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Pharmaceutical Nanotechnology

Zinc(II) phthalocyanine loaded PLGA nanoparticles for photodynamic therapy use

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Abstract

Sophisticated delivery systems, such as nanoparticles, represent a growing area in biomedical research. Nanoparticles (Np) were prepared using a solvent emulsion evaporation method (SEEM) to load zinc(II) phthalocyanine (ZnPc). Np were obtained using poly (D,L latic-co-glycolic acid) (PLGA). ZnPc is a second generation of photoactive agents used in photodynamic therapy.

ZnPc loaded PLGA nanoparticles were prepared by SEEM, characterized and available in cellular culture. The process yield and encapsulation efficiency were 80 and 70%, respectively. The nanoparticles have a mean diameter of 285 nm, a narrow size distribution with polydispersive index of 0.12, smooth surface and spherical shape. ZnPc loaded nanoparticles maintains its photophysical behavior after encapsulation. Photosensitizer release from nanoparticles was sustained with a moderate and burst effect of 15% for 3 days. The photocytotoxicity of ZnPc loaded PLGA Np was evaluated on P388-D1 cells what were incubated with ZnPc loaded Np (5 μM) by 6 h and exposed to red light (675 nm) for 120 s, and light dose of 30 J/cm^2. After 24 h of incubation, the cellular viability was determined, obtaining 61% of cellular death. All the physical–chemical, photophysical and photobiological measurements performed allow us conclude that ZnPc loaded PLGA nanoparticles is a promising drug delivery system for photodynamic therapy.

Keywords: Nanoparticles; PLGA; ZnPc; Preparation; Characterization; Phototoxicity assay

1. Introduction

Sustained delivery systems have been emerging as a main goal to provide a modified release of drugs. These systems avoid plasmatic fluctuations, low side effects, facilitate dosage administration, reduce the required frequency of administration, and enhance the specific uptake of drug by targeted tissue increasing patient compliance (Madan, 1985; Hans and Lowman, 2002; Konan et al., 2002). Advances in nanobiotechnology have resulted in evolution of several novel colloidal carriers systems such as liposomes, polymeric micelles, microemulsions and nanoparticles to achieve these multiple objectives (Barratt, 2003; Shenoy and Amiji, 2005).

Among them, nanoparticles (Np) have been studied in the last decade and have clearly shown the capacity to be utilized to maintain adequate therapeutic levels of drugs in the body and still address towards a target. Np of biodegradable polymers can offer an ideal solution for sustained release of anticancer agents because they are easily manufactured and represent an attractive alternative improves modulation of drug delivery. Due to their small diameter (<1000 nm), nanoparticles can be used as injectable carriers for drug targeting (Soppimath et al., 2001; Hans and Lowman, 2002; Shenoy and Amiji, 2005). Nanoparticles can accumulate at the tumor site due to enhanced endocytotic activity and leaky vasculature in the tumor (Saxena et al., 2004). PLGA nanoparticles have been used as drug carrier systems for antibiotics (Naraharisetti et al., 2005; Pinõn-Segundo et al., 2005), anticancer agents (Huo et al., 2005; Shenoy and Amiji, 2005); anti-inflammatory (Lagarce et al., 2005) and hormone replacement (Kwon et al., 2001) and photosensitizer for PDT use (Konan et al., 2003).

PDT, first developed for cancer treatment, is being actively exploited in many clinical applications such as the treatment of age related macular degeneration, hardening arteries, sun induced precancerous skin lesions, and wound infections (Bonnett, 1995; Dougherty, 1998; Castano et al., 2005). In

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most of the applications studies, the first step in the treatment involves the administration of a photosensitive drug or photosensitizer which has high affinity to malignant tissues and others non-normal cells. In a second step, the biological systems are irradiated with appropriated low doses light, in special at the maximum absorption wavelength of the photoactive compound, leading to the production of many reactive oxygen species such as $^1O_2$, H_2O_2 and O_2. Singlet oxygen ($^1O_2$) is reported to be the predominant cytotoxic agent when photosensitizers are excited by light of appropriated wavelength and power (Davies, 2003; Stief, 2003). Among the various photosensitizer drugs investigated, phthalocyanines have been found to be highly promising (Ali and Lier, 1999; Konan et al., 2003; Sibata et al., 2004; Castano et al., 2005).

Zinc(II) phthalocyanine (ZnPc), a second generation photosensitizer much utilized in PDT, was used in our experiments due its high selectivity for tumor targets and enhanced cytotoxic efficiency due to singlet oxygen photogeneration. In addition, hydrophobic character of ZnPc facilitates the encapsulation of this photosensitizer in many drug delivery systems such as liposomes (Rodal et al., 1998; Derycke and De Witte, 2004) and polymeric micelles (Sibata et al., 2004; Van Nostrum, 2004; Sharman et al., 2004). However, these delivery systems have low loading efficiency and stability for photosensitizers (Leroux et al., 1996; Soppimath et al., 2001). Nanoparticles have better stability than liposomes in biological systems and during storage (Quintanar-Guerrero et al., 1998). Our group has investigated the feasibility to apply poly (D,L latic-co-glycolic acid) (PLGA) Np as an alternative delivery system for PDT use.

Much attention has been focused on biodegradable polymers such as PLGA. This polymer has emerged as the most widely used and studied biodegradable polymers for pharmaceutical use due to their biocompatibility and biodegradability (Soppimath et al., 2001). PLGA has been approved by FDA and has been used in humans for many years as suture material.

In the present work, ZnPc as a classical photosensitizer for PDT loaded with PLGA nanoparticles was prepared and characterized by physical–chemical and photophysical methods. In vitro release studies were carried out on all systems to evaluate the drug availability. Phototoxicity assays were carried out to evaluate the capability of the delivery system against cells cancer.

2. Materials and methods

2.1. Materials

Zinc(II) phthalocyanine (MW = 577.91) was purchased from Aldrich (Milwaukee, USA), poly (D,L latic-co-glycolic acid) (PLGA 50:50), Resomer RG 502 (MW = 8000) from Boehringer Ingelheim (Germany), polyvinyl alcohol (PVA) (MW = 26000) from Mallinckrodt Chemical (French), sodium dodecysulphate (SDS) from Calbiochem (USA). Dichloromethane (DCM) and pyridine, all of analytical grade, were purchased from Merck (Germany). NaH_2PO_4 and Na_2HPO_4 were purchased from Nuclear (São Paulo, Brazil). Tripan Blue and 3-(4,5 dimethylthiazol-2-yl-2,5 biphenyl) tetrazolium bromide were purchased by Sigma (USA). Culture medium RPMI 1640 was purchased from Gibco BRL (Brazil).

2.2. Methods

2.2.1. Preparation of nanoparticles

Nanoparticles containing ZnPc was prepared from PLGA according to the methodology previous describes by Scholes et al. (1993). Briefly, 100 mg of PLGA and 0.5 mg of ZnPc were dissolved in dichloromethane. This solution was added dropping to an aqueous PVA (3%, w/w) solution under mixing in an Ultraturrax T25 laboratory emulsifier (Ika, Germany), at 15,000 rpm. The resulting emulsion was sonicated for 10 min using a Thornton soniprobe at a 60 W output (Thornton, Brazil). The solvent (DCM) was evaporated at room temperature (28 °C) for 8 h, under magnetic stirring. ZnPc loaded nanoparticles were purified by 30 min centrifugation at 3000 rpm and resuspension in water. The suspension was added trehalose (trehalose:particules mass ratio of 2:1) (crioprotector) and placed glass vial and frozen in a liquid nitrogen bath. Freeze-drying was carried out in a lyophilizer Freezone 4.5 (Labconco) at 0.001 bar, yielding powdered nanoparticles. The samples were stored at 25 °C before analysis.

2.2.2. Process yield (%)

Powdered nanoparticles were obtained after freeze-drying. The process yield was calculated by Eq. (1):

$$Y(\%) = \frac{M_{NP}}{M_T} \times 100 \quad (1)$$

where $Y(\%)$ is the process yield, M_{NP} the mass of nanoparticles recovered after freeze-drying and M_T is the mass of PLGA plus mass of ZnPc in formulation. The encapsulation method was accomplished in triplicate ($n = 3$).

2.2.3. Quantification of ZnPc by the fluorescence emission method

ZnPc (MW = 577.91) was dissolved in pyridine at 1.73 mM. This solution was diluted with isotonic phosphate buffer saline (PBS) at pH 7.4, containing 2% sodium dodecylsulphate (SDS), for preparation of the standard curve. The standard curve was prepared in triplicate ($n = 3$) covering the range of 0.02–0.82 μM ZnPc. The standard solutions were excited at 610 nm and the fluorescence emission spectra recorded between 650 and 800 nm using a Spex Fluorog 3 (Perkin-Elmer, UK) spectrofluorimeter, at the conditions described above. The area of fluorescence emission spectrum was correlated with the ZnPc concentration (μM) to obtain the standard curve. Precision and linearity were calculated from the coefficient of variation (CV%) and linear regression of the standard curve, respectively.

2.2.4. ZnPc encapsulation efficiency

Ten milligrams of freeze-dried nanoparticles were dissolved in 10 ml of pyridine, and 0.5 ml samples were diluted with 2% SDS buffer saline (PBS), and magnetically stirred for 1 h at 28 °C. PLGA is insoluble in aqueous media and the suspension obtained was centrifuged at 10,000 × g for 10 min. The

supernatant was collected and its ZnPc content measured by the area on the fluorescence emission spectrum as described above; 2% SDS in PBS was used as reference solvent. Samples were excited at 610 nm and the fluorescence emission spectra recorded between 650 and 800 nm. The ZnPc concentrations were measured from the standard curve. Encapsulation efficiency was calculated from Eq. (3):

$$\mathrm{EE} = \frac{M_1}{M_t} \times 100 \quad (3)$$

where EE is the ZnPc encapsulation efficiency, M_1 the mass of ZnPc in nanoparticles and M_t is the mass of ZnPc used in formulation. The experiments were accomplished in triplicate ($n = 3$).

2.2.5. Physical–chemical characterization

2.2.5.1. Particle size analysis. Particle size and size distribution were measured by laser light scattering using a particle size analyzer (Zetasizer, Malvern, UK). Size distribution was analyzed over the range of 1–3000 nm and mean diameter was calculated for each sample. The equipment also gives a polydispersive index (PI), which should be low (0.1) for a monodisperse system. The nanoparticles suspension was measured in a 1.0 cm quartz cell, at 28 °C. Np were analyzed three times ($n = 3$) with 30 readings taken for each sample.

2.2.5.2. Scanning electron microscopy (SEM). To examine PLGA Np morphology, freeze-dried particles were observed using SEM (Stereoscan 440, Leica). Dried Np were prepared on glass slides and coated with gold prior to examination by SEM. The voltage was ranged from 10 to 25 kV during scanning.

2.2.6. Photochemical characterization

ZnPc standard was dissolved in pyridine and diluted in ethanol. A known amount of ZnPc loaded PLGA Np was extracted with pyridine and diluted in ethanol to result in a 5 μM photosensitizer solution. Solutions were analyzed for absorption spectroscopy, fluorescence emission, time-resolved fluorescence measured (τ_f), fluorescence quantum yield (ϕ_f) and time-resolved triplet measured (τ_t).

2.2.6.1. Absorption spectroscopy and fluorescence emission. Absorption measurements of ZnPc standard and extracted from PLGA nanoparticles were made on a Perkin-Elmer Lambda 20 setup whose background was corrected using matched quartz cells with scanning over the wavelength range from 300 to 800 nm. Ethanol was used as reference solvent (Eichwurzel et al., 2000; Sibata et al., 2004).

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 fluorescence emission spectra recorded between 650 and 800 nm (Cuccovia et al., 1982; Sibata et al., 2004).

The samples were measured in a 1.0 cm quartz cell. The systems were equipped with stirring and temperature control of the sample (28 °C).

2.2.6.2. Time-resolved fluorescence measurement (τ_f). Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was an Edinburgh Analytical Instruments FL-900 lifetime spectrometer, operating with hydrogen-filled nanosecond flash lamp at 40 kHz pulse frequency. The exponential decay fitting was judged by statistical parameters such as the reduced Chi-square function (χ^2). Chi-square values of 1 ± 0.2 indicate a monoexponential fitting to decay curves and, in this case photosensitizer has one fluorescence lifetime (ns) (Valduga, 1987).

The excitation and emission wavelength used were setup at 610 and 682 nm, respectively. All measurements were made in the presence of oxygen, at 25 °C. The experiments were carried out in triplicate ($n = 3$).

2.2.6.3. Fluorescence quantum yield (ϕ_f). The fluorescence quantum yield (ϕ_f) of ZnPc was measured using the ratio method described by Eaton (1988), utilizing ZnPc in ethanol as a standard ($\phi_f = 0.28$). Optical densities were set below 0.1 A.U. in ZnPc solutions at the wavelength of maximum absorbance (675 nm) and the corrected fluorescence emission spectra recorded on a Fluorog 3 spectrofluorimeter between 650 and 800 nm, following the technique described by Eaton (1988). Fluorescence quantum yield was calculated from Eq. (3):

$$\phi_u = \left[\frac{A_s F_u n^2}{A_u F_s n_o^2}\right] \phi_s \quad (3)$$

where u is the unknown, s the standard, ϕ the fluorescence quantum yield, A the absorbance, F the fluorescence area and n is the refractive index. The experiments were carried out in triplicate ($n = 3$).

2.2.6.4. Time-resolved triplet measured (τ_t). Solutions of ZnPc standard and extracted from Np were set to 0.3 A.U. at the wavelength of 610 nm using a spectrophotometer F-4500 (Hitachi). Samples were measured in a 1.0 cm quart cell, at 25 °C (Owens et al., 1998; Sibata et al., 2004). Time-resolved triplet measured (τ_t) were made using a laser flash photolysis spectrometer. The system uses the third harmonic (355 nm) of Nd-YAG laser, Continuum, model SURELITE I-10. The exponential decay fitting was judged by statistical parameters such as the reduced Chi-square function (χ^2). Chi-square values of 1 ± 0.2 indicate a monoexponential fitting to decay curves and, in this case ZnPc triplet excited state has one fluorescence lifetime (ns). All measurements were made in the presence of oxygen, at 25 °C. The experiments were carried out in triplicate ($n = 3$) (Sibata et al., 2004).

2.2.7. In vitro releasing studies

A known amount of freeze-dried particles (5 mg, equivalent to 17.5 μg ZnPc) was dispersed in 50 ml of 2% SDS phosphate buffered saline, pH 7.4, at 37 °C. The acceptor solution was stirred with a paddle at a constant rate of 100 rpm, using dissolution equipment (SR8 Plus, Hanson Research). Paddles were fitted to 100 ml glass dissolution vessels supplied by Hanson Research. At given time intervals, six samples ($n = 6$) of 3 ml

were withdrawn and centrifuged at 10,000 × *g* for 10 min. The precipitates were re-suspended in 3 ml of fresh medium and placed in the respective dissolution vessels. The fluorescence emission spectra of the supernatant were measured under the conditions described above, and the areas of the spectra were utilized to calculate the concentrations of ZnPc released from the PLGA nanoparticles, and used to draw the release profile of ZnPc. The experiments were carried out to six determinations ($n = 6$).

2.2.8. Toxicity and photocytotoxicity assay

2.2.8.1. Cells line. P388-D1 cells were purchased American Type Culture Collection (ATCC). Cells line was derived from a 3-methyl chlolanthrene injected in the thorax of the DBA/2 mouse and adapted to in vitro culture (Fisher et al., 1958). The cells were maintained in RPMI 1640 medium (Gibco BRL), supplemented with 10% fetal bovine serum, 100 mM L-Glutamine, 100 U/ml penicillin-streptomycin and 1 ml 2-mercaptoethanol (Gibco BRL) at 37 °C in 5% CO_2 atmosphere. Aliquots of 2×10^4 cells were placed into 96-well dishes in 100 μl of culture medium and incubated for 24 h at 37 °C in 5% CO_2 atmosphere before dark toxicity and photocytotoxicity assays (Rodal et al., 1998; Konan et al., 2003). The P388-D1 cells grew adhered on well surface, forming a cell layer.

2.2.8.2. Photocytotoxicity. Nanoparticles suspension was prepared in culture medium at 5 μM ZnPc. The cells were washed and incubated with ZnPc loaded PLGA Np (freeze-dried) for 6 h. Following incubation, cells were washed twice with phosphate buffer saline (Gibco BRL) and 100 μl fresh culture medium was added in each well. Finally, after cellular uptake of ZnPc or nanoparticles, the cells P388-D1 (macrophages) were exposed to red light (675 nm) for 120 s, and light dose of 30 J/cm^2 (Rodal et al., 1998; Konan et al., 2003). After light exposure, cellular culture was incubated for 24 h at 37 °C in 5% CO_2 atmosphere and the cellular viability was determined by MTT assay.

The dark toxicity was also determined. The P388-D1 cells were incubated with 100 μl of PBS, PLGA nanoparticles without photosensitizer and ZnPc loaded PLGA nanoparticles (5 μM) for 6 h. Cells were washed twice with phosphate buffer saline (Gibco BRL) and 100 μl fresh culture medium was added in each well. Cellular culture was incubated for 24 h at 37 °C in 5% CO_2 atmosphere. The cellular viability was determined by MTT assay (Rodal et al., 1998; Konan et al., 2003).

Light photocytotoxicity, without photosensitizer, was evaluated. P388-D1 cells were exposed to light (675 nm) for 120 s, with different light dose (0–100 J/cm^2). Cells were washed twice with phosphate buffer saline (Gibco BRL) and 100 μl fresh culture medium was added in each well. Cellular culture was incubated for 24 h at 37 °C in 5% CO_2 atmosphere. The cellular viability was determined by MTT assay (Rodal et al., 1998; Konan et al., 2003).

2.2.8.3. MTT assay. The cellular viability was determined by 3-(4,5 dimethyl-thiazol-2-yl-2,5 biphenyl) tetrazolium bromide (MTT, Sigma, Germany) (Mosmann, 1983; Vistica, 1991). Briefly, after removing the cellular medium, 50 μl of MTT solution (1 mg/ml) was added to each well and incubated for 3 h. Then, the formazan crystals were dissolved by adding 100 μl of 10% sodium dodecylsulphate (Sigma–Aldrich) in 0.01 M HCl to each well. The absorbance was determined at 595 nm by a microplate reader. For each sample, average cellular viability was calculated from the data of 10 wells ($n = 10$) and expressed as a percentage, compared to untreated cells (100%).

3. Results and discussion

3.1. Determination of ZnPc by fluorescence emission

Fluorescence emission spectrum of ZnPc in 2% SDS PBS was measured from 650 to 800 nm. ZnPc was dispersed in aqueous media by SDS micelles, keeping its emission behavior. Previous studies indicate that ZnPc keeps its fluorescence emission behavior in polymeric micelles and ethanol (Reddi et al., 1987; Valduga, 1987; Sibata et al., 2004), allowing measurements of the photosensitizer in aqueous medium.

The area of fluorescence emission spectrum of ZnPc correlated linearly with the concentration over the 0.02–0.82 μM range. The precision of the standard curve was below 5%. The correlation coefficient exceeded 0.9998, with excellent linearity. This method for the determination of ZnPc in an aqueous medium has sensitivity and reproducibility. The simplicity and short analysis time of the proposed method allows for the analysis of a large number of samples providing a useful, reliable means for the determination of ZnPc in aqueous medium.

3.2. Physical–chemical characterization

The analysis of encapsulation methods and the results of physical–chemical characterization are contained in Table 1, and each of the experiments of physical–chemical characterization of delivery system is discussed below.

3.2.1. Method of preparation

PLGA Np were prepared with success by the solvent emulsion evaporation method (SEEM). No changing was observed in the particle after freeze-drying. Satisfactory yields and encapsulation efficiency of ZnPc were obtained (Table 1).

Soppimath et al. (2001) suggest that the encapsulation methods should produce nanoparticles with high drug loading effi-

Table 1
Results of physical–chemical characterization

Sample	ZnPc loaded Np
Yield (%)[a]	80 ± 3.2
E.E. (%)[b]	70 ± 1.7
Diameter (nm)[c]	285 ± 5.1
Polydispersity index[c]	0.12 ± 0.02

[a] Yield of the dried PLGA nanoparticles, Mean ± D.S. ($n = 3$).
[b] EE = encapsulation efficiency (%) of ZnPc in PLGA nanoparticles, Mean ± D.S. ($n = 3$).
[c] Mean ± S.D. Mean ± D.S. ($n = 3$).

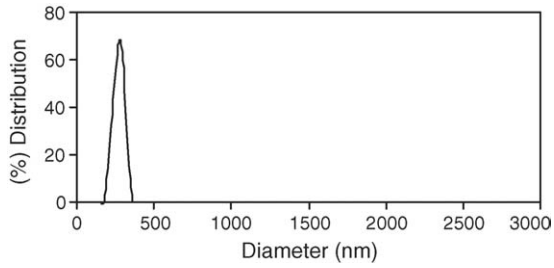

Fig. 1. Size distribution of ZnPc loaded PLGA nanoparticles (aqueous suspension). Particles were measured by laser light scattering using a particle size analyzer (Zetasizer).

ciency to reduce the quantity of the carriers required for administration. ZnPc loaded PLGA Np has satisfactory encapsulation efficiency of 70%.

3.2.2. Size and size distribution

Nanoparticles have a further advantage over large microparticles, because they are better suited for intravenous delivery. The smallest capillaries in the body are 5–6 μm in diameter. The size of particles into the bloodstream must be significant smaller than 5 μm to ensure that the particles do not form an embolism. Thus, it is important to measure the size particles. The average diameter and polydispersity index of the Np prepared by SEEM is shown in Table 1, and the size distribution of the nanoparticles is presented in Fig. 1.

Fig. 1 indicates that over 95% of the nanoparticles manufactured by SEEM are in the range between 228 and 290 nm, with a mean value of 285 ± 5.1 nm. ZnPc loaded Np prepared by SEEM has nanometric size and this size is considered satisfactory to intravenous administration.

Polydispersive index is used for judging the sample quality. PI near 0.1 indicates that samples have excellent quality with narrow size distribution to monomodal systems (Scholes et al., 1993). PI in the range of 0.5–1.0 indicates samples with bad quality, constituting polyimodal systems. Polydispersive index is shown in Table 1. ZnPc loaded nanoparticles obtained by SEEM has PI of 0.12.

3.2.3. Scanning electron microscopy

The morphology of the particles was examined by SEM and is shown in Fig. 2. The PLGA nanoparticles have spherical shape, smooth regular surface (Fig. 2a). Fig. 2b shows PLGA nanoparticles, which were isolated and magnified. PLGA nanoparticles have an almost spherical shape and smooth surface because these characteristics depend on the polymer.

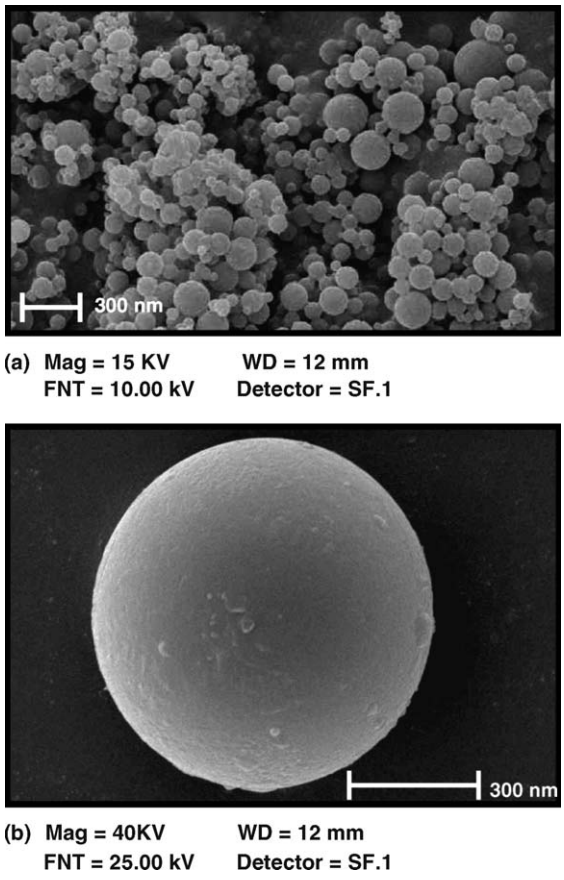

Fig. 2. Scanning electronic micrograph of ZnPc loaded PLGA nanoparticles: (a) panoramic view of ZnPc loaded PLGA Np (Mag. = 15 KV, WD = 12 mm, FNT = 10.00 kV, detector = SF.1) and (b) isolated magnified particle (Mag = 40 KV, WD = 12 mm, FNT = 25.00 kV, detector = SF.1).

3.3. Photophysical characterization

The results of photophysical characterization are contained in Table 2. Each experiment is discussed below. The results contained in Table 2 show that the photosensitizer loaded in nanoparticles does not suffer changing in its photophysical properties after the encapsulation process. ZnPc standard and loaded nanoparticles have similar photophysical behaviors, so these results show that encapsulation process is suitable.

Table 2
Results of photophysical characterization

Samples	UV–vis (nm)[a]	λ_{EX} (nm)[b]	λ_{EM} (nm)[c]	τ_f (ns)[d]	ϕ_f (%)[e]	τ_t (μs)[f]
Free ZnPc	666	610	680	4.2 ± 0.04	0.280 ± 0.005	0.260 ± 0.006
ZnPc loaded NP	666	610	679	4.1 ± 0.10	0.255 ± 0.010	0.250 ± 0.002

[a] Maximum wavelength UV–vis absorption.
[b] Excitation wavelength.
[c] Maximum wavelength fluorescence emission.
[d] Fluorescence lifetime, Mean ± D.S. (n = 3).
[e] Fluorescence quantum yield (%), Mean ± D.S. (n = 3).
[f] Triplete lifetime, Mean ± D.S. (n = 3).

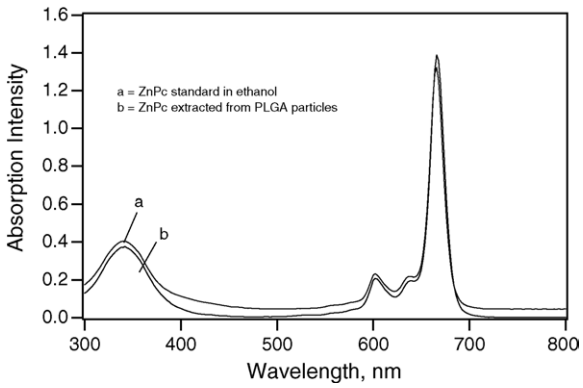

Fig. 3. Absorption spectra of ZnPc (5 μM) in ethanol: (a) ZnPc standard and (b) photosensitizer extracted from PLGA nanoparticles.

The absorption spectra of ZnPc (5 μM) standard and extracted from PLGA particles in ethanol are illustrated in Fig. 3. ZnPc exhibits a strong absorbance in the red region, with a maximum wavelength at 666 nm. Furthermore, it presents a group of Q bands in the region from 600 to 710 nm and a Soret band at 340 nm. In all the studied systems (ZnPc in ethanol) the photosensitizer was not dimer aggregation state form. This can be observed clearly in Fig. 3 behind of the spectral profile Q and Soret bands (monomeric state) in ethanol. ZnPc maintains monomeric state in ethanol or in hydrophobic environment (Owens et al., 1998; Sibata et al., 2004). Based on Sibata et al. (2004), and other pieces of 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 with critical spectroscopy changes when aggregation process takes place.

One of the best manners to investigate the behavior of heterogeneous or biological systems consists of the study of their photophysical properties using fluorescent compounds as probe. The fluorescence emission of the photosensitizers is sensitive in its environment yielding much information (Cuccovia et al., 1982). The fluorescence emission spectra shown in Fig. 4 indicate the position of the maximum emission 680 nm of the classical ZnPc emission maximum, after light excitation at 610 nm in ethanol.

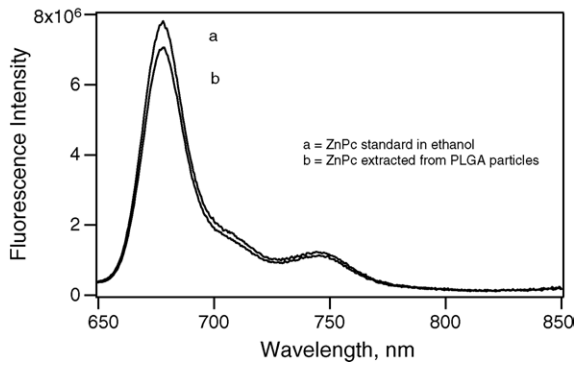

Fig. 4. Fluorescence emission spectra of ZnPc (5 μM) in ethanol: (a) ZnPc standard and (b) photosensitizer extracted from PLGA nanoparticles.

Singlet excited state lifetime (τ_f) was determined through the decay curves profiles of ZnPc in ethanol (data not shown). ZnPc in ethanol has best fit to monoexponential function with Chi-square (χ^2) values of 1.02 and 1.05 to ZnPc standard and extracted from nanoparticles, respectively. The Chi-square value (χ^2) is monoexponential to range 1 ± 0.2 (Valduga, 1987; Sibata et al., 2004). ZnPc standard in ethanol has lifetime of 4.2 ns. Sibata et al. (2004) obtained similar results ($\tau_f = 4.06$ ns). The singlet lifetimes of ZnPc standard and extracted from nanoparticles are similar.

The fluorescence quantum yields (ϕ_f) for ZnPc standard and extracted from nanoparticles are similar (Table 2), and we believe that the photosensitizer does not suffer degradation during the encapsulation method. ZnPc has low fluorescence quantum yield, so greater part absorbed energy is used to reach the triplet excited state. Thus, when excited triplet loses absorbed energy, it comes back to ground state. However, if excited triplet meets an oxygen molecule, it is able to transfer energy to this oxygen. The oxygen molecule absorbs energy and changes to oxygen singlet which is toxic to cells (Sibata et al., 2000; Castano et al., 2005). In ethanol was obtained fluorescence quantum yield value about 0.28. This value is still low enough to allow this class of phthalocyanine to be used as photodiagnostic and photosensitizer in neoplasic diseases (Foote, 1991).

Triplet excited state lifetime (τ_t) was determined through the decay curves profiles of ZnPc in ethanol (data not shown). ZnPc in ethanol has best fit to monoexponential function with Chi-square (χ^2) values of 0.98 and 0.95 to ZnPc standard and extracted from nanoparticles, respectively. The Chi-square value (χ^2) is monoexponential to range 1 ± 0.2 (Owens et al., 1998; Sibata et al., 2004). ZnPc standard in ethanol has lifetime of 260 ns. The singlet lifetimes of ZnPc standard and extracted from nanoparticles are shown in Table 2. We should notice that those values have similar lifetime. The ZnPc loaded nanoparticles does not suffer degradation during the encapsulation process. 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.

3.4. In vitro releasing studies

Nanoparticles were added directly into the dissolution medium (without membrane and dialysis bag). The same technique was adopted by Calvo et al. (1996), Naraharisetti et al. (2005) and Pinõn-Segundo et al. (2005). However, this method is not sensitive enough to study the rapid release formulations, but can only be used for the release of formulations having the long release time (Soppimath et al., 2001).

In this study, in vitro release of ZnPc from PLGA nanoparticles was carried out to dissolution equipment SR8 Plus Hanson Research, utilizing paddles. An amount of lyophilized nanoparticles equivalent to 17.5 μg ZnPc was suspended in a 2% SDS phosphate buffer saline (pH 7.4) solution (50 ml), allowing the

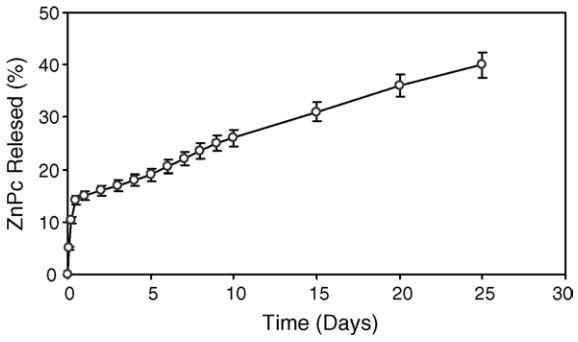

Fig. 5. The in vitro release profile of ZnPc loaded PLGA nanoparticles. Mean values and their standard deviations (bars), $n = 6$ determinations.

experiments to be carried out under sink conditions. The release rate was not controlled by a membrane or dialysis bag and the PLGA nanoparticles were in direct contact with the dissolution medium. It is important to point out that the selection of the sink conditions and dissolution medium were established in previous works (Pinõn-Segundo et al., 2005; Ricci et al., 2005; Feng and Huang, 2001).

The values, mean and standard deviation of six determinations ($n = 6$), on the in vitro studies are shown in Fig. 5. ZnPc has 15% burst effect for 3 days, slow releasing during a period of 25 days, and nearly 40% of drug was released from PLGA Np. The releasing rate was low and neared zero order for the sustained releasing. These results are similar to the results observed in previous works. Feng and Huang (2001) obtained sustained releasing to paclitaxel loaded PLGA Np produced by SEEM. The method to study the in vitro release was reverse dialysis technique, and in this method, nanoparticles were added directly into the dissolution medium. In all release profiles, 25% burst effect was observed for 2 days, slow releasing rate for the 80th day, and 50% of drug was released from PLGA nanoparticles. Naraharisetti et al. (2005) obtained sustained releasing to gentamicin loaded PLGA Np produced by SEEM. In the release profile, 25% burst effect was observed for 2 days, and this effect was followed by a slow releasing for 90 days, and 50% of drug was released from PLGA nanoparticles.

Since the particles had been prepared to remove existing free drug, the initial burst could not be due to free drug. It could probably be caused by molecules in the outer layer of the PLGA nanoparticles (Huang and Brazel, 2001; Soppimath et al., 2001; Pinõn-Segundo et al., 2005). After the third day, ZnPc had a very low releasing rate into the solution medium.

Drug release from nanoparticles and subsequent degradation are important for developing the successful formulations. The mechanisms of drug release depend upon: (1) desorption through the nanoparticles matrix, (2) diffusion through the nanoparticles matrix, (3) diffusion, in case of nanocapsules, through the polymer wall, (4) nanoparticles erosion, (5) a combined erosion and diffusion process and (6) polymer degradation (chemical or enzymatic hydrolysis). Thus, diffusion and biodegradation govern the process of the drug release (Soppimath et al., 2001).

The releasing rate of the drugs from nanoparticles depends upon the nature of the release system. In the case of a polymeric matrix, drug is uniformly distributed in the matrix and the release occurs by diffusion and erosion of the matrix. If the diffusion of the drug is faster than matrix degradation (polymer hydrolysis), then the mechanism of the drug release occurs mainly by diffusion (Soppimath et al., 2001).

Since the degradation time of polymer can usually take many months, its effect on the release in the first month is little significant. Therefore, the main releasing mechanism during this period might be diffusion alone (Jain et al., 1998; Feng and Huang, 2001).

We believed that ZnPc releasing from matrix may be governed by many factors and the most important are drug's diffusion and polymer erosion. ZnPc has hydrophobic behavior requiring SDS micelles for dissolution; this process became the determining step in the releasing process. The drug's diffusion occurs through pores and water filled channels, what probably were formed during the process of Np preparation as well as by polymer erosion and degradation.

3.5. Photocytotoxicity

Nanoparticles have been used as a possible method to target and control drug delivery and to prevent the side effects associated with undesirable biodistribution of the free form of the drugs (Soppimath et al., 2001; Hans and Lowman, 2002; Konan et al., 2003). They are known to be appropriated for selective uptake by neoplasic cells and thus, could improve the phototherapeutic response during cancer treatment for PDT (Dunne et al., 2000; Konan et al., 2003). The carrier with this typical size (nanoparticles, polymeric micelles, liposomes) may overflow into solid tumor tissue because the capillaries are defective with endothelium lack (holes). This drug target is passive because it does not depend on delivery system (Konan et al., 2003).

The in vitro photocytotoxicity was evaluated at the cellular level using P388-D1 cells line, neoplasic cells. The results obtained from these studies demonstrated that the phototoxicity of ZnPc against P388-D1 cells occurred after 24 h light exposure because the cellular damage resulting to photophysical reactions is not immediately lethal. This phenomenon could be due to apoptosis response (slow process) since it has been reported that PDT can lead to an apoptotic response in tumor cells (Argawal et al., 1991; Sibata et al., 2000; Konan et al., 2003). The time required for initiation of apoptosis varies widely (He et al., 1994).

The dark cytotoxicity of the ZnPc loaded PLGA Np and drug free nanoparticles was assessed (Fig. 6). As expected, P388-D1 cells viability was not affected after treatment with these particles. Drug free nanoparticles have high cellular viability (>97%). The results obtained are similar to those published by other authors who studied the toxicity of PLGA nanoparticles. Jalón et al. (2003) assessed toxicity of PLGA nanoparticles obtained by solvent evaporation emulsification method in cellular cultures. They obtained satisfactory cellular viability (88%).

ZnPc loaded PLGA nanoparticles is biocompatible because it has low dark toxicity in cellular culture (cellular viability > 92%). The toxicity of free ZnPc is not assessed because it has hydrophobic behavior and requires hydrophobic delivery

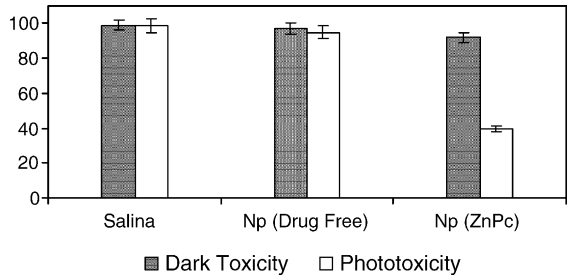

Fig. 6. Dark toxicity: cells P388-D1 were incubated with saline; drug free PLGA Np and ZnPc loaded PLGA Np (5 μM ZnPc) for 6 h. Photocytotoxicity: cells P388-D1 were incubated with saline, drug free PLGA Np and ZnPc loaded PLGA Np (5 μM ZnPc) for 6 h and exposed at the red light (675 nm), for 120 s, light dose of 30 J/cm^2. MTT assay was carried out after 24 h after light exposure for determination of cellular viability. Each data represent the Mean ± S.D. of six determinations.

system such as liposomes, micelles and nanoparticles to administration.

The photocytotoxicity of the red light (675 nm), without photosensitizer, was also assessed and cellular viability was not affected (figure not shown). Red light ($\lambda = 675$ nm, exposure time = 120 s, light dose = 100 J/cm^2) has low toxicity (cellular viability > 97%). The results obtained are consistent with those obtained by other authors. Kolárová et al. (2003) irradiated cellular cultures (G361, human melanoma), without photosensitizer, with red light (600–700 nm) and light dose in the range 0–150 J/cm^2. They obtained high cellular viability (95% to 150 J/cm^2).

PDT is a complex therapy and its efficiency dependent on several parameters including: photophysical properties of photosensitizer, delivery systems, biological systems (cells), drug uptake to tumor tissue, drug light activation, oxygen concentration, light dose, wavelength and exposure time (Konan et al., 2003). We used data of previous works to establish parameters which were utilized in the photocytotoxicity assays. The parameters were: photosensitizer concentration, incubation time, light dose and irradiation time. The photocytotoxicity of the ZnPc loaded PLGA Np and drug free nanoparticles was assessed (Fig. 6). The P388-D1 cells viability was affected after treatment with delivery system and red light exposure. Saline and drug free nanoparticles have low photocytotoxicity (cellular viability >95%). However, ZnPc loaded PLGA Np has high photocytotoxicity (30% cellular viability). The results obtained here are consistent with those obtained by other authors who studied the incorporation of ZnPc into hydrophobic delivery systems. Rodal et al. (1998) irradiated cellular cultures (NHIK 3025, neoplasic cells) containing ZnPc loaded liposomes (1 μg/ml) with red light (600–700 nm) for 120 s, and light dose 16 J/cm^2. They obtained reduction of the cellular viability to 10%.

Delivery systems for PDT use are incubated with cells in the photocytotoxicity assay. Nanoparticles normally enter the cells by endocytosis and can deliver photosensitizer in the cell cytoplasm. The intracellular localization of photosensitizer, an important factor in PDT, can induce different damage in treatment cells. Due to its hydrophobic behavior, ZnPc tends to localize in the cytoplasmic membrane, lysosomal compartment and mitochondria (Gantchev et al., 1994; Zhou et al., 1996; Rodal et al., 1998). After irradiation with red light, different photodynamic mechanism of cytotoxicity could also explain the good efficiency of photosensitizer loaded PLGA Np. Generation of singlet oxygen and additional free radicals may both act as potent cytotoxic agents (Gantchev et al., 1994; Rodal et al., 1998).

PLGA nanoparticles can enter the cells and release ZnPc. This photosensitizer could localize in the cytoplasmic membrane, lysosomal compartment and mitochondria. After light exposure, the cells died by photodynamic mechanism.

4. Conclusions

The encapsulation method was suitable to the preparation of PLGA nanoparticles because the release system has nanometric size and regular shape. After incorporation of ZnPc into PLGA nanoparticles, no significant spectroscopic changes of the photosensitizer properties were observed. ZnPc loaded with PLGA Np presented suitable conditions for its utilization in PDT and photodiagnostic procedures. ZnPc loaded with PLGA nanoparticles had a sustained release rate.

Toxicity and phototoxicity in cell culture has demonstrated that the encapsulation of ZnPc in PLGA nanoparticles should be considered as an effective strategy for targeting and releasing ZnPc to tumor cells. The relative low drug concentration, which can be increased, and short incubation time required by induced satisfactory photodynamic damage after cell treatment indicate that these pharmaceutical formulations offer good photodynamic activity.

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