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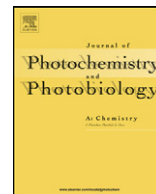
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## An exhaustive study of a novel sulfur-linked adamantane tetrasubstituted zinc(II) phthalocyanine incorporated into liposomes

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### ABSTRACT

The lipophilic tetrasubstituted sensitizer 2,9(10),16(17),23(24)-tetrakis-(1-adamantylsulfanyl)phthalocyaninatozinc(II) (ZnPc) was incorporated into different liposomal formulations in order to evaluate its photophysical properties, as well as the size, incorporation efficiency, thermotropic properties and stability of liposomes during a time period. Liposomes were prepared by the thin-layer evaporation technique followed by hydration with buffer at pH 7.4 and 0.15 M NaCl. Small unilamellar vesicles (SUV) were obtained after sonicating the mixture. Phthalocyanine was incorporated efficiently into all the liposomal formulations. The shape of ZnPc spectra depended on the environment. The S-PEG liposomal formulation showed the lowest aggregation value. The average diameter of liposomes with different lipid compositions varied from  $49.70 \pm 0.09$  to  $223.10 \pm 0.36$  nm, with significant differences between the various liposomal preparations. The inclusion of cholesterol (CHOL) and polyethylene glycol (PEG) on the lipid bilayer did not influence the size of the particles. The formulations with PEG increased the stability of liposomes for at least 9 months. After a long storage, the most stable formulations were those without CHOL. Singlet molecular oxygen values ranged between 0.10 and 0.26. These values were higher than other zinc(II) phthalocyanines incorporated into dimyristoyl phosphatidylcholine (DMPC) liposomes or Tween 80.

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### 1. Introduction

Phthalocyanines have been found to have applications as phototoxic drugs for photodynamic therapy (PDT) [1–6]. Most phthalocyanines are hydrophobic and in many cases, the selectivity of tumor targeting can be enhanced by association of the sensitizer with suitable delivery systems before systemic injection [7–9]. Thus, the design of optimal delivery systems for the administration of phthalocyanines has become an important aim in PDT.

Liposomes with various lipid compositions are widely used as delivery systems for water-insoluble compounds [10–12]. Drugs in liposomal formulations are known to be taken up very efficiently by the macrophages of components of the reticuloendothelial system. This feature can be changed by the incorporation of polyethylene glycol in liposomal formulations, since liposomes with the hydrophilic cover have a longer circulation time in the blood stream [13].

Cancer cells exhibit high levels of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) receptors [14,15], which

present a highly competitive mechanism to uptake LDL, thus leading to high rates of cholesterol (CHOL) incorporation. These features can be used to formulate an efficient drug delivery system [12].

Based on these results, studies have been initiated to investigate the incorporation of the lipophilic 2,9(10),16(17),23(24)-tetrakis-(1-adamantylsulfanyl)phthalocyaninatozinc(II) (ZnPc) into different formulations of liposomes to evaluate not only its photophysical properties, but also the size and stability of liposomes during a time period.

### 2. Materials and methods

#### 2.1. Materials

2,9(10),16(17),23(24)-Tetrakis-(1-adamantylsulfanyl)phthalocyaninatozinc(II) (ZnPc) [16] (Fig. 1) and tetra-*t*-butyl phthalocyaninatozinc(II) [17] were synthesized in our laboratory. L- $\alpha$ -Phosphatidylethanolamine from egg yolk Type III (PEEY); L- $\alpha$ -phosphatidylcholine from egg yolk, Type XVI-E (PCEY); 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE); L- $\alpha$ -phosphatidylethanol-amine distearoyl methoxypolyethylene glycol conjugate (DSPE-PEG); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); Cholesterol BioReagent, suitable for cell

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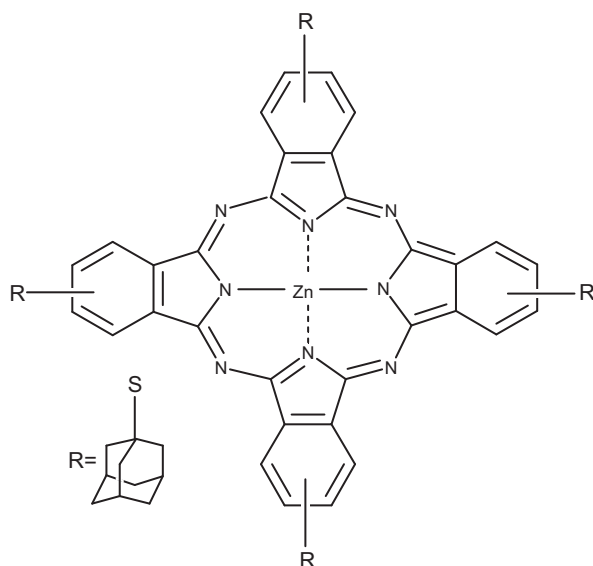


Fig. 1. Chemical structure of phthalocyanine (ZnPc).

culture (CHOL); and HEPES BioUltra, for molecular biology, were purchased from Sigma–Aldrich, Germany (Schnelldorf, Germany). Sodium chloride was obtained from Mallinckrodt (Phillipsburg, NJ, USA). Imidazole BioUltra and Methylene Blue Hydrate (MB) were from Fluka (Sigma–Aldrich, India); N,N-diethyl-4-nitrosoaniline 97%, and tetrahydrofuran (THF) spectrophotometric grade were from Sigma–Aldrich (Steinheim, Germany). Diethyl ether was from Carlo Erba (Rodano, Italy), Chloroform from Merk–Química Argentina (Buenos Aires, Argentina) and polyethylene glycol 400 (PEG 400) from Parafarm, Droguería Saporiti S.A.C.I.F.I.A. (Buenos Aires, Argentina). All chemicals were of reagent grade and used without further purification. Distilled water treated in a Milli-Q system (Millipore) was used.

## 2.2. Instrumentation

Electronic absorption spectra were determined with a Shimadzu UV-3101 PC spectrophotometer and fluorescence spectra were monitored with a QuantaMaster Model QM-1 PTI spectrofluorometer. pH was measured with a Thermo pH meter Altronix TPX-1. Static light scattering (SLS) experiments were carried out using an SLS 90 Plus/BI-MAS (MultiAngle Particle Sizing Option) equipped with a He–Ne laser operating at 632.8 nm and 15 mW. Transmission electron microscopy (TEM) liposome images were obtained by means of an EM 301 Phillips operating at 65 kV.

Differential scanning calorimetry was carried out using a Shimadzu DSC-50. Vortex-mixing was performed by means of a VELP Scientific ZX Classic. The sonicator used was MSE Soniprep 150.

**Table 1**  
Liposome solvent preparation, composition and incorporation efficiency (IE).

Liposome type	Lipid composition	Molar relation	Stabilizer	Solvent (v/v)	ZnPc/liposome (μmol/mg)	<sup>a</sup> IE (%)
D1	DPPC:CHOL	24:1	–	Diethyl ether/chloroform (3:1)	0.0739	76.29 ± 0.98
D2	DPPC	1	–	Diethyl ether	0.0765	75.48 ± 1.76
D1-PEG 400	DPPC:CHOL	24:1	PEG 400 (10%)	Diethyl ether/chloroform (3:1)	0.0739	76.29 ± 0.98
D2-PEG 400	DPPC	1	PEG 400 (10%)	Diethyl ether	0.0765	75.48 ± 1.76
M1	PCEY:PEEY:CHOL	16:16:1	–	Diethyl ether/chloroform (3:1)	0.0496	69.34 ± 7.25
M2	PCEY:PEEY	1:1	–	Diethyl ether	0.0494	72.02 ± 2.33
S	DPPC:PCEY:PEEY:DSPE:CHOL	1:8:7:1:3	–	Diethyl ether/chloroform (3:1)	0.0467	66.37 ± 7.40
S-PEG	DPPC:PCEY:PEEY:DSPE-PEG:CHOL	1:8:7:1:3	–	Diethyl ether/chloroform (3:1)	0.0467	60.26 ± 0.03

<sup>a</sup> Data represent the mean value standard error of four independent experiments.

## 2.3. Preparation of unilamellar liposomes

Large unilamellar vesicles (LUV) and SUV were prepared as follows: an aliquot of lipid organic solvent solution (Table 1) was evaporated at 25 °C to form a thin film of lipid and then hydrated with 10 mM HEPES (pH 7.4) and 0.15 M sodium chloride. All excess of organic solvent was removed during evaporation. This was followed by 15 min vortex-mixing at 25 °C to obtain large multilamellar vesicles (LMV), after sonicating the mixture using a probe-style sonicator for 10 min in an ice bath to obtain a translucent solution.

After adding ZnPc stock solution, samples were incubated for at least 20 min to allow dye incorporation into the liposomes. For 1,2-dipalmitoyl-sn-glycero-3-phosphocholine:cholesterol, 24:1 (D1) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (D2), the above step was followed by the incorporation of 10% PEG 400 solution to stabilize the liposomal formulations [18].

## 2.4. Sample preparation. Determination of incorporation efficiency (IE)

Stock solutions of ZnPc were prepared in THF, kept at 4 °C, and carefully protected from ambient light. The dye and carrier concentrations are indicated in each experiment.

Liposomes with ZnPc incorporated were disrupted using Triton-X 100 to fully release the dye incorporated into the liposomes, and the absorbance of samples measured after leaving them at room temperature for 24 h. The absorbance of ZnPc at  $\lambda_{max}$  indicated in Table 2 for each sample was measured to determine the concentration of ZnPc incorporated. The IE was calculated by Eq. (1).

$$IE = \left[ \frac{\text{incorporated ZnPc}}{\text{initial ZnPc loaded}} \right] \times 100 \quad (1)$$

## 2.5. Static light scattering (SLS)

SLS was then used to determine the average size of liposomes. Scattering was detected at an angle of 90°. All measurements were made at 25 °C, using different concentrations of liposomes in HEPES to obtain a better reproducible unimodal population. Experiments were carried out without photosensitizer to prevent fluorescence interference in the SLS signals.

## 2.6. Differential scanning calorimetry

Differential scanning calorimetry, from –60 °C to 80 °C at a 10 °C/min rate, except for liposomes type S and S-PEG, for which the temperature ranged from –60 °C to 120 °C, was used to determine the temperature of phase transitions ( $T_m$ ) and the associated change of enthalpy ( $\Delta H$ ) of liposomes.

**Table 2**  
Photophysical parameters of ZnPc in homogeneous media and inside liposomes.

Photophysical parameters	THF	D1	D2	M1	M2	S	S-PEG
<sup>b</sup> $\Phi_{\Delta}$	0.66 ± 0.10	0.20 ± 0.03	0.17 ± 0.03	0.11 ± 0.02	0.20 ± 0.04	0.26 ± 0.07	0.10 ± 0.03
<sup>b</sup> $\Phi_F$	0.310 ± 0.040	0.040 ± 0.006	0.060 ± 0.01	0.010 ± 0.002	0.010 ± 0.002	0.030 ± 0.008	0.003 ± 0.001
$\lambda_{\text{max absorb}}$ (nm)	680	684–653 <sup>a</sup>	683–653 <sup>a</sup>	683–652 <sup>a</sup>	683–652 <sup>a</sup>	686–652 <sup>a</sup>	688–652 <sup>a</sup>
$\lambda_{\text{max emission}}$ (nm)	687	685	687	685	685	684	688

<sup>a</sup> Absorption corresponding to monomer and oligomer species.

<sup>b</sup> Data represent the mean value ± standard error of three independent experiments.

## 2.7. Photophysical properties

### 2.7.1. Spectroscopic studies

Absorption and emission spectra were recorded with a 10 mm × 10 mm quartz cuvette with a 500 µl capacity at room temperature.

Emission spectra of ZnPc were recorded at an excitation wavelength ( $\lambda_{\text{exc}}$ ) of 610 nm (Q-band) between 630 and 800 nm; a cut-off filter was used to prevent the excitation beam from reaching the detector (Schott RG 630).

Emission and absorption spectra of liposomal phthalocyanines were corrected for light scattering by subtracting the spectra from empty liposomes.

Spectroscopic experiments were carried out at ZnPc concentrations ranging between  $1.2 \times 10^{-7}$  M and  $1.5 \times 10^{-6}$  M.

### 2.7.2. Fluorescence quantum yields

Fluorescence quantum yields ( $\Phi_F$ ) were determined by comparing with those of tetra-*t*-butyl phthalocyaninatozinc(II) ( $\Phi_F = 0.30$  in toluene) as a reference at  $\lambda_{\text{exc}}$  610 nm and calculated as described elsewhere [17].

### 2.7.3. Quantum yield of singlet oxygen production

The quantum yield of singlet oxygen generation rates ( $\Phi_{\Delta}$ ) was determined using standard chemical monitor bleaching rates [19].

Imidazol (8 mM) and *N,N*-diethyl-4-nitrosoaniline (40–50 µM) in HEPES was used for liposomal ZnPc [20]. *N,N*-diethyl-4-nitrosoaniline decay was monitored at 440 nm.

A projector lamp (Philips 7748SEHJ, 24V–250 W) and a cut-off filter at 610 nm (Schott, RG 610) were used to generate polychromatic irradiation and a water filter to prevent infrared radiation. The liposomal samples of ZnPc and the reference (MB:  $\Phi_{\Delta} = 0.56$  in buffer) [19] were irradiated within the same wavelength interval  $\lambda_1 - \lambda_2$ , and  $\Phi_{\Delta}$  was calculated according to Amore et al. [21].

### 2.7.4. Aggregation studies of ZnPc in liposomal formulations

The intensity absorption ratio of the two bands corresponding to the monomer and oligomers was calculated. The higher values of the ratio indicated a disaggregated dye form [22,23]. This ratio was calculated for all liposomal formulations using the  $\lambda_{\text{max}}$  indicated in Table 2. These values were compared with those obtained in THF (7.0), where aggregation was not observed, and with those obtained in phosphate buffered saline (PBS) pH 7.4 and dimethyl sulfoxide (DMSO) 95:5 (1.0). The value obtained in HEPES was 0.9.

## 2.8. Release studies in HEPES

Liposomes were incubated at room temperature and the release of the dye calculated as a function of time using Eq. (2).

$$\text{Release (\%)} = \frac{[\text{Abs}_{\text{day1}} - \text{Abs}_{\text{dayX}}]}{[\text{Abs}_{\text{day1}}]} \times 100 \quad (2)$$

## 3. Results

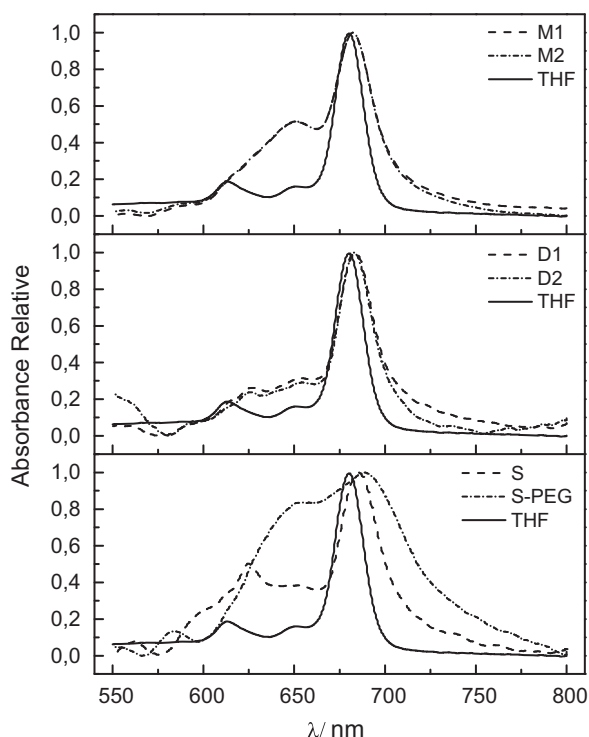
### 3.1. Spectroscopic studies

The spectroscopic characterization of the absorption and emission spectra of free and incorporated phthalocyanine ZnPc was used to establish the incorporation of the dye into liposomal formulations. Fig. 2 shows the incorporation of ZnPc in homogeneous media (THF) and into liposomes. The shape of ZnPc spectra in liposomes was the same as that of other zinc(II) phthalocyanines in organic solvents and liposomes. The spectra in Fig. 2 shows a wider Q-band and a new band at 652 nm, for dimer/oligomer ZnPc (see Tables 1 and 2). The shape of the spectra in M1, M2 and S liposomes presents an intermediate behavior between hydrophilic and lipophilic media [16,23].

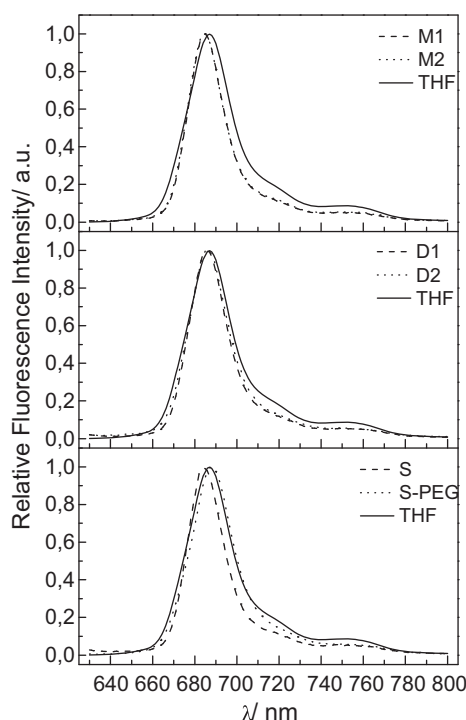
Fig. 3 shows the fluorescence spectra of ZnPc in solution (THF) and in the presence of liposomes. Neither significant wavelength shifts nor relevant modifications in shape were observed. This evidences that only the monomer is the emitting species.

### 3.2. Liposomal formulation: lipid/phthalocyanine ratio

By monitoring the fluorescence after addition of ZnPc, we found that 20 min of incubation was sufficient to achieve incorporation. This equilibration time was maintained after the addition of each amount of dye.



**Fig. 2.** Absorption spectra of ZnPc in a homogeneous medium (THF) and inside liposomes.



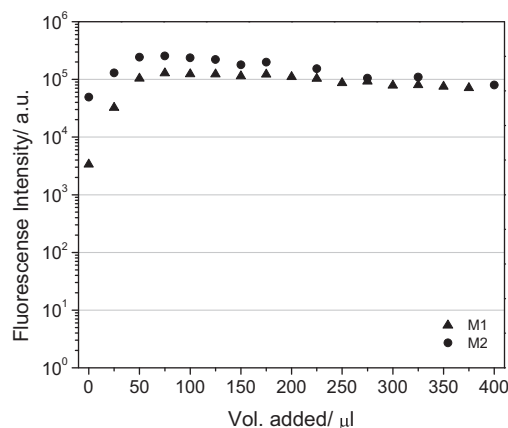
**Fig. 3.** Emission spectra of ZnPc at different liposomal formulations,  $\lambda_{\text{exc}} = 610$  nm.

### 3.2.1. Titration of liposomes with ZnPc

To establish the optimal ZnPc/liposomal ratio concentration, the following procedure was used: measurements were carried out at a fixed lipid concentration and increasing ZnPc concentrations. In these experiments, the concentrations of liposomes were kept constant at 0.723 mg/mL for D1, 0.698 mg/mL for D2, 0.727 mg/mL for M1, 0.703 mg/mL for M2, 0.857 mg/mL for S, and 0.400 mg/mL for S-PEG. [ZnPc] was in the range of  $1.01\text{--}5.70 \times 10^{-5}$  M,  $1.01\text{--}5.70 \times 10^{-5}$  M,  $4.26 \times 10^{-7}\text{--}5.89 \times 10^{-5}$  M,  $4.26 \times 10^{-7}\text{--}5.89 \times 10^{-5}$  M,  $1.01\text{--}6.16 \times 10^{-5}$  M,  $4.17 \times 10^{-6}\text{--}6.51 \times 10^{-5}$  M respectively at 25 °C. The emission experiments showed that the fluorescence intensity increased linearly with ZnPc incorporation, until a non-linear saturation pattern.

### 3.2.2. Titration of ZnPc with liposomes

To determine the concentration of the carrier that induces monomerization, fluorescence spectra were recorded at a fixed amount of ZnPc and different carrier concentrations. In these experiments, the concentration of ZnPc was kept constant at  $6.16 \times 10^{-5}$  M for D1,  $5.70 \times 10^{-5}$  M for D2,  $5.89 \times 10^{-5}$  M for M1,  $5.89 \times 10^{-5}$  M for M2,  $6.16 \times 10^{-5}$  M for S, and  $6.51 \times 10^{-5}$  M



**Fig. 4.** Fluorescence titration plots. Measurements were carried out at a fixed ZnPc concentration. [ZnPc] =  $6.16 \times 10^{-5}$  M and  $6.51 \times 10^{-5}$  M for M1 and M2 respectively. Experiments were carried out within the following ranges: [M1] = 0.703–3.364 mg/mL and [M2] = 0.857–3.429 mg/mL, at 25 °C and  $\lambda_{\text{exc}} = 610$  nm.

for S-PEG. The lipid concentration ranged between 0.723 and 2.764 mg/mL for D1, 0.698 and 2.669 mg/mL for D2, 0.727 and 3.419 mg/mL for M1, 0.703 and 3.364 mg/mL for M2, 0.857 and 3.429 mg/mL for S, and 0.400 and 1.731 mg/mL for S-PEG.

The lipid concentrations used correspond to the maximum incorporation of ZnPc (in the plateau region, obtained as described above) to prepare dye formulations (Fig. 4 and Table 1).

### 3.2.3. Determination of incorporation efficiency (IE)

ZnPc was incorporated into liposomes as described in the Section 2. The IE of the ZnPc incorporated into liposomes, as determined by UV spectroscopy, reached about 70% IE (Table 1).

### 3.2.4. Determination of ZnPc release

At predetermined intervals of 2, 7, 15, and 30 days, the stored samples were analyzed for changes in the percentage of encapsulation. To evaluate the amount of ZnPc released from the liposomes, the concentrations were monitored over 1 month (Fig. 5A and B). A significant increase in the extent of the release was observed after 7 days in all liposomal formulations except in the S-PEG formulation. Further changes in the extent of the release after 7 days showed a non-significant change. The same behavior was observed for the different ZnPc concentrations. On the other hand, the amount of ZnPc released in the S-PEG formulation was lower, probably because of the incorporation of the dye not only into the lipidic bilayer but also into PEG tails.

**Table 3**

Influence of CHOL and PEG on the size distribution of the liposomes, in formulations without ZnPc. Effect of storage (at 4 °C) on the size distribution and polydispersity of the liposome preparations.

Liposome type	After preparation		After 7 days of storage		After 14 days of storage		After 9 months of storage	
	Mean diameter (nm)	Polydispersity	Mean diameter (nm)	Polydispersity	Mean diameter (nm)	Polydispersity	Mean diameter (nm)	Polydispersity
D1	52	0.346	89.7	0.451	150.4	0.588	3735.1	0.206
D2	49.7	0.088	72.1	0.136	80.6	0.476	1794.4	0.134
D1-PEG 400	103	0.613	93.1	0.371	75.9	0.488	443.3	1.125
D2-PEG 400	47.2	0.116	45.5	0.105	51.4	0.082	490.1	0.335
M1	155.5	0.688	255.9	0.601	226.9	0.431	1876.1	0.974
M2	147.7	0.323	160.3	0.369	194	0.380	835.5	0.809
S	223.1	0.355	307.3	0.869	268.8	0.605	653.6	0.573
S-PEG	216.1	1.455	156.6	0.104	341.1	2.463	435.8	0.462



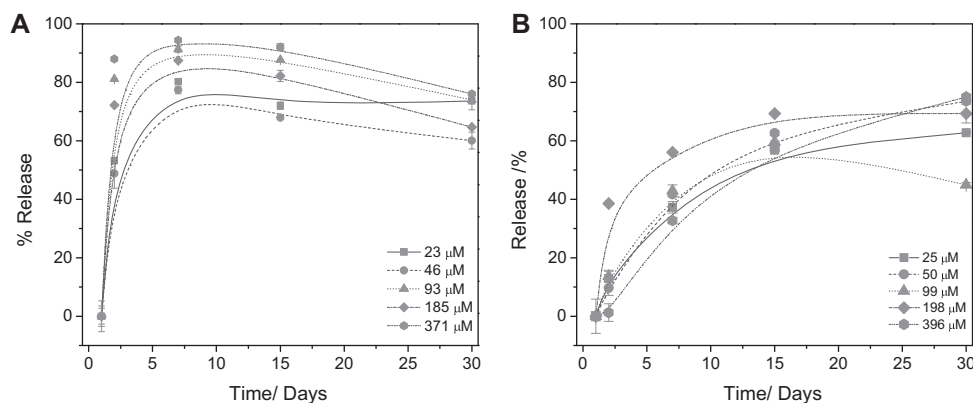


Fig. 5. ZnPc release from M1 and S-PEG liposomes.

### 3.2.5. Aggregation studies of ZnPc in liposomal formulations

The aggregation values observed in liposomes were lower than those observed in PBS-DMSO and HEPES, but higher than those obtained in THF. Except for S-PEG, all liposome formulations showed a similar behavior (Fig. 6A).

Fig. 6B shows the S-PEG intensity absorption ratio values for different concentrations of ZnPc (25 μM, 50 μM, 99 μM, 198 μM, and 396 μM). Except for 198 μM, and 396 μM, all the range of concentration sample aggregation values exhibited a tendency toward those obtained in THF. In all cases, the intensity absorption ratio values were higher than those obtained in HEPES. Furthermore, phthalocyanine molecules might present an equilibrium between PEG tails and the aqueous media.

### 3.3. Fluorescence and singlet molecular oxygen quantum yields

The  $\Phi_F$  and  $\Phi_\Delta$  values in homogeneous and microheterogeneous media are listed in Table 2. The  $\Phi_\Delta$  values were calculated against MB as a reference incorporated into different liposomal formulations at a concentration where only the monomer is present (0.1 μM). In all the experiments, samples were prepared with HEPES pH = 7.4 instead of Milli-Q water. The MB  $\Phi_\Delta$  values in liposomes were calculated using MB as a reference in HEPES.

### 3.4. Size and stability of the liposomes

The well-dispersed liposomes were characterized by SLS and TEM. The average diameter of liposomes with different lipid compositions varied from  $49.70 \pm 0.09$  to  $223.10 \pm 0.36$  nm, with significant differences between the various liposomal formulations.

The polydispersity (i.e., the measurement of size heterogeneity) for the size distribution of liposomal formulations ranged from  $\pm 0.088$  to  $\pm 1.455$ . Fig. 6 shows electron micrographs of LUV ZnPc-SPEG liposomal samples, which appear as unilamellar spherical-shaped vesicles. In full agreement with light scattering size measurements, liposomes presented a uniform size around 100 nm in diameter.

CHOL inclusion into the lipid bilayer did not influence the size of the particles (Table 3). In the presence of PEG, size changes in the liposomes were not significant.

The formulations with PEG increased the stability of liposomes, either in the presence or the absence of CHOL. After a long storage period, the most stable formulations were those without CHOL. For formulations with CHOL, storage showed an increase in the effective mean particle diameter, favoring the formation of vesicles with higher diameters due to fusion of the smaller particles.

### 3.5. Calorimetric measurements

Differential scanning calorimetry was used to determine the effect of the incorporation of ZnPc in the thermotropic behavior of the liposomal bilayers. The thermotropic profiles of the aqueous suspensions of all the liposomal bilayers showed two endothermic processes: one low peak between  $-24$  and  $-18^\circ\text{C}$ , with a maximum at  $-22^\circ\text{C}$ , and a second higher peak, between  $-3$  and  $10^\circ\text{C}$ , corresponding to the fusion of the aqueous medium. The transition temperature of liposomes with ZnPc was not significantly from that of empty liposomes. The thermodynamic parameters corresponding to each thermogram are shown in Table 4. The incorporation of ZnPc into liposomes decreased the phase transition enthalpy. These results indicate that as expected, hydrophobic dyes were

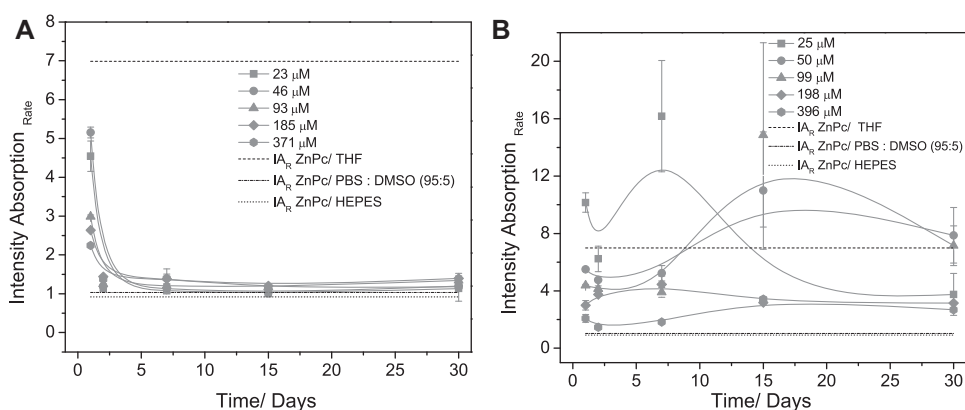


Fig. 6. Intensity absorption rate of M1 and S-PEG liposomes.

**Table 4**

Effect of phthalocyanine liposome incorporation on the thermodynamic parameters of the aqueous dispersions of different bilayer compositions.

Liposome type	$T_m^a$ (°C)	$\Delta H^b$ (J g <sup>-1</sup> )	$\Delta S^c$ (J g <sup>-1</sup> K <sup>-1</sup> )	$\Delta T_{1/2}^d$ (°C)
D1 without ZnPc	2.2	370	1.3	14.6
D1 with ZnPc	-0.2	330	1.2	15.8
D2 without ZnPc	2.0	380	1.4	18.0
D2 with ZnPc	-0.3	330	1.2	15.1
M1 without ZnPc	2.1	370	1.3	14.4
M1 with ZnPc	0.7	330	1.2	13.7
M2 without ZnPc	1.9	380	1.4	14.6
M2 with ZnPc	-0.4	340	1.2	13.3
S without ZnPc	1.7	380	1.4	14.1
S with ZnPc	0.1	340	1.2	14.1
S-PEG without ZnPc	2.3	380	1.4	14.9
S-PEG with ZnPc	0.1	340	1.2	13.9

<sup>a</sup> Temperature corresponding to the maximum calorimetric peak.

<sup>b</sup> Calorimetric enthalpy calculated from the area under the peak.  $\Delta H$  errors are approximately 10%.

<sup>c</sup> Calorimetric entropy calculated as  $\Delta H/T_m$ .

<sup>d</sup> Width of the calorimetric peak at half peak height.

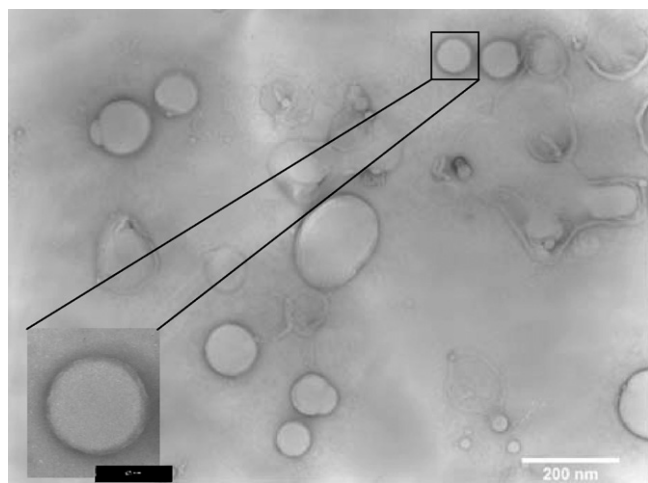
incorporated into the bilayers, thus causing changes in the bilayer organization [24,25] (Fig. 7).

#### 4. Discussion

Our results showed that the stability of liposomal formulations was improved by the addition of PEG 400 since, after a 9-month storage period, liposomes without PEG 400 showed a mean diameter larger than 1000 nm. Other liposomes with different lipid compositions varying from 50 to 200 nm in diameter and a homogeneous size distribution were prepared. The best molar ratio for the lipids PCEY and PEEY to obtain stable SUV liposomes was 1:1 (M1, M2), although other molar relations achieved multilamellar vesicles. On the other hand, CHOL inclusion into the lipid bilayer did not influence the size of the particles, whereas after a long storage period, the most stable formulations were those without CHOL.

The ZnPc incorporation efficiency is between 66% and 76% in most liposomal formulations. These values are lower than those obtained for no peripheral substituted zinc(II) phthalocyanine [26].

Aggregation values were studied in three different polarity media: THF, PBS/DMSO and HEPES. S-PEG showed the lowest aggregation values of all liposomal formulations.



**Fig. 7.** TEM micrographs of the liposome formulation S-PEG (DPPC:PCEY:PEEY:DSPE-PEG:CHOL (1:8:7:1:3)) with ZnPc 0.0467 μg/mg of lipid. Panoramic view, in which the size uniformity of the liposomes can be observed (70,000×). Inset: a magnification of one liposome, where the lipid bilayer and a slight drain of the vesicles can be observed.

The ZnPc incorporated into all liposomal formulations generated singlet molecular oxygen. The decrease in the  $\Phi_\Delta$  values, as compared with those found in homogeneous media, suggests that ZnPc is incorporated into the lipid bilayer as aggregates. The results suggest that the reduction of singlet oxygen efficiency photoproduction is due not only to the presence of dimers but also to the interaction between the dye and the neighboring lipids in the bilayer. This is supported by several factors including: the high incorporation efficiency of ZnPc into liposomal bilayers, the environmental dependence on the absorption spectra, the  $\Phi_\Delta$  values obtained for the ZnPc incorporated into the liposomal bilayers and the modification of the thermodynamic parameters  $T_m$  and  $\Delta T_{1/2}$ . Besides, the  $\Phi_\Delta$  values were higher than those obtained with other zinc(II) phthalocyanines incorporated into DMPC liposomes or Tween 80 [7]. In general, the results showed that the incorporation and aggregation of zinc(II) phthalocyanines into liposomes were dependent of the chemical structure of the peripheral substituents of the macrocycle [7,25,27].

The above results allow us to consider liposome formulations as good photosensitizers to generate singlet molecular oxygen and as a very useful method to incorporate other hydrophobic drugs that can be used as photo-therapeutic agents.

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