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PHASE BEHAVIOUR OF POLYMER-GRAFTED DPPC MEMBRANES FOR DRUG DELIVERY SYSTEMS DESIGN

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DPPC dispersions containing DPPE with attached PEG of molecular masses 350, 2000 and 5000 were investigated by DSC in order to determine their phase behaviour and potential use as drug delivery systems. In comparison with previously obtained ESR data, DSC provided a definition of the lipid composition and temperature at which the vesicles are in a liquid crystalline phase. For DPPC DPPE-PEG 350 the composition range is at molar fractions $0<\chi_{PEG350}<0.5$.For DPPC DPPE–PEG 2000 the range of applicability is $0<\chi_{PEG2000}<0.07$ and for DPPC/DPPE–PEG 5000 system it is $0<\chi_{PEG5000}<0.05$.

Keywords: *DPPC dispersions, drug delivery systems, phase behaviour, polymer grafted lipids*

Introduction

Aqueous dispersions containing phospholipids that are derivatized in their polar head group with different hydrophilic moieties, such as long chain saccharides [1, 2], proteins [3, 4] and synthetic polymers [5–7] are of special interest both for basic biomembrane research and for biotechnological applications [8]. In particular, surface-modified liposomes with poly(ethylene glycol) (PEG) chains, known as sterically stabilized liposomes (SSLs), act as very effective drug encapsulation and delivery systems [8–10]. SSLs have an enhanced circulation time in vivo, this being mainly ascribed to the steric barrier provided by brushes of end-grafted PEG chains that are highly resistant to protein absorption in aqueous media. The most widely used formulation of SSLs contains phosphatidylcholines (PCs) and micelle-forming lipids in which poly(ethylene glycol) polymers of different molecular masses are covalently coupled to the polar head of phosphatidylethanolamines.

To improve the design of PEG-grafted SSLs, it is essential to study both the ways in which the polymer-grafted lipids interact with the structural lipid matrix and the thermodynamics of the resulting bilayer. These factors ultimately determine the stability of SSLs in serum and the efficiency with which they are able to deliver and release their content upon interaction with cells. In this study, differential scanning calorimetry (DSC) was used to investigate the stability of fully hydrated aqueous dispersions of zwitterionic dipalmitoyl phosphatidylcholine (DPPC) mixed with poly-(ethylene glycol)s dipalmitoyl phosphatidylethanolamine (DPPE–PEGs) over the entire range of relative compositions (0÷100 mol%). PEGs of different chain length were used in order to investigate the effect of the polymer headgroup size on the lyotropic and thermotropic phase behaviour of DPPC/DPPE–PEGs mixtures. The PEGs used were as follows: PEG:350 (degree of polymerization, n_p, of ~8 oxyethylene monomeric units and Flory radius $R_F \approx 1.5$ nm), PEG:2000 (n_p ~45 and $R_F \approx 3.5$ nm), and PEG:5000 (n_p ~114 and $R_F \approx 6.7$ nm). In order to characterize the structure of the different lipid systems, the DSC data obtained were analyzed in the light of previously obtained Electron Spin Resonance (ESR) spectra for the same spin-labelled systems [11, 12].

Materials and methods

Materials

Synthetic1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was purchased from Sigma (St. Louis, MO). High-purity (>99%) poly(ethylene glycol)-lipids (PEG-lipids), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) covalently linked with PEGs of average molecular masses of 350 Da (PEG:350–DPPE), 2000 Da (PEG:2000–DPPE) and 5000 Da (PEG:5000–DPPE), were obtained from Avanti Polar Lipids (Birmingham, AL). Reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH 7.5 were purchased from Merck (Darmstadt, Germany).

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Sample preparation

Lipid dispersions were prepared by dissolving the required amounts of DPPC and PEG–DPPE lipids in chloroform. The solvent was evaporated in a nitrogen gas stream and then kept under vacuum overnight. The dried lipid samples were fully hydrated with PBS at pH 7.4 by heating at 60°C and periodically vortexing for 40 min until homogeneous lipid dispersions were obtained (final lipid concentration 2 mg mL^{-1}). Three preparations were produced for each sample. DSC analysis of different preparations of the same lipid system consistently showed that any differences were negligible.

DSC measurements

DSC scans were carried out using a second generation high-sensitivity SETARAM micro-differential scanning calorimeter (microDSC III) with 1 mL stainless steel sample cells, interfaced with a BULL 200 Micral computer. The sampling rate was 1 point/s in all measurement ranges. The same solution without the sample was used in the reference cell. Both sample and reference were heated in a temperature range 30–50°C at a scanning rate of 0.5 K min^{-1}. In order to obtain excess heat capacity (Cp_{exc}) curves, buffer–buffer base lines were recorded at the same scanning rate and then subtracted from sample curves as described elsewhere in the literature [13]. Average noise level was about ±0.4 μW and reproducibility at refilling was about 0.1 mJ K^{-1} mL^{-1}. Calibration in energy was obtained by providing a definite power supply, electrically generated by an EJ2 SETARAM Joule calibrator within the sample cell. In order to check the reproducibility of the results, three identical samples were scanned. For each sample, three heating and two cooling scans were recorded. 0.7 mL of lipid dispersion were used in each experiment. Kinetic effects were not evident when all DSC experiments were repeated at 24 and 48 h. Multicomponent DSC peaks were deconvoluted into Gaussians by using the Microcal Origin 7 software, which is based on the simplex minimization algorithm.

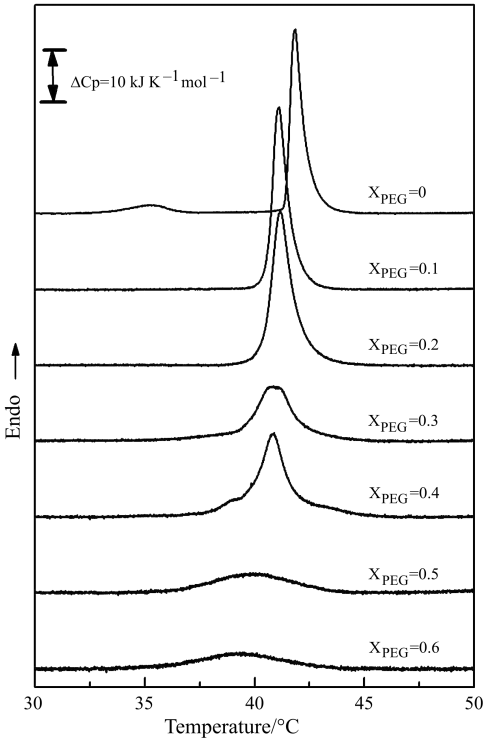

Fig. 1 DSC curves of DPPC/PEG350–lipid liposomes at molar fractions $\chi_{PEG-350}$ ranging from 0 to 0.6

Results

DSC of DPPC/DPPE–PEG 350 vesicles

Figure 1 shows the DSC scans of DPPC/DPPE–PEG 350 mixtures at different DPPE–PEG 350: DPPC molar χ_{PEG} ratios. It can be noted that, for all χ_{PEG} values, the incorporation of DPPE–PEG 350 lipid into DPPC liposome causes the disappearance of the gel–ripple phase pretransition and decreases the temperatures of the main transition of DPPC. In particular, a unique DSC peak centered at T_m=41.1±0.1°C is observed at $\chi_{PEG-350}$=0.1. At a molar fraction of $\chi_{PEG-350}$=0.2 the DSC peak is located at 41.2±0.4°C. At molar fractions of $\chi_{PEG-350}$=0.3 and $\chi_{PEG-350}$=0.4 two multicomponent peaks can be observed. Deconvolution of the DSC curves (as an example the deconvolution of the DSC transition of the system $\chi_{PEG-350}$=0.3 is reported in Fig. 2) revealed the presence of at least three components associated with molar fraction $\chi_{PEG-350}$=0.3

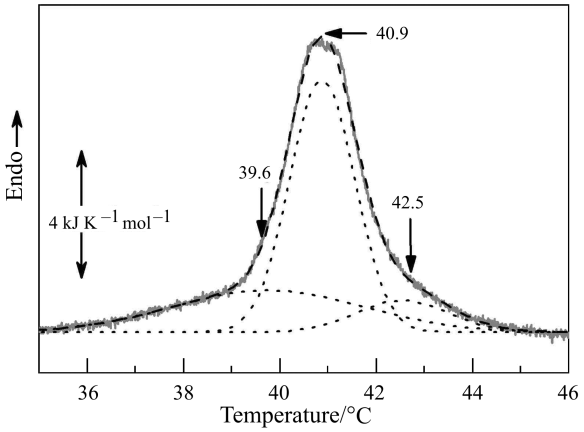

Fig. 2 Deconvolution of the DSC curve relative to the system DPPC/DPPE–PEG 350 at a molar fraction $\chi_{PEG-350}$=0.3; gray line – experimental DSC curve; dotted lines – deconvoluted peaks; dashed line – sum of the deconvoluted peak

Table 1 Phase transition temperatures obtained from the DSC curves reported in Fig. 2 obtained at different of DPPC/PEG–350 lipid at different molar ratios. Where necessary, the main transition was deconvoluted according to the procedure reported in experimental section

$X_{DPPE–PEG}$	Gel→ripple phase transition*	Component of the main transition	
	T_1/°C	T_2/°C	T_3/°C
0.0	–	41.8±0.2	–
0.1	–	41.1±0.3	–
0.2	–	41.2±0.4	–
0.3	39.6±0.3	40.9±0.1	42.5±0.5
0.4	40.4±0.2	40.8±0.5	43.0±0.4
0.5	–	39.9±0.1	-
0.6	–	39.5±0.6	-

*Each value is an average of three experiments ± standard deviation

and $\chi_{PEG–350}$=0.4. At molar fractions $\chi_{PEG–350}$=0.5 and $\chi_{PEG–350}$=0.6 only a broad peak was observed. The temperatures of all the DSC peaks obtained at different $\chi_{PEG–350}$ values are shown in Table 1.

DSC of DPPC/DPPE–PEG 2000

DSC analysis of DPPC/DPPE-PEG 2000 lipids (Fig. 3) showed that grafted DPPE lipids greatly modify the *Cp* profile of DPPC even at low molar fractions. A narrower range of molar fractions was thus investigated, since mixing the two components at molar fractions $\chi_{PEG–2000}$>0.4 provides lipid aggregates which do not exhibit thermal transitions. At $\chi_{PEG–2000}$=0.07 the main transition is centred at 41.4±0.03°C. A weak transition which resembles the gel–ripple phase transition at T=32.6±0.2°C is observed. The system at a molar fraction of $\chi_{PEG–2000}$=0.1 shows a peak centered at T=41.8±0.5°C. A small peak appears at 33.9±0.3°C. Both lipid systems at molar fractions of $\chi_{PEG–2000}$=0.12 and $\chi_{PEG–2000}$=0.15 show a broad peak resembling the main transition. This can be deconvoluted into two components as reported in Table 2. At molar fraction $\chi_{PEG–2000}$=0.18 the main transition can be deconvoluted in at least two components at T=41.1±0.4°C and 43.1±0.5°C. At molar fraction $\chi_{PEG-2000}$ = 0.2 two components located at T=41.6±0.1°C and T=42.6±0.5°C may be observed. At molar fractions $\chi_{PEG–2000}$=0.3 and $\chi_{PEG–2000}$=0.4 only broad transitions located at T=44.8±0.3°C and 45.8±0.4°C, respectively, were observed.

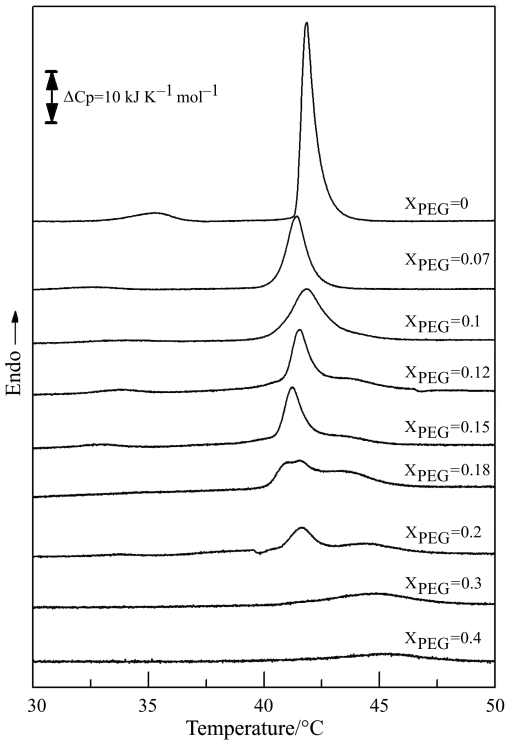

Fig. 3 DSC curves of DPPC/PEG2000–lipid liposomes at molar fractions $\chi_{PEG–2000}$ ranging from 0 to 0.4

Table 2 Phase transition temperatures obtained from the DSC curves reported in Fig. 3 obtained at different of DPPC/PEG–2000 lipid at different molar ratios. Where necessary, the main transition was deconvoluted according to the procedure reported in experimental section

$X_{DPPE-PEG}$	Gel→ripple phase transition*	Component of the main transition	
	T_1/°C	T_2/°C	T_3/°C
0.00	35.2±0.1	41.8±0.2	–
0.07	32.6±0.2	41.4±0.3	–
0.10	33.9±0.3	41.8±0.5	–
0.12	33.6±0.2	41.5±0.4	42.3±0.1
0.15	32.6±0.6	41.2±0.6	41.8±0.2
0.18	–	41.1±0.4	43.1±0.5
0.20	33.6±0.4	41.6±0.1	42.6±0.5
0.30	–	44.8±0.3	–
0.40	–	45.8±0.4	–

*Each value is an average of three experiments ± standard deviation

DSC of DPPC/DPPE–PEG 5000 vesicles.

The incorporation of DPPE–PEG 5000 into DPPC model membranes up to $\chi_{PEG–5000}$<0.1 does not eliminate the gel→ripple pretransition. However, as shown in Fig. 4, the area of the DSC peak relative to this transition becomes smaller as the percentage of poly-

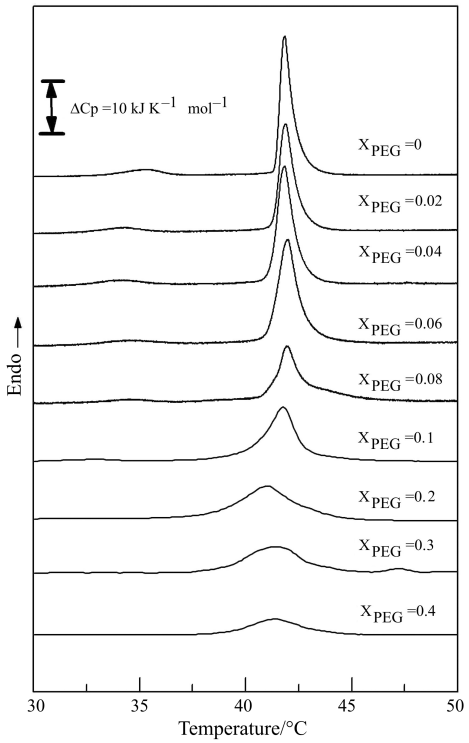

Fig. 4 DSC curves of DPPC/PEG5000-lipid liposomes at molar fractions $\chi_{PEG\text{-}5000}$ ranging from 0 to 0.4. ΔH and T_m of the deconvoluted peaks are reported in Table 3

mer-grafted DPPE lipids increases. The gel→ripple phase transition disappears at $\chi_{PEG-5000}$>0.1, and only a broad main transition that can be deconvoluted into two components was observed (Table 3). All the systems at molar fractions $\chi_{PEG-5000}$=0.02, $\chi_{PEG-5000}$= 0.04 and $\chi_{PEG-5000}$=0.06 exhibit a similar thermotropic behaviour. The main transition is located at about 41.9±0.1°C and the gel→ripple phase transition is hardly visible. At molar fraction $\chi_{PEG-5000}$=0.08 the gel→ripple phase transition is almost undetectable and the main transition is deconvoluted in two components located at 41.9°C and 42.6±0.1°C. At molar fractions $\chi_{PEG-5000}$=0.1 and $\chi_{PEG-5000}$=0.2, the gel→ripple phase transition disappears and only a broad peak, constituted by two components is observed. At higher molar fractions, i.e. $\chi_{PEG-5000}$=0.3 and $\chi_{PEG-5000}$=0.4, only a broad peak located at about 41°C can be detected.

Discussion

Phase diagram of DPPC/DPPE–PEG 350

Optimizing the content release of liposomes requires detailed knowledge of the phase behaviour of the system adopted, particularly in terms of the molar fraction of the PEG-grafted lipid and temperature.

It has already been shown that, depending on temperature and composition, DPPC/DPPE–PEG

Table 3 Phase transition temperatures obtained from the DSC curves reported in Fig. 4 obtained at different of DPPC/PEG-5000 lipid at different molar ratios. Where necessary, the main transition was deconvoluted according to the procedure reported in experimental section

$X_{DPPE-PEG}$	Gel→ripple phase transition*	Component of the main transition	
	T_1/°C	T_2/°C	T_3/°C
0.00	35.2±0.1	41.8±0.2	–
0.02	34.2±0.1	41.9±0.1	–
0.04	34.2±1.1	41.8±0.1	–
0.06	34.5±0.1	41.9±0.1	–
0.08	34.8±0.1	41.9±0.1	42.64±0.1
0.10	32.6±0.1	41.3±0.1	41.75±0.1
0.20	31.5±0.1	40.9±0.1	41.28±0.1
0.30	–	41.2±0.1	–
0.40	–	41.3±0.1	–

*Each value is an average of three experiments ± standard deviation

liposomes prepared in a large excess of water (lipid concentration ~25 mM) can be found in four different phases: gel, ripple, liquid-crystal or micellar phase [11, 12]. Spin label ESR measurements have been yet used to identify different phases of the bilayer in which the motions of the lipid chain are limited (e.g. gel and ripple phase) or not (e.g. liquid crystal state and micellar phase). However ESR measurements cannot distinguish between gel and ripple phases or liquid crystal and micellar phase. Optical density measurements at 400 nm provide qualitative information about the formation of micelles. In particular, the bilayer→micelles transition is accompanied by a decrease in OD measurements. Combining spin-label ESR spectra with OD measurements, several authors have thus observed that for the DPPC/PPE–PEG 350 system at $0.2<\chi_{PEG-350}<0.6$ at temperature of T=30°C, the micelles and gel phases coexist [11, 12]. At $\chi_{PEG-350}$<0.2 only the gel or ripple phase is present and at $\chi_{PEG-350}$>0.6 there are only micelles or bilayers in a liquid crystalline phase.

The DSC results reported in this paper extend the scope of these previously published data allowing to make a clear distinction between the gel and ripple phases and to distinguish the liquid crystal from the micellar state. In fact, DSC measures the gel→ripple and gel→liquid crystal transition temperatures, thus clarifying the border lines separating the different phases.

The informations provided by ESR, OD and DSC data presented here can be combined in the phase diagram reported in Fig. 5. In the phase diagram, points along the y-axis represent the tempera-

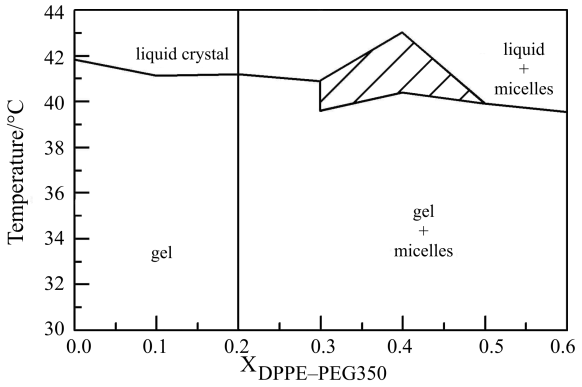

Fig. 5 Phase diagram of DPPC/PEG350-lipid liposomes. The solid lines are drawn to guide the eye through the transition temperatures obtained from DSC peaks. The hatched regions represent the regions in which the main transition can be deconvoluted in multiple components which, on turns, are due to phase segregations

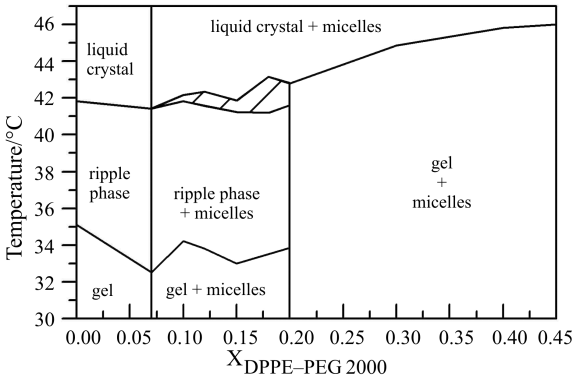

Fig. 6 Phase diagram of DPPC/PEG2000-lipid dispersions. The solid lines are drawn to guide the eye through the transition temperatures obtained from DSC peaks. The hatched regions represent the regions in which the main transition can be deconvoluted in multiple components which, on turns, are due to phase segregations

ture-induced phase transitions of pure DPPC. In particular the point located at 35.2°C represent the gel–ripple phase transition, as well as point at 41.8°C. The point at 41.8°C is related to the gel→liquid-crystal phase transition. At molar fraction $\chi_{PEG-350}$=0.1 the gel→ripple phase transition cannot be observed and the gel→liquid crystal phase transition occurs at 41.6°C. At molar fraction $\chi_{PEG-350}$=0.2 the point located at 41.2°C indicates the transition from lamellar gel phase to lamellar liquid crystal. At molar fraction $\chi_{PEG-350}$=0.3 previously published ESR [11] measurements have shown the presence of micelles and the coexistence of micelle and lamellar structures below the temperature of phase transition located at 39.6°C. This phase transition temperature has been ascribed to the gel-liquid crystal of lamellar structures. The two components of the DSC curves shown at this molar fraction are probably due to the phase transition of boundary lipids, which originate phase segregation. At molar fraction $\chi_{PEG-350}$=0.4, the DPPC/DPPE–PEG 350 system exhibits a similar thermotropic behaviour, even if the transition temperatures are slightly higher. At molar fraction $\chi_{PEG-350}$=0.5, only a broad transition was detected at 39.9°C as shown in Fig. 4. This is ascribable to the gel→liquid crystal transition of the residual bilayer. The phase diagram shows a point at a temperature of 39.5°C and a molar fraction of $\chi_{PEG-350}$=0.6. This indicates a gel→liquid crystal transition similar to that observed at $\chi_{PEG-350}$=0.5. In the $0.7<\chi_{PEG-350}<1$ concentration range DSC analysis did not reveal any transitions. This is in agreement with ESR data which indicate only the presence of micelles alone at this molar fraction [11].

Phase diagram of DPPC/DPPE–PEG 2000

Previously reported ESR and OD data have demonstrated that for the DPPC/DPPE–PEG 2000 system at molar fraction $0.007<\chi_{PEG-2000}<0.45$ micelle and bilayer (in a gel or ripple phase) coexist [11]. The DSC data here reported demonstrates that at molar fraction $\chi_{PEG-2000}<0.07$ the liposomes exhibit a thermotropic behaviour similar to the pure DPPC system. At molar fraction $\chi_{PEG-2000}$=0.07 it is possible to determine two temperatures, T=32.6 and 41.4°C, corresponding to phase transitions gel→ripple, and liquid crystal→gel as observed for pure DPPC (Fig 6). However gel→crystal phase transition, which is present up to molar fraction $\chi_{PEG-2000}$= 0.2, shifts to lower temperature values because of the destabilizing effect of the polymer on the surface of the bilayer. If compared to DPPC/DPPE PEG 350 system, in the DPPC/DPPE PEG 2000 system the gel phase is destabilized at lower molar fractions of PEG due to the longer polymer chain. At a molar fraction of $\chi_{PEG-2000}$=0.1 the temperature-induced gel→ripple phase transition occurs at 33.9 °C and the ripple→liquid crystal phase transition is detected at 41.8°C. A third transition is shown at 42.1°C which is probably ascribable to the gel→liquid crystal phase transition of the DPPC boundary lipids surrounding the polymer-grafted DPPE. At molar fractions ranging from $\chi_{PEG-2000}$=0.10 to $\chi_{PEG-2000}$=0.2, the systems exhibit the same thermal behaviour. At molar fractions $\chi_{PEG-2000}$=0.3 and $\chi_{PEG-2000}$=0.4 the DPPC/DPPE–PEG 2000 system does not exhibit the gel→ripple phase. A residual amount of bilayer mixed with micelles is still present as evidenced by the gel→liquid crystal transitions occur-

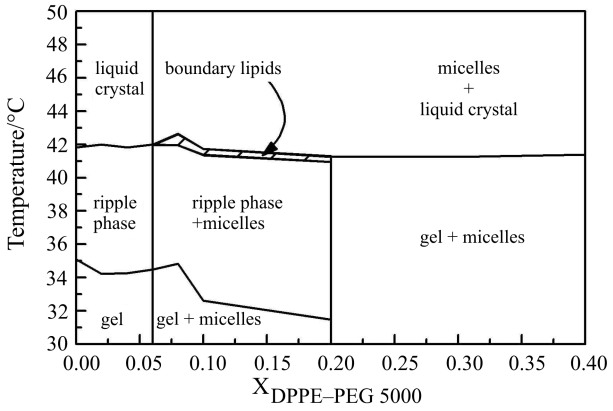

Fig. 7 Phase diagram of DPPC/PEG 5000-lipid liposomes. The solid lines are drawn to guide the eye through the transition temperatures obtained from DSC peaks. The hatched regions represent the regions in which the main transition can be deconvoluted in multiple components which, on turns, are due to phase segregations

ring at temperatures of 44.8 and 45.8°C, respectively. At molar fractions $\chi_{PEG-2000}>0.45$ the DSC curves do not show thermal transitions because at these molar fractions only micelles are present, in agreements with previously reported ESR data [11].

Phase diagram of DPPC DPPE-PEG 5000

In Fig. 7 the phase diagram of DPPC/DPPE–PEG 5000 is reported. Previously reported ESR and OD measurements [11, 12] have shown that at molar fractions $\chi_{PEG-5000}<0.06$ micelles are absent, at $0.06<\chi_{PEG-5000}< 0.2$ bilayers and micelles coexist, and at $\chi_{PEG-5000}>0.4$ only micelles are present. DSC data reported here show that at $\chi_{PEG-5000}< 0.06$ the system exhibits a thermal behaviour similar to that of pure DPPC. At molar fractions $0.06<\chi_{PEG-5000}<0.2$ the gel→ripple phase transition is less evident because of the presence of micelles. In this concentration range, the DSC curve of the main transition is a convolution of multiple transitions, probably due to the presence of boundary lipids. At molar fractions $0.2<\chi_{PEG-5000}<0.4$ the gel→ripple phase transition disappears and a broad DSC peak ascribable to the gel→liquid crystal phase transition of the residual bilayer is evident. At $\chi_{PEG-5000}>0.4$ DSC there is no evidence of a transition, in agreement with spectroscopic data that have shown that micelles alone are present in this concentration range.

Conclusions

The phase behaviour of the DPPC/DPPE–PEG 350, DPPC/DPPE–PEG 2000 and DPPE–PEG 5000 systems as a function of temperature and lipid composition has been investigated. It has been evidenced that the presence of polymer-grafted lipids, although necessary for increasing the lifetime of liposomes *in vivo*, causes a destabilisation of the bilayer, this being more evident as molar fractions and length of the polymer-grafted lipid increase. Since liposomes release the encapsulated drug only when they are in a liquid crystalline phase, it is possible to use phase diagrams to establish with precision, in terms of lipid composition and working temperature, the correct formulation for an effective design drug delivery systems. For the DPPC/DPPE–PEG 350 system the useful composition range is at a molar fraction of $0<\chi_{PEG-350}<0.5$. Outside this range only small-sized micelles are present and it is thus not possible to encapsulate drugs. For the DPPC/DPPE–PEG 2000 system the useful range of applicability is even smaller (molar fraction within $0<\chi_{PEG-2000}<0.07$). The range of applicability of DPPC/DPPE–PEG 5000 is much smaller ($0<\chi_{PEG-5000}<0.05$) as a result of the severe destabilization of the bilayer due to the length of the polymer chain.

Acknowledgements

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References

1 A. Gabizon and D. Papahadjopoulos, Biochim. Biophys. Acta, 1103 (1992) 94.
2 T. M. Allen, J. L. Ryan and D.Papahadjopoulos, Biochim. Biophys. Acta, 818 (1985) 205.
3 A. Abuchowski, T. van Es, N. C. Palczuk and F. F. Davis, J. Biol. Chem., 252 (1977) 3578.
4 A. L. Klibanov, B. A. Khaw, N. Nossiff, S. M. O'Donnell, L. Huang, M. A. Slinkin and V. P. Torchilin, Am. J. Physiol., 261 (1991)
5 D. Marsh, L. I. Horvath, M. J. Swamy, S. Mantripragada and J. H. Kleinschmidt, Mol. Membr. Biol., 19 (2002) 247.
6 P. S. Uster, T. M. Allen, B. E. Daniel, C. J. Mendez, M. S. Newman and G. Z. Zhu, FEBS Lett., (1996) 386.
7 G. Blume and G. Cevc, Biochim. Biophys. Acta, 1029 (1990) 91.
8 M. C. Woodle and D. D. Lasic, Biochim. Biophys. Acta, 1113 (1992) 171.
9 G. Blume, G. Cevc, M. D. Crommelin, I. A. Bakker-Woudenberg, C. Kluft and G. Storm, Biochim. Biophys. Acta, 1149 (1993) 180.
10 D. D. Lasic, Trends. Biotechnol., 16 (1998) 307.
11 S. Belsito, R. Bartucci, G. Montesano, D. Marsh and L. Sportelli, Biophys. J., 78 (2000) 1420.
12 G. Montesano, R. Bartucci, S. Belsito, D. Marsh and L. Sportelli, Biophys. J., 80 (2001) 1372.
13 D. Grasso, D. Milardi, C. La Rosa and E. Rizzarelli, New J. Chem., 25 (2001) 1543.