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Carboplatin liposomes coated with *O*-palmitoylpullulan: in vitro characterization

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Abstract

Encapsulation and stability of carboplafin in large unilamellar liposomes (LUV) of L-α egg phosphatidylcholine (PC), uncoated and coated with *O*-palmitoylpullulan (OPP), were studied. Synthesized OPP was characterized by infrared (IR) spectroscopy, revealing the existence of a covalent bond between the pullulan and palmitoyl groups, and by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, allowing the calculation of the substitution degree of palmitoyl groups in pullulan (0.4%). Moreover, encapsulated carboplatin does not change the fluorescence polarization of either 1,6-diphenyl-1,3,5-hexatriene (DPH) or 3-[p-(6-phenyl-1,3,5-hexatrienyl]phenylpropionic acid (DPH-PA), suggesting that this drug does not modify the membrane lipid organization either in the bilayer core or in the outer domains, as monitored by DPH and by DPH-PA, respectively. Carboplatin encapsulation in LUV and their stability seem to be simultaneously improved by hydration in a medium without chloride ions and with an ionic strength equivalent to 0.6% NaCl. Additionally, it is shown that stability of carboplatin liposomes, as measured by the platinum remaining associated with liposomes (platinum latency), is improved by coating with OPP, for an OPP/PC weight ratio of 3. Higher OPP/PC weight ratio values (e.g. 7.5) induce an opposite effect. © 1997 Elsevier Science B.V.

Keywords: Carboplatin; Liposomes; *O*-palmitoylpullulan; Drug-carrier

1. Introduction

Liposomes as drug delivery systems have been applied to several fields of interest, namely, cancer chemotherapy, antimicrobial therapy, vaccines and diagnostic imaging (Gregoriadis and Flo-

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rence, 1993). One major issue of this technology is the physical stability of liposomes, a critical parameter that affects their in vitro and in vivo behaviour.

Over recent years, attempts have been made to improve either the in vitro or the in vivo stability of liposomes as drug delivery systems, for example, by charge modification (Nakamori et al., 1993) and by including additional compounds in the phospholipid bilayer, e.g. cholesterol (Kirby et al., 1980) or bovine serum albumin (Law et al., 1994). Additionally, according to Barenholz and Crommelin (1994), physical stability of liposomes could be improved by increasing their surface hydrophilicity by using polar lipids with large and highly hydrophilic head groups, such as polyethyleneglycol. Accordingly, Sunamoto and collaborators developed a strategy, that consisted of coating the liposomal surface with natural modified polysaccharides, namely amylopectin, pullulan and mannan, with the aim of improving the physical and biochemical stability of liposomes (Sunamoto et al., 1984a,b, 1992) and the ability to target liposomes to specific organs and cells (Takada et al., 1984; Hirota et al., 1988; Sato et al., 1988; Akiyoshi et al., 1990). This strategy has been applied in the present work to improve the physical stability of LUV containing carboplatin.

Carboplatin is a cisplatin analog, usually used in the treatment of ovarian cancer, small cell lung cancer, squamous cell carcinoma of the head and neck and seminomas (Smith et al., 1985; Koch, 1986; Wagstaff et al., 1989). It was developed with the goal of circumventing the nephrotoxicity of cisplatin, while retaining the broad antitumoral activity of cisplatin. Within the second generation of platinum complexes, only carboplatin fulfilled all the requirements for marketing approval (Weiss and Christian, 1993). However, the toxicity of carboplatin is dose-related myelosupression, with severe thrombocytopenia and leucopenia, more severe in older patients with renal impairment or previously submitted to chemotherapy (Wagstaff et al., 1989). Thus, the encapsulation of carboplatin in liposomes is justified, on one hand, in order to prevent its side effects and, on the other hand, in order to increase the therapeutic efficiency of the drug.

The intraperitoneal administration of freeze dried liposomes with encapsulated carboplatin to rats bearing ascites hepatoma (AH 130 tumors) has been shown to be therapeutically more effective than free carboplatin (Yasui et al., 1992), regarding the increased life span of animals and decreased toxic side effects of the drug. Moreover, Fichtner et al. (1993) have proposed the use of liposomes with entrapped carboplatin as activators of hematopoiesis, associated with the treatment of cancer. These studies emphasize the therapeutic potential of carboplatin entrapped in liposomes, that may be improved by coating the liposomes with polysaccharides, regarding liposome stability and drug action specificity.

Therefore, the aim of this work was to synthesize and characterize a modified polysaccharide, *O*-palmitoylpullulan (OPP) with the purpose of being used for coating LUV containing carboplatin. Possible alterations of the bilayer organization promoted by carboplatin were evaluated by fluorescence polarization of DPH and DPH-PA. The influence of the hydration medium composition on carboplatin encapsulation in uncoated liposomes and the stability of carboplatin liposomes, either coated or not with OPP, were also studied.

2. Materials and methods

2.1. Materials

Pullulan, palmitoyl chloride, egg phosphatidylcholine (PC), carboplatin and DPH were obtained from Sigma. The DPH-PA fluorescent probe was obtained from Molecular Probes. Sephadex G-25 was purchased from Pharmacia. All other reagents were of analytical grade for biochemical purposes.

2.2. Synthesis and characterization of O-palmitoylpullulan

O-palmitoylpullulan was prepared as described by Sunamoto et al. (1992). Briefly, 1 g of pullulan was dissolved in 11 ml of dry dimethylformamide at 60°C. To the resulting solution, 1 ml of dry

pyridine and 0.1 g of palmitoyl chloride dissolved in 0.24 ml of dry dimethylformamide, were added. The mixture was stirred at 60°C for 2 h followed by 1 h at room temperature. Then, it was slowly poured into 70 ml of absolute ethanol under vigorous stirring. The precipitate was collected and washed with 80 ml of absolute ethanol and 60 ml of dry diethyl ether. The white solid material obtained was dried in vacuum at 50°C for 2 h; the yield was about 800 mg.

The polymer was characterized by IR and ^{1}H-NMR. The IR spectrum of OPP (1%), incorporated in a KBr tablet, was run on a Fourier transform infrared simple beam spectrometer (Nicolet 740). The ^{1}H-NMR spectrum was obtained in deuterated dimethylsulfoxide solution (DMSO-d_6) (50 mg/ml), containing tetramethylsilane (TMS) as internal standard and operating at a frequency of 499.843 MHz (Spectrometer Varian Unity-500). Additionally, a ^{1}H-NMR spectrum of palmitic acid was also obtained in the previously described conditions, at a concentration of 2.56 mg/ml.

2.3. Preparation of liposomes

LUV were prepared by combination of reverse phase evaporation and extrusion through polycarbonate membranes (Szoka et al., 1980; Sato, 1990). In each preparation, 30 mg (39 μmol) of PC were dissolved in 4 ml of diethyl ether. Then, 2 ml of an aqueous solution containing carboplatin (27 μM) at pH 6.5 was added to the organic solution and the mixture was sonicated under a nitrogen atmosphere in a water bath for 10 cycles of 20 s each, alternated with intervals of 10 s. Ether was evaporated from the homogeneous suspension under controlled reduced pressure in a rotary evaporator, under nitrogen atmosphere, at 20–25°C. Following gel formation, the tube was vortexed briefly to break up the gel and the evaporation was repeated. When the mixture turned into a homogeneous aqueous suspension, 2 ml of aqueous solution without carboplatin was further added and the evaporation was carried out under a pressure of 5 mm Hg, until residual diethyl ether was completely removed. Formed liposomes were twice extruded through polycarbonate membranes of 0.4-μm pore diameter (Nucleopore Polycarbonate Costar). Non-encapsulated carboplatin was removed either by filtration through a Sephadex G-25 (medium) column (30 × 1 cm) or by ultracentrifugation (described later), according to the type of study to be carried out. In Sephadex G-25 filtration, the applied liposome vesicles elute at a volume between 7 and 13 ml, while free carboplatin elutes at a volume between 22 and 36 ml. Therefore, a complete separation of free and liposome entrapped carboplatin was achieved.

Coating of liposomes with OPP was carried out by addition of 1 ml OPP dissolved in the liposomes hydration media to 1.5 ml previously formed liposome dispersion, in order to obtain OPP/PC weight ratios of 3 or 7.5, and further submitted to stirring below 20°C for 1 h before use (Sunamoto et al., 1987). The separation of any free polysaccharide that might exist in the medium was not performed since, as already mentioned by Takada et al. (1984), it did not exert any relevant effect on the liposomes behaviour, namely, in size variation. The size of both uncoated or coated liposomes was about 245 nm.

The concentration of phospholipids was determined by measuring the inorganic phosphate (Bartlett, 1959) after acid hydrolysis at 180°C in 70% $HClO_4$ (Böttcher et al., 1961).

2.4. Fluorescence polarization measurements

Fluorescent probes of membrane organization were incorporated into liposomes as described elsewhere (Custódio et al., 1993). DPH (2 mM in tetrahydrofuran) or DPH-PA (2 mM in dimethylformamide) were injected, while vortexing for 10 s, into liposome dispersions (345 μM in phospholipid), either prepared with 27 μM carboplatin or without the drug (control sample), to obtain a final phospholipid/probe molar ratio of about 400. The mixtures were then incubated, in the dark, overnight. The fluorescence measurements were carried out at 37 and 50°C (the temperature of the samples was checked with an accuracy of $\pm$0.1°C with a thermistor thermometer) in a Perkin-Elmer LS-50 B computer controlled spectrometer, provided with a thermostated cell

holder. The excitation and emission wavelengths were set at 357 and 428 nm, respectively, and the excitation and emission bandwidths were 3 and 4 nm, respectively. Adequate control experiments were carried out without the probes to correct for the contribution of scattered light and/or background fluorescence of sample or solvents. Neither solvents of drug and probes, nor carboplatin, exhibit by themselves any measurable fluorescence under our experimental conditions.

2.5. Platinum determination

The concentration of platinum (Pt) was determined by atomic absorption spectroscopy (Perkin Elmer 305 AAS) at 265.9 nm (bandwith 0.7 nm) with an oxidizing air/acetylene flame. The samples were prepared by mixing 0.1 ml of a 20% Triton X-100 aqueous solution and 1 ml of a lanthane nitrate ($La(NO_3)_3 \cdot 6H_2O$) aqueous solution (14.4 mg/ml) with 0.6 ml of liposome dispersion; the final volume was adjusted to 3 ml with 1% HCl (v/v). Standard solutions (0–100 μM Pt) were prepared in 1% HCl containing Triton X-100, lanthane nitrate and liposomes without carboplatin in similar concentrations.

2.6. Influence of sodium chloride content of the hydration medium on the encapsulation parameters of carboplatin

This study was carried out with uncoated liposomes containing carboplatin hydrated with several hydration media (defined as the aqueous solution of carboplatin added to the lipids dissolved in organic solvent), at pH 6.5, containing different amounts of NaCl: 0, 0.3, 0.6 and 0.9%. Except for 0.9% NaCl, the hydration media were adjusted to physiological osmolarity with dextrose. The evaluated encapsulation parameters of carboplatin were the binding capacity and the encapsulation efficiency.

The binding capacity (BC) is defined as the mass of carboplatin (μg) entrapped in liposomes per μmol of phospholipid in the final liposome dispersion (obtained after extrusion and filtration through Sephadex G-25) (Steerenberg et al., 1987):

$$\mathrm{BC}(\mu g/\mu mol) = \frac{\mu g \text{ carboplatin}}{\mu mol \text{ phospholipid}} \text{ in the final liposome dispersion} \quad (1)$$

The encapsulation efficiency (EE) is the percentage of the ratio between the carboplatin to lipid molar ratio in the final liposome dispersion and that in the initial mixture (liposome dispersions before extrusion and filtration through Sephadex G-25) (Cruz et al., 1993)

$$\mathrm{EE}(\%) = \frac{[\text{carboplatin}]/[\text{phospholipid}]_{\text{final liposome dispersion}}}{[\text{carboplatin}]/[\text{phospholipid}]_{\text{initial mixture}}} \times 100 \quad (2)$$

2.7. Stability studies

Uncoated LUV were prepared in different media containing carboplatin and different concentrations of NaCl (0 and 0.6%), adjusted to physiological osmolarity with dextrose. The nonencapsulated carboplatin was removed by ultracentrifugation (135 000 × g, 30 min at 4°C), in a Beckman rotor (type TLA 100.3, SN 1600), and the liposomes were washed twice with the aqueous solution used as hydration medium. The final pellet was resuspended in 2.5 ml of the respective hydration medium. Stability studies were then carried out under gentle magnetic stitring at 50°C for 6 days, in order to decrease the time of the experiment. At different incubation times, aliquots of liposomal dispersion were removed and filtrated through a Sephadex G-25 (medium) column to remove free carboplatin. Then, the platinum (Pt) latency was calculated according to Steerenberg et al. (1987), and expressed as the percentage of drug remaining associated with liposomes:

$$\text{Pt latency}(\%) = \frac{[\mathrm{Pt}]/[\text{phospholipid}]_{\text{at an appropriate incubation time}}}{[\mathrm{Pt}]/[\text{phospholipid}]_{\text{before starting stability study}}} \times 100 \quad (3)$$

where [Pt] and [phospholipid] are the molar concentrations of Pt and PC in liposomal dispersions.

Stability studies of carboplatin liposomes coated with OPP (OPP/PC weight ratios of 3 and 7.5) were performed in the same conditions as described above.

2.8. Measurement of liposome size

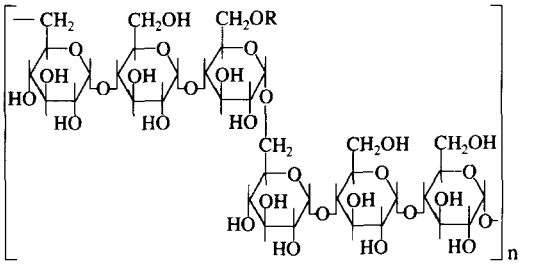
Mean particle size of liposomes was measured by photocorrelation spectroscopy with an Autosizer II C apparatus (Malvern Instruments, UK) equipped with a 5 mW He-Ne laser at a wavelength of 633 nm. The measurements were carried out at 20 ± 1°C with a scattering angle of 90° using a correlator Malvern Multi-8 (type 7032 CE).

3. Results and discussion

3.1. Characterization of OPP

Carboplatin is activated intracellularly into reactive aquated complexes that cross-link deoxyribonucleic acid (DNA) (Knox et al., 1986; Kim, 1993). Hence, one of the best putative strategies to improve drug targeting to tumor cells is the attachment of a monoclonal antibody to the polysaccharide coated liposomes to promote specificity to tumor cells and further cellular internalization of carboplatin. Among the natural polysaccharides mentioned before, pullulan is probably the best choice to conjugate a monoclonal antibody due to its high coating efficiency (Sunamoto et al., 1992) and reduced cell specificity, thus preventing any kind of interference with the specificity of a monoclonal antibody (Sato, 1990).

Pullulan is a linear α-glucan, produced by the yeastlike fungus *Pullularia pullulans*, in which about 480 maltotriose units are linked by $1 \rightarrow 6$ glycosidic bonds (Bender and Wallenfels, 1966). Like other naturally occurring polysaccharides mentioned before, pullulan is known to protect plasma membranes against physicochemical stimuli, such as osmotic pressure and ionic strength. However, when adsorbed to the liposomal surface



R = H (pullulan)
R = C(O)$(CH_2)_{14}CH_3$ (O-palmitoylpullulan)

Fig. 1. Chemical structure of pullulan and *O*-palmitoylpullulan.

it is easily removed by dilution. Therefore, a chemical modification of pullulan is required by conjugating a hydrophobic group which allows the polysaccharide to tightly interact with the liposomal membrane (Sato and Sunamoto, 1992). Therefore, pullulan was chemically modified by esterification which palmitoyl chloride which yields the polysaccharide derivative, OPP (Fig. 1). This product was characterized by IR and ^{1}H-NMR.

3.1.1. Infrared spectroscopy

OPP was characterized by IR spectroscopy to identify carbonyl groups and, thus, to ascertain that palmitoyl moieties are covalently bonded to pullulan. According to Pavia et al. (1979), the most characteristic carbonyl band, due to the stretching vibration of the C=O bonds, is found at about 1735 cm^{-1}. However, the synthesized OPP shows a stretching vibration band relative to a

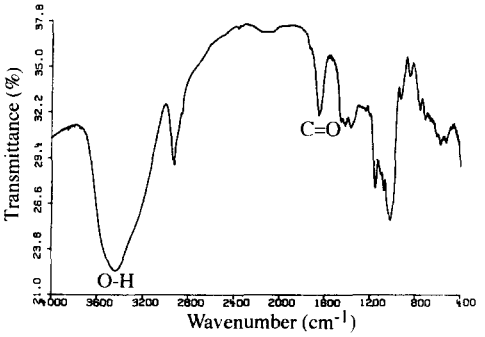


Fig. 2. IR Spectrum of OPP obtained from a KBr tablet (1%).

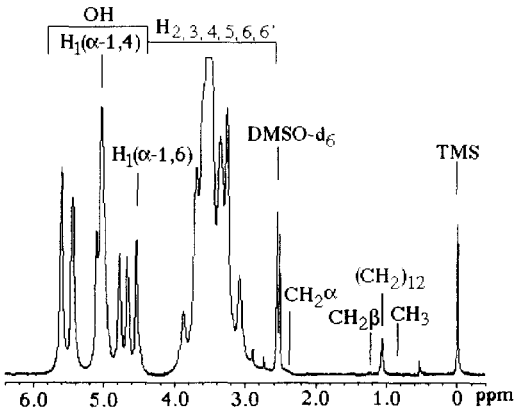


Fig. 3. ^{1}H-NMR Spectrum of OPP (50 mg/ml) in DMSO-d_6, using TMS as internal reference.

C=O bond at about 1664 cm^{-1} (Fig. 2). This frequency shift may be a consequence of intramolecular hydrogen bonds between carbonyl and hydroxyl groups, which lower the stretching force constant of the C=O bond. The establishment of hydrogen bonds was confirmed by the lower frequency stretching vibration of the O–H bond (at about 3456 cm^{-1}) and also by its higher intensity and larger band width (Fig. 2), as compared to the situation where no hydrogen bonding takes place. In the range from 1300 to 1000 cm^{-1}, the bands characteristic of the stretching vibration of the C–O bond were found (Pavia et al., 1979). These observations are consistent with the existence of an ester bond between pullulan and palmitoyl residues, indicating that the two compounds are not just physically mixed.

3.1.2. Proton nuclear magnetic resonance spectroscopy

The OPP characterization was complemented with the determination of the degree of substitution of palmitoyl residues per 100 glucose units in OPP by ^{1}H-NMR. The OPP ^{1}H-NMR spectrum (Fig. 3) permits the identification of the protons corresponding to the palmitoyl chain at: 0.858 (terminal methyl group), 1.062 (12 methylene groups), 1.234 (β-methylene group) and 2.380 ppm (a methylene group). According to a previous assignment made by Akiyoshi et al. (1990), it was possible to identify in the range of 2.60–4.20 ppm protons of the glucose residues (at positions C_2, C_3, C_4, C_5, C_6 and $C_{6'}$) of the OPP molecule. In conformity with the same authors, the signals displayed at 4.545 and 5.048 ppm correspond to the protons of C_1 position of α-1,6 and α-1,4 glycosidic bonds, respectively. Finally, according to the assignment elaborated by Casu et al. (1966), the signals within the range of 4.20–5.80 may be ascribed to the hydroxyl protons of maltotriose units.

To evaluate the substitution degree of palmitoyl residues in OPP, it is mandatory to confirm whether the signal at 1.062 ppm corresponds or not to the protons of 12 methylene groups, since Sato (1990) reported this signal as being the resonance of the protons of 13 methylene groups. To elucidate this point, the ^{1}H-NMR spectrum of palmitic acid was recorded, since palmitic acid is a molecule similar to palmitoyl chloride (both with the same number of carbon atoms) (Fig. 4). From this spectrum, it is possible to distinguish a triplet corresponding to the protons of the terminal methyl group (in the range of 0.80–0.90 ppm), a multiplet corresponding to the protons of β methylene group (in the range of 1.40–1.50 ppm) and a triplet corresponding to the protons of the α methylene group (in the range of 2.10–2.20 ppm). At 3.344 ppm the resonance signal corresponds to the carboxylic proton of paimitic acid. It can, therefore, be concluded that the peak at

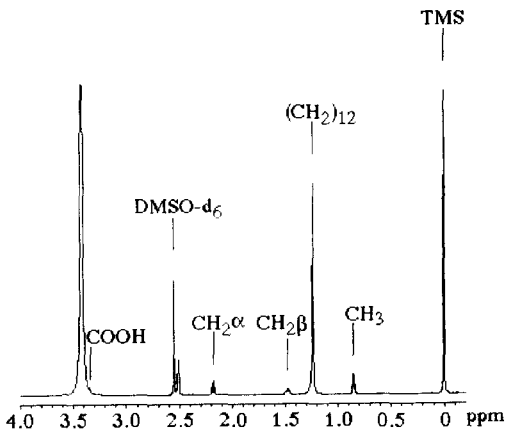


Fig. 4. ^{1}H-NMR Spectrum of palmitoyl chloride (2.56 mg/ml) in DMSO-d_6, using TMS as internal reference.

1.237 ppm, and thus, the signal at 1.062 ppm in the OPP ^{1}H-NMR spectrum (Fig. 3), corresponds, in fact, to the resonance of the protons of the remaining 12 methylene groups.

To calculate the substitution degree of palmitoyl residues in OPP, the integral value of the peak displayed at 1.062 ppm in Fig. 3 (designated as A) and the total integral value of the peaks displayed in the range of 4.20–5.80 ppm (designated as B) were estimated. The integral value correspondent to A was the result of the resonance of 24 protons from 12 methylene groups of palmitoyl chain attached to substituted maltotriose units. The integral value correspondent to B was the result of the resonance of 12 protons (9 from hydroxyl groups, 2 from α-1,4 and 1 from α-1,6 glycosidic bonds, being these last three protons located at C_1 position) on non substituted maltotriose units (whose number was designated as X) and the resonance of 11 protons (8 from hydroxyl groups, 2 from α-1,4 and 1 from α-1,6 glycosidic bonds, being these last three protons located at C_1 position) on substituted maltotriose units (whose number was designated as Y). Thus, symbolically it can be written:

$$A = 24Y \Leftrightarrow Y = \frac{A}{24} \tag{4}$$

$$B = 12X + Y \Leftrightarrow X = \frac{24B - 11A}{288} \tag{5}$$

The substitution degree of palmitoyl residues in OPP is defined by the ratio between the substituted maltotriose units (Y) and the total number of maltotriose units, both substituted (Y) and non substituted (X):

$$\text{Substitution degree} = \frac{Y}{Y + X} \tag{6}$$

Replacing in the previous Eq. (6) Y and X by their values (Eqs. (4) and (5), respectively), the following equation is obtained:

$$\text{Substitution degree} = \frac{12\text{A}}{24\text{B} + \text{A}} \tag{7}$$

Finally, substituting A and B for their correspondent integral values, the substitution degree of palmitoyl residues in OPP per maltotriose unit (each containing three glucose units) is obtained and, subsequently, per 100 glucose units:

$$\text{Substitution degree} = 0.012/\text{maltotriose unit}$$
$$= 0.012 \times \tfrac{1}{3} \times 100 = 0.4\% \tag{8}$$

Thus, the OPP characterization revealed the existence of an ester bond between pullulan and the palmitoyl group, indicating that the two compounds were covalently bonded, and allowed the computation of a substitution degree of palmitoyl residues in OPP of 0.4 per 100 glucose units (0.4%).

3.2. Effect of carboplatin on the organization of liposomal bilayer

The effect of carboplatin on lipid bilayer organization was investigated by fluorescence polarization of DPH and DPH-PA, two probes located in different regions of the lipid bilayer. These probes report similar physical parameters dependent on the organization of probe environment. Their locations across the membrane have been relatively characterized, being DPH located in the bilayer core (Mulders et al., 1986) and DPH-PA displaced to the outer regions since its charged propionic acid chain is positioned at the membrane surface (Trotter and Storch, 1989). The term bilayer organization is used here in an operational sense and aproximately proportional to the fluorescence polarization, that depends on the rotational diffusion of the probes. Disordering of the lipid bilayer environment is detected as an increase of the rotational diffusion which results on a decreased polarization of DPH probes.

The effect of entrapped carboplatin on the fluorescence polarization of DPH probes in liposomes of PC, at 37 and 50°C is shown in Table 1. Carboplatin at 27 μM (therapeutic concentration, according to Nijker, 1990), does not induce a significant change in the fluorescence polarization values of either DPH or DPH-PA at 37 and 50°C, suggesting that this drug does not modify the lipid organization (membrane fluidity) neither of the bilayer core nor of the outer domains, where the probes are located, respectively.

Table 1
Fluorescence polarization of DPH and DPH-PA in LUV with carboplatin

Sample	Fluorescence polarization			
	DPH		DPH-PA	
	37°C	50°C	37°C	50°C
Liposomes without carboplatin (control)	0.119 (± 0.003)	0.085 (± 0.009)	0.186 (± 0.002)	0.159 (± 0.006)
Liposomes with entrapped catboplatin	0.101 (± 0.004)	0.084 (± 0.004)	0.190 (± 0.005)	0.163 (± 0.002)

Liposomes were prepared with a 27 μM carboplatin aqueous solution.
Data are the mean ± S.D. for three independent determinations.

3.3. Influence of sodium chloride of the hydration medium on the encapsulation of carboplatin

The composition of the hydration medium is a major parameter regarding the optimization of liposomes containing drugs. The effects of pH (Crommelin et al., 1983) and of sodium chloride concentration (Steerenberg et al., 1987) of the hydration medium on the encapsulation of several drugs have been reported. Carboplatin has revealed an increased stability in 5% dextrose relatively to 0.9% NaCl (Wenfu et al., 1992). Therefore, liposomes were prepared by hydrating the lipids in media, at pH 6.5, containing NaCl ranging from 0 to 0.9%, and adequate amounts of dextrose to adjust to the physiological osmolarity. For example, the concentration of dextrose in the medium without NaCl was 5%.

The encapsulation efficiency (Fig. 5A) and binding capacity (Fig. 5B) of carboplatin in liposomes increase as the NaCl concentration in the hydration medium increases, reaching the highest values at 0.6% NaCl (32.8% and 187.7 μg/mol, for encapsulation efficiency and binding capacity, respectively). Further increase of NaCl to 0.9% leads to a decrease of the carboplatin encapsulation efficiency and binding capacity, to values similar to those in the absence of NaCl (16.5% and 89.4 μg/μmol). It is worthy of notice that Fichtner et al. (1993) observed a lower encapsulation efficiency of carboplatin (between 15–20%) for liposomes of hydrated PC and cholesterol, prepared by reverse phase evaporation (with a diameter ranging from of 110–1600 nm) in phosphate buffered saline at pH 7.2, when compared with the highest encapsulation efficiency value obtained in the present work (32.8%).

The variation profiles of the encapsulation parameters may be related to the variation of liposome size, as a function of the NaCl concentration in hydration medium (Fig. 5A, B). In fact, the maximum size value is observed at 0.6% NaCl (274.7 nm) and when the NaCl content increases to 0.9% the size decreases (256.5 nm). The variation of liposomes size may affect the fraction of encapsulated aqueous volume, resulting in a similar variation pattern for the encapsulation efficiency and the binding capacity of carboplatin.

3.4. Stability of liposomes with entrapped carboplatin: effect of coating

Since the maximum encapsulation efficiency and binding capacity of carboplatin in uncoated liposomes was observed at 0.6% NaCl, the stability of carboplatin liposomes prepared in this hydration medium was compared with that of carboplatin liposomes obtained in the absence of NaCl, but in a medium with the same osmolarity.

The results in Fig. 6 show that uncoated liposomes are more stable when prepared in a medium without chloride ions. In fact, after 6 days, liposomes in the absence of NaCl, but in a medium with 5% dextrose retain about 50% of its initial content in Pt, but in 0.6% NaCl only 25% of the initial Pt is retained. It has been shown that the degradation of carboplatin is accelerated in aqueous solutions containing chloride ions, leading to the formation of an ionic specimen (diamminodiaquoplatin complex) and cisplatin

(Cheung et al., 1987; Allsopp et al., 1991). Allsopp et al. (1991) found that the time required for 5% carboplatin degradation, at 25°C, in 0.9% NaCl, is 29.2 h, whereas in water it is 52.7 h. Thus, the low hydrophilicity and the increased lipophilicity of the resultant cisplatin may facilitate its passive diffusion through liposomal membranes (Freise et al., 1982; Steerenberg et al., 1987), contributing to the low stability of carboplatin liposomes prepared in the presence of chloride ions.

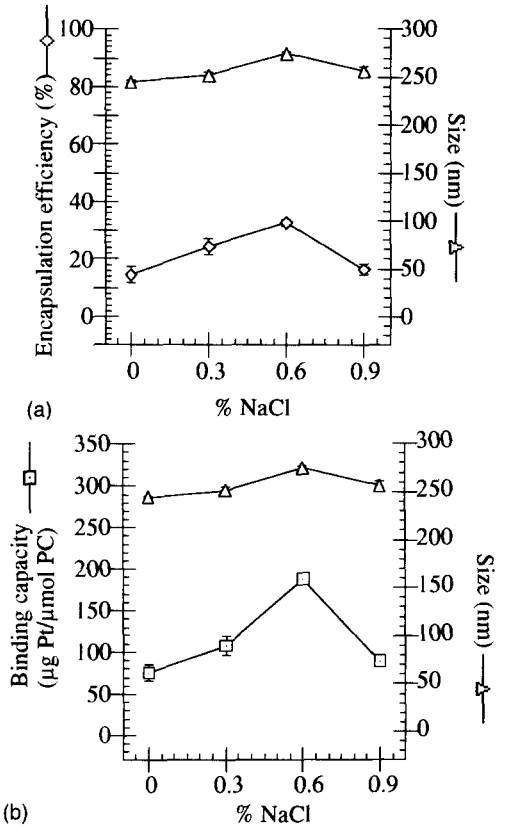


Fig. 5. Effect of NaCl concentration (0–0.9%) of the hydration medium (pH 6.5) on the encapsulation efficiency (◇) (A) and on the binding capacity (□) (B) of carboplatin in LUV of L-α egg PC. The change of liposomes size (△) is also described. All hydration media, except with 0.9% NaCl, were adjusted to physiological osmolarity with dextrose. The encapsulation efficiency is expressed as the percentage of the ratio between the carboplatin to lipid molar ratio in the final liposome dispersion and that one in the initial mixture (before extrusion and filtration through Sephadex G-25), and the binding capacity is defined as the mass of carboplatin (μg) entrapped in liposomes per μmol of phospholipid in the final liposome dispersion (after extrusion and filtration through Sephadex G-25), as indicated in Materials and Methods. Data are the mean ± S.D. for three independent determinations.

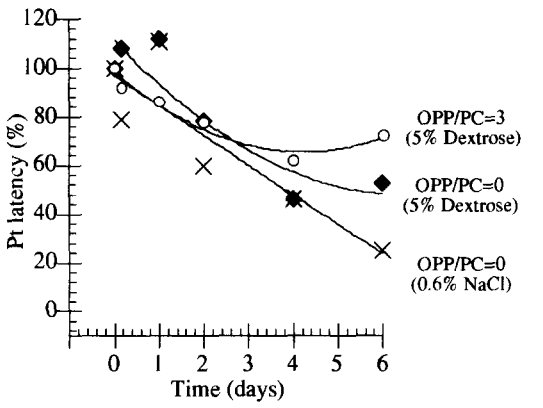


Fig. 6. Typical stability experiments of uncoated carboplatin liposomes prepared in hydration media without (◆) and with 0.6% NaCl (X), and also coated with OPP (for an OPP/PC weight ratio of 3), prepared in a hydration medium without NaCl (○), at 50°C during 6 days. All hydration media were adjusted to physiological osmolarity with dextrose. The stability is expressed as the percentage of platinum remaining associated with liposomes (platinum latency), as indicated in Materials and Methods.

The results shown in Figs. 5 and 6 suggest that either encapsulation of carboplatin in LUV or the stability of these drug containing vesicles, may be improved by the use of a hydration medium without chloride ions but with an ionic strength similar to that of a 0.6% NaCl solution.

The stability of LUV with entrapped carboplatin was further improved by coating the liposomes with OPP. Recently, it has been shown that the stability of LUV containing 5(6)-carboxyfluorescein, coated with OPP for an OPP/PC weight ratio of 3, is probably improved by decreasing the permeability and the fluidity of the outer region of the liposomal membranes (Moreira et al., 1996).

Carboplatin LUV coated with OPP and prepared in a medium without chloride ions show a higher stability than that of uncoated liposomes prepared in the same medium (Fig. 6). In fact, coated liposomes, after 6 days of incubation, retain about 70% of their initial content in Pt, a value 20% higher than that presented by uncoated

liposomes, for the same period of time. Therefore, besides the correct choice of the composition of the hydration medium, the stability of LUV with entrapped carboplatin is improved by coating the surface with OPP, at a OPP/PC weight ratio of 3.

3.5. Effect of the OPP/PC weight ratio on the stability of carboplatin liposomes

The effect of several polysaccharides on the stability of small unilamellar liposomes has been extensively studied and the relevance of the relative proportion of polysaccharide in liposomes to its stability has been described (Sato, 1990; Sunamoto et al., 1992). In fact, coating of liposomes with *O*-palmitoylamylopectin (OPAp), prepared with a substitution degree of 4.9 palmitoyl groups per 100 glucose units, for an OPAp/PC weight ratio of 3, led to a decrease of liposomal stability by enhancing membrane permeability. This effect was ascribed to the penetration of an excessive amount of palmitoyl groups into the liposomal membrane (Sato, 1990). Regarding OPP, it was also found that an excessive increase in the OPP/PC weight ratio in LUV with entrapped carboplatin, induced a drastic decrease of the liposome stability, as estimated from the Pt remaining associated with liposomes (Fig. 7). In liposomes coated with an OPP/PC weight ratio of 7.5 and prepared in a medium without chloride ions, Pt latency decreases rapidly over the time, reaching a value of about 21% after 6 days of incubation at 50°C, which is significantly lower than the one observed for liposomes coated with an OPP/PC weight ratio of 3 (70%) and also lower than that for uncoated liposomes (50%).

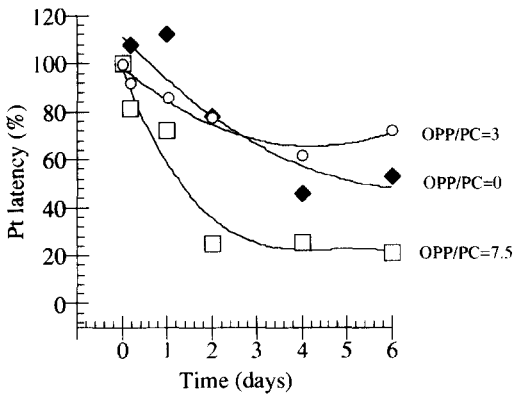


Fig. 7. Typical stability experiments of carboplatin liposomes (expressed in terms of platinum latency), uncoated (◆) and coated with OPP/PC weight ratios of 3 (○) and 7.5 (□), prepared in a hydration medium without chloride ions and adjusted to physiological osmolarity with dextrose, at 50°C for 6 days.

The lowest stability observed for those liposomes with the highest OPP/PC weight ratio may be explained by the penetration of an excessive number of palmitoyl groups in the liposomal bilayer, which could disturb the lipid packing reflected in increased permeability to carboplatin, as suggested previously by Sato (1990) for OPAp.

During the stability studies (referred to in Figs. 6 and 7), the effect of coating on the variation of liposomes size as a function of time was also evaluated. Coated liposomes with an OPP/PC weight ratio of 3 show a minor size variation (8%) as compared to uncoated liposomes (38%). However, for coated liposomes with an OPP/PC weight ratio of 7.5, it is observed the highest size variation (65%). This size variation refers to a decrease in size. Considering that the size is a parameter that plays a very important role in the in vivo distribution of colloidal systems (Müller, 1991), data presented here is relevant regarding liposomes as pharmaceutical dosage forms, once they may be stored as a dispersion where there is the real possibility of size variation upon storage.

In conclusion, carboplatin does not modify the lipid organization of liposomal membranes. Moreover, a hydration medium without chloride ions and with an ionic strength similar to that one of an aqueous solution of 0.6% NaCl may improve the encapsulation of carboplatin (encapsulation efficiency and binding capacity) and also the stability (expressed in terms of drug retention) of uncoated liposomes with entrapped carboplatin. Additionally, it is shown that coating of liposomes with OPP is an important strategy in order to improve the stability of liposomes, not only in terms of drug retention but also by preventing the size variation upon storage. It is also pointed out that the polysaccharide/lipid weight ratio must be strictly settled, as high ratios can lead to undesirable effects.

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