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Thermotropic phase behavior of DPPC liposome systems in the presence of the anti-cancer agent 'Ellipticine'

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Abstract This letter presents our first results on the structural changes in DPPC multilamellar vesicles dispersed in water in the presence of the anti-cancer agent Ellipticine. The thermotropic phase transitions of the lamellar packing inside lipid vesicles were characterized in situ by small angle X ray diffraction. The results lead to the determination of a critical concentration value for drug loading on the vesicle system around 4% molar fraction of Ellipticine, an indication of the localization of the drug in the alkyl chains and the influence of the drug on the decreasing rate of the bilayer period after the main phase transition.

Keywords Liposome · Membrane · DPPC · Ellipticine · X-ray diffraction · Drug delivery

Introduction

Phospholipid bilayers represent an efficient structural model for the study of biological membranes, and multilamellar liposomes, in particular, are the issue of intense research in several areas, notably in the development of cosmetics or medical applications such as drug material encapsulation, the subject of the present letter.

Ellipticine, 5,11-dimethyl-6H-pyrido[4,3-b]carbazole ($C_{17}H_{14}N_2$, 246.3 g/mol), originally isolated from the Ochrosia Elliptica Tree, is a highly hydrophobic alkaloid and consists of a planar heterocyclic ring system with maximum dimension of 10 Å. A precise mechanism of action has not yet been explained but it has been suggested that its antitumoral activity is due to intercalation between base pairs of helical nucleic acids (Kohn et al. 1975) and inhibition of DNA topoisomerase II activity (Singh et al. 1994). Although the antitumor specificity of this molecule is still under investigation (Stiborová et al. 2001), it was found that ELPT shows excellent antitumoral activity against experimental and human tumors when studied in vitro. Some derivatives were selected for pre-clinical studies and they inhibited the proliferation of serious metastasis processes caused by different types of leukemia, carcinoma, melanoma and sarcoma. It was also demonstrated that ELPT has anti-HIV activity (Mathe et al. 1998). Despite of these great advantages, clinical tests were discontinued because the drug appeared not to reach the desired site of action satisfactorily, apparently due to its insolubility in aqueous media (Sainsbury 1990).

The hydrophobic region of lipid vesicles can easily incorporate this kind of hydrophobic molecule for delivery to the desired site of action. For the development of liposome preparations for drug delivery or drug targeting the compositional and structure parameters relevant for drug incorporation, (e.g., drug critical concentration, liposome critical size and stability)

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and the way to program the drug release using the appropriate trigger (e.g., pH, liposome phase transition temperature or lipid bilayer interaction with target cells) must be studied.

In this work, the structural changes in the thermotropic lamellar phases of liposomes in the presence of the drug ELPT were investigated by measuring the diffraction patterns of large multilamellar vesicles in water dispersions heated in situ.

Experimental

Synthetic L-α-dipalmitoylphosphatidylcholine (DPPC) (Sigma) was first dissolved in chloroform and ELPT (Sigma) was dissolved in methanol. The two solutions were mixed together in appropriate proportions to achieve the desired molar fractions. We used two methods of preparing vesicles in solution based on the literature (Torchillin and Weissig 2003; Wack and Webb 1989; Bota and Kriechbaum 1998). In the first method, the evaporation of the solvent was done through a N_2 stream. Then, dried samples were dispersed in a BIS-TRIS buffer solution (0.01 M, pH 7.4) under different dilutions and stored at 4°C for 12 h to promote swelling of the lipids. After that, the lipid system was stirred by vortex at ~50°C and stored again at 4°C for at least 4 weeks before data collection. During this period, each sample was periodically heated and stirred by vortex. In the second method we used a rotoevaporator to mix, to remove the solvent (through a water-jet pump connected to a tap) and also to disperse the samples with the same buffer solution cited before. X ray diffraction data were collected at the Brazilian Synchrotron Light Laboratory (LNLS) using the SAXS (D11) beamline facility (Bernardes et al. 1992; Kellermann et al. 1997). The X ray beam energy used was 8.4 keV. The scattered intensity as a function of momentum transfer $I(q)$ was registered in the range of $0.009 < q < 0.305$ Å^{-1} ($q = 4\pi \sin\theta/\lambda$, where θ was one half of the scattering angle). The experiments were performed with transmission geometry. A hollow copper chamber surrounding the sample holder coupled it to a thermal bath. The temperature control ranged from 25 to 50°C. The samples were heated at a rate of (0.6 ± 0.1)°C/min and up to 25 successive 1 min-exposure diffraction patterns were recorded in situ. Five fixed temperatures were selected to repeat the measurements with a longer exposure time in order to confirm the results obtained during the heating scan.

Results and discussion

We studied two series of large multilamellar vesicles (LMLV) with different DPPC/ELPT molar ratio. The first one was vortex stirred and had the following DPPC/ELPT molar ratio (or %ELPT): 20:1 (5%), 30:1 (3.3%) and the pure DPPC (100:0 or 0%). The second series was stirred through a rotoevaporator and had the following molar ratio (or %ELPT): 10:1 (10%), 20:1 (5%), 30:1 (3.3%), 40:1 (2.5%), 50:1 (2%), 70:1 (1.4%), 100:0 (0%). Three concentrations of the dispersions were studied: 3, 10 and 19 mg/ml of lipid in water.

Figure 1 shows a heating scan from room temperature up to 50°C with three distinct thermotropic phases, Lβ', Pβ' and Lα of pure DPPC. This heating scan was taken as an example for explaining the general behavior of all studied systems. The room temperature Lβ' lamellar gel phase is characterized by a first order diffraction peak from a periodicity of 66 Å. In this phase the hydrocarbon chains must be rigid and the polar groups laid on a two-dimensional surface. As temperature increases a ''pre-transition'' appears after 35°C as found by Tardieu et al. (1973). The peak position shifts toward to lower values with a periodicity around 77 Å. This characterizes the Pβ' lamellar gel phase. For temperatures above ~42°C during the P$\beta' \rightarrow$ Lα lamellar phase transition (main transition-T_m), we observe in the graph that the peak becomes sharper and is shifted toward higher values of q. The starting point is with a periodicity of 68 Å that decreases continuously with the increase of temperature up to ~50°C. As already known, the main characteristics of the Lα lamellar liquid crystal phase is the fact that the polar groups come back to the two-dimensional-plane conformation without any ripple pattern as found in Pβ' phase and the hydrocarbon chains become completely disordered and fill homogeneously the space between polar groups.

The diffraction patterns highlighted in Fig. 1 were taken to determine the phase transition temperature T_m with the precision of half of the temperature step between two subsequent scans (error bar in Fig. 2).

With the same procedure we determined the phase transition as a function of drug concentration for each sample of the two studied series. Even though these two series were not prepared under the same conditions, which can influence in several aspects as stability, poly-dispersity of size and shape, they exhibited similar behavior shown in Fig. 2. The T_m started to decrease around 2% of added ELPT. A minimum T_m was observed around 4% of ELPT (in between the measured points at 3.3% and 5%) and after 5% the value of T_m

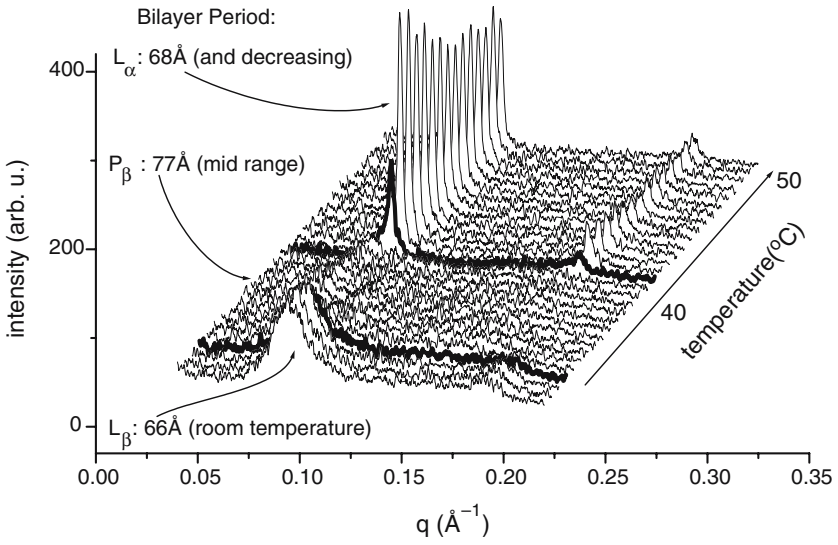

Fig. 1 Temperature dependent evolution of the lamellar diffraction peaks of pure DPPC liposomes in aqueous media (19 mg/ml) heated from 25 to 50°C at a rate of (0.6 ± 0.1)°C/min. Each diffraction pattern was recorded with 1 min of exposure time

increased coming back to values close to the pure DPPC system suggesting that there is saturation for ELPT incorporation. After the saturation, the drug content might be expelled from the bilayers; that is why the system comes back with the same behavior of the pure system. On account of this experimental fact, the critical value of ELPT that produces the minimum T_m could be considered the loading critical concentration of the liposome system. The same value for the critical concentration was found to produce ELPT crystalline phase in a similar multilayer system deposited on a glass substrate (Cavalcanti et al. 2006). This fact corroborates with the hypothesis of an expelled material, which could crystallize in aqueous media after the saturation.

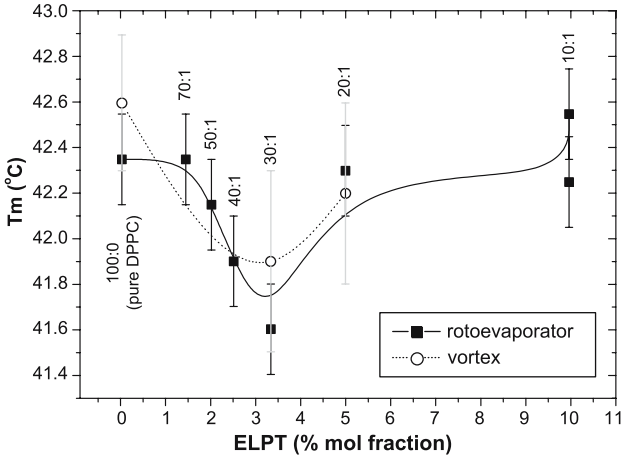

Fig. 2 Main phase transition temperature ($P\beta' \rightarrow L\alpha$) of the LMLV of DPPC/ELPT systems for two kinds of series prepared by rotoevaporator (*filled rectangle*) and prepared by vortex (*open circle*). The concentration of the dispersions is 10 mg/ml of lipid in water. The DPPC:ELPT molar ratio is indicated for each point. The profile suggests a minimum T_m around 4% (in between the measured points at 3.3% and 5%) of ELPT (mol) for both series. The continuous lines are eye guides only. The two points at 10% of ELPT concentration are two samples from the same stock solution measured one after the other

For temperatures above T_m, the system is in the Lα phase and presents a sharper low angle peak corresponding to the $d = 67$ Å (see Fig. 3a–c). At room temperature the system is in the lamellar-gel Lβ' phase and presents a low angle diffraction peak corresponding to $d = 66$Å lamellar spacing (see Fig. 3d–f). For both phases, the position of the peak changes only some tenths of angstrom as a function of drug concentration or dilution, which is comparable with the error bar size. However, concerning the peak width of all curves, we can observe two different behaviors: above T_m the measured width is around 0.0025 Å^{-1} for all curves of Fig. 3a–c; below T_m, at room temperature, the peak width increases significantly with minor changes in drug concentration for all the three dilutions of the system as we can see in Fig. 3d–f. This result reinforces the hypothesis that the ELPT is incorporated entirely into the hydrophobic region of the lipid matrix. At room temperature, the rigid β' conformation of the alkyl chains can induce defects and, consequently, disturb the long-range order of the lamellar packing. However, the same does not occur above T_m, in the Lα lamellar phase, where there is no important change in the shape and width of the diffraction peaks for different concentration of ELPT. Since no rigidity from the highly disordered hydrocarbon chain in the α conformation is offered to the positioning of the drug, no disturbance of the lamellar

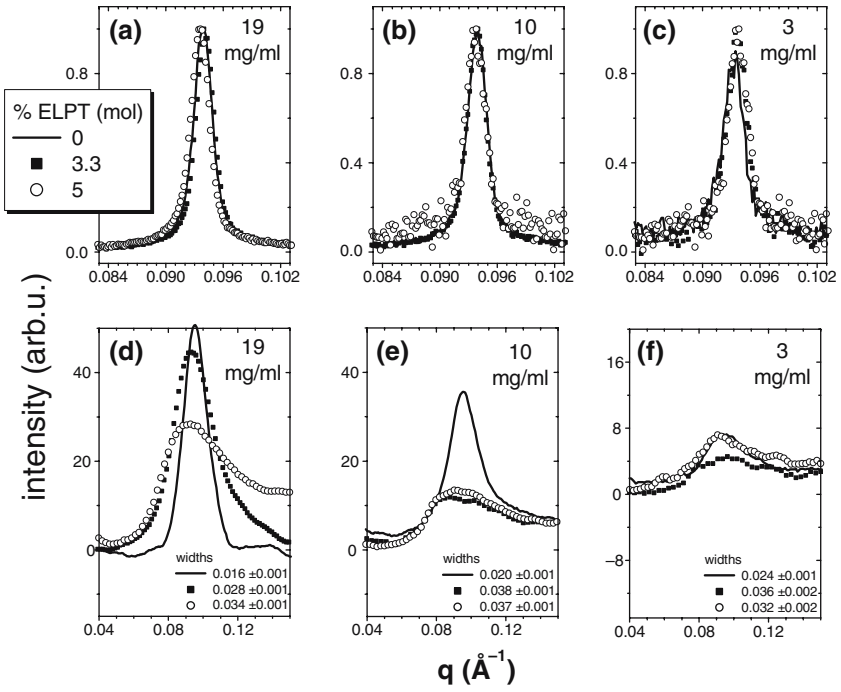

Fig. 3 X ray diffraction patterns for DPPC/ELPT lamellar systems. Measurements were done with an exposure time of 20 min for each curve. Graphs **a**, **b** and **c** refer to the Lα liquid crystalline phase (50°C); the intensities are normalized to the unity; the widths for these curves are around 0.0025Å^{-1}. Graphs **d**, **e** and **f** refer to Lβ' gel phase (25°C); intensities are not normalized; the widths are changing with drug concentration and dilution of the system

packing can be observed in this phase. The hydration also affects the lamellarity of the system at room temperature as we can see from the comparison of the peak widths in Fig. 3d–f. As the intensities are very weak for these diluted systems, further measurements would be necessary to determine more accurate parameters.

Analyzing the bilayer thickness evolution of the phase transition for each sample, we observed that above T_m, the hydrocarbon chains become highly disordered and the period of the liposome bilayers decreases continuously in a phenomena that we could call thermal compressibility (Fig. 4). The decreasing rate for the Lα lamellar phase of DPPC has an average linear value of 10^{-3}/°C (0.1% per degree) in agreement with results reported by Tardieu et al. (1973). A curve fitting was made in the range of 43–48°C and we found that the relaxation obeys the first order exponential decay $d_0 + Ae^{k(t-t_0)}$, where d_0 and t_0 are the values of periodicity and temperature, respectively, just after the main transition (see parameter values in Table 1). We observed that adding ELPT into the system attenuates the decreasing rate, k, of liposome bilayer period making the phase transition less abrupt. The DPPC liposome sample with higher ELPT concentration showed a decreasing rate of 15% less than the pure system. Although it seems there is no influence of the drug inside the membrane after T_m by looking at the results pointed in Fig. 3, we have evidence that the drug is still in the system by looking how the decreasing rate changes as a function of ELPT concentration at the Lα lamellar phase.

Summary and final remarks

In order to study how the structure of lipid model membranes can be affected by the presence of a hydrophobic drug in some different thermotropic phases we prepared samples of DPPC multilamellar vesicles mixed with the anticancer agent ELPT to be

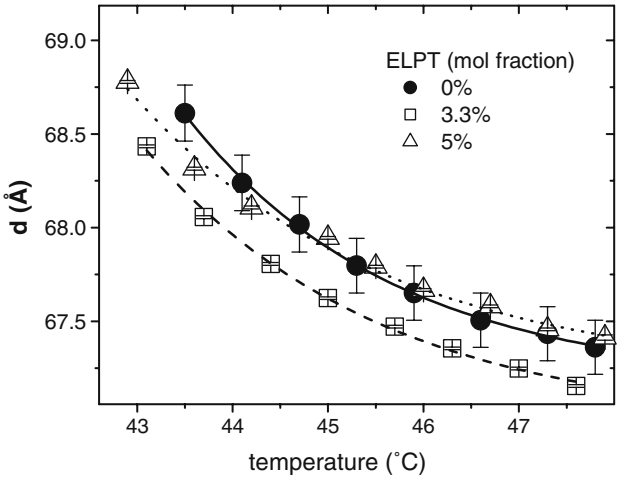

Fig. 4 Decreasing rate of bilayer period at concentration of 19 mg/ml. The lines correspond to an exponential decay function fitting for each case (see parameters in Table 1)

Table 1 Decreasing rate of bilayer period fitted by a first order exponential decay

ELPT (%)	Water dilution (mg/ml)	Temperature range used for fitting		Fitting curve parameters $(d_0 + Ae^{k(t-t_0)})$,		
		t_0	t_f	d_0	A	k
0	19	43.5	47.8	67.16 ± 0.03	1.44 ± 0.03	0.45 ± 0.02
	10	43.2	47.9	67.37 ± 0.03	1.55 ± 0.03	0.45 ± 0.02
3.3	19	43.1	47.6	66.94 ± 0.06	1.47 ± 0.06	0.41 ± 0.04
	10	43.3	47.6	67.04 ± 0.06	1.54 ± 0.06	0.42 ± 0.04
5	19	42.9	47.9	67.2 ± 0.1	1.55 ± 0.09	0.38 ± 0.05
	10	43.0	46.7	–	–	–

The drug concentration is indicated both in percentage of ELPT in relation to DPPC

t_0 and t_f are the initial and final temperature of the considered range; d_0 is the bilayer period at t_0; A is a fitting constant; k is the decreasing rate

investigated by X ray diffraction when heated from room temperature up to 50°C in situ.

The first observation was that the main thermotropic phase transition, T_m, change as a function of drug concentration in the system. The results pointed to a critical concentration of the drug around 4% of added ELPT for loading the vesicle system. The quantification of a critical concentration is mainly important for development of pharmaceutical formulations.

Next we compared the influence of the drug on the lamellarity of the system in two different phases by monitoring the diffraction peak width. A significant change in the peak width was observed when the alkyl chains are in the β' conformation (room temperature) when compared to the α conformation (at 50°C, after T_m). Consequence of this result was the hypothesis of the insertion of the drug, when in the molecular form, into the hydrophobic region of the vesicles disturbing the order of the lamellar packing when facing the rigidity offered by the alkyl chains in the β' conformation.

Finally, we also observed that, even though influencing less on the order of the lamellar structure, there is an evidence that the drug is present into the system, after T_m. This evidence is a change in the decreasing rate of the bilayer period as a function of ELPT concentration at the Lα lamellar phase.

Further studies associating calorimetric measurements are foreseen to complement the present results.

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