower size distribution pattern of its particles and a lower photobleaching quantum yield.

All these results suggest that LTCM may be promise system to vehicle ZnPc for PDT use. Understanding the physical chemistry properties of ZnPc loaded LTCM is the first step for a future study of their biodistribution in cells neoplasic.

A study in vitro for ZnPc quantification in aqueous media will be developed briefly.

Acknowledgements

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References

Ali, H., Van Lier, J.E., 1999. Metal complexes as photo- and radiosensitizers. Chem. Rev. 99, 2379–2450.

Aveline, B.M., Sattler, R.M., Redmond, R.W., 1998. Environmental effects on cellular photosensitization: correlation of phototoxicity mechanism with transient absorption spectroscopy measurements. Photochem. Photobiol. 7, 51–62.

Balasubramaniam, E., Natarajan, P., 1997. Photophysical properties of protoporphyrin IX and thionine covalently attached to macromolecules. J. Photochem. Photobiol. A: Chem. 103, 201–211.

Ben Hur, E., Rosenthal, I., 1985. The phthalocyanines: a new class of mammalian cell photosensitizers with a potential for cancer phototherapy. Int. J. Radiat. Biol. 47, 145–147.

Bonnett, R., 2000. Chemical Aspects of Photodynamic Therapy. Gordon & Breach, Amsterdam.

Demas, J.N., Crosby, G.A., 1971. Measurement of photoluminescence quantum yields. J. Phys. Chem. 75, 991.

Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbelik, M., Moan, J., Peng, Q., 1998. Photodynamic therapy. J. Natl. Cancer Inst. 12, 889–905.

Duncan, R., 2001. Polymer therapeutics. Drug. Deliv. 1, 178–184.

Eaton, D.F., 1988. International union of pure and applied chemistry organic Chemistry division commission on photochemistry. Reference materials for fluorescence measurements. J. Photochem. Photobiol. B 4, 523–531.

Foote, C.S., 1991. Definition of type I and II photosensitized oxidation. Photochem. Photobiol. 54, 659.

Fu, J., Li, X., Ng, D.K.P., Wu, C., 2002. Encapsulation of phthalocyanines in biodegradable poly(sebacic anhydride) nanoparticles. Langmuir A–E.

Gantchev, T.G., Sharman, W.M., Van Lier, J.E., 2003. Metallophthalocyanines photosensitize the breakdown of (hydro)peroxides in solution to yield hydroxyl/lkoxyl and peroxyl free radicals via different interaction pathways. Photochem. Photobiol. 77 (5), 1–46.

Genov, N.C., Shopova, M., Boteva, R., Ricchelli, F., Jori, G., 1983. Intramolecular distances between tryptophan residues and the active-site serine residue in alkaline bacterial proteinases as measured by fluorescence energy-transfer studies. Biochem. J. 215 (2), 413–416.

Kataoka, K., Kwon, G.S., Yokoyama, M., Okano, T., Sakurai, Y., 1993. Block copolymer micelles as vehicles for drug delivery. J. Contr. Rel. 24, 119–132.

Kessel, D., 1982. Determinants of hematoporphyrin-catalyzed photosensitization. Photochem. Photobiol. 36, 99–101.

Kwon, G.S., Kataoka, K., 1995. Block copolymer micelles as long circulating drug vehicles. Adv. Drug. Deliv. Rev. 16, 295–309.

Lamola, A.A., Asher, I., Muller-Eberhard, U., Poh-Fitzpatrick, M., 1981. Fluorimetric study of the binding of protoporphyrin to haemopexin and albumin. Biochem. J. 196, 693–698.

Liu, L., Chenxi, L., Xuechen, L., Zhi, Y., Yingli, A., He, Binglin, 2001. Biodegradable polylactide/poly(ethylene glycol)/polylactide triblock copolymer micelles as anticancer drug carriers. J. Appl. Pol. Sci. 80, 1976–1982.

Madan, P.L., 1985. Sustained release drug delivery systems. Pharm. Manuf. 1, 23–27.

Morgan, W.T., Smith, A., Koskelo, P., 1980. The interaction of human serum albumin. Biochim. Biophys. Acta 24, 271–285.

Nunes, S.M.T., Sguilla, F.S., Tedesco, A.C., 2004. Photophysical studies of zinc phthalocyanine and chloroaluminium phthalocyanine incorporated into liposomes in the presence of additives. Braz. J. Med. Biol. Res., in press.

Oldham, T.C., Phillips, D., 2000. Triplet-state photophysics of aluminium phthalocyanine sensitizer in murine cancer cells. J. Photochem. Photobiol. B 55, 16–19.

Reddi, E., Ricchelli, F., Jori, G., 1981. Interaction of human serum albumin with hematoporphyrin and its Zn^{2+} and Fe^{3+} derivatives. Int. J. Pept. Prot. Res. 18, 402–408.

Reddi, E., Zhou, C., Biolo, R., Menegaldo, E., Jori, G., 1990. Liposome- or LDL-administered Zn(II) phthalocyanine as a photodynamic agent for tumors. I. Pharmacokinetic properties and phototherapeutic efficiency. Braz. J. Cancer 61, 407–411.

Ringsdorf, H., Sackmann, E., Simon, J., Winnik, F.M., 1993. Interactions of liposomes and hydrophobically-modified poly-(*N*-isopropylacrylamides): an attempt to model the cytoskeleton. Biochim. Biophys. Acta 1153, 335–344.

Sharman, W.M., Allen, C.M., Van Lier, J.E., 1999. Photodynamic therapeutics: basic principles and clinical applications. Drug Discov. Today 4, 507–517.

Siemiarczuk, A., Ware, W.R., 1990. Pyrene lifetime broadening in SDS micelles. Chem. Phys. Lett. 167, 263–268.

Spikes, J.D., Van Lier, J.E., Bommer, J.C., 1985. A comparison of the photoproperties of zinc phthalocyanine and zinc naphthalocyanine tetrasulfonates: model sensitizers for the photodynamic therapy of tumors. J. Photochem. Photobiol. 91, 193–198.

Tedesco, A.C., Rotta, J.C.G., Lunardi, C.N., 2003. Nitric oxide release from the *S*-nitrosothiol zinc phthalocyanine complex by flash photolysis. Braz. J. Med. Biol. Res. 36, 587–594.

Torchilin, V.P., 1999. Polymeric micelles in diagnostic imaging. Coll. Surf. B: Biointerf. 16, 305–319.

Torchilin, V.P., 2001. Structure and design of polymeric surfactant-based drug delivery systems. J. Contr. Rel. 73, 137–172.

Valduga, G., Nonell, S., Reddi, E., Jori, G., Braslavsky, S.E., 1988. The production of singlet molecular oxygen by zinc(II) phthalocyanine in ethanol and in unilamellar vesicles. Chemical quenching and phosphorescence studies. Photochem. Photobiol. 18, 1–5.

Viswanathan, K., Natarajan, P., 1996. Photophysical properties of thionine and phenosafranine dyes covalently bound to macromolecules. J. Photochem. Photobiol. A: Chem. 95, 245–253.

Weissig, V., Whiteman, K.R., Torchilin, V.P., 1998. Accumulation of protein-loaded long-circulating micelles and liposomes in subcutaneous Lewis lung carcinoma in mice. Pharm. Res. 15, 1552–1556.