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Sterically stabilized liposomes of DPPC/DPPE-PEG:2000. A spin label ESR and spectrophotometric study

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Abstract

The chain dynamics and the thermotropic phase behavior of sterically stabilized liposomes obtained introducing in the host bilayer matrix of DPPC up to 7 mol% of the polymer-lipid DPPE-PEG:2000 were investigated by spin label electron spin resonance spectroscopy and spectrophotometry. The experimental data indicate that the dispersions have the dynamic and thermotropic characteristics of normal lamellar phase. Moreover, using spin labels that locate both in the interfacial and in the hydrocarbon regions, namely TEMPO-stearate, 5- and 16-PCSL, we find that relative to the unmodified DPPC bilayers, the polymer-grafted bilayers are loosely packed in the interfacial region and have reduced chain mobility in the gel phase. From the temperature dependence of the partition coefficient (P_c), of the spin probe DTBN between the aqueous and the fluid hydrophobic regions of the bilayers and from the melting curves of the absorbance at 400 nm, we observe a slight influence on the endothermic phase transitions when increasing the concentration of the polymer-lipid in the DPPC bilayers, the influence being more evident in the pre-transition.

Keywords: Liposomes; Polymer-lipids; Spin label; Electron spin resonance; Spectrophotometry

1. Introduction

In the last years interest in liposomes both for basic research and for biotechnological applications has notably increased [1]. For example, a novel class of liposomes with peculiar properties has been developed. They are obtained from the swelling in water of bilayer forming lipids and polymer-lipids, i.e. lipids having water-soluble polymers covalently attached on the polar head. These polymer-grafted membranes, under particular conditions, act as very effective drug encapsulation and delivery systems [1–4]. In particular, it has already been experimentally established that liposomes containing small percentage of poly(ethylene glycol)-linked lipids (PEG-lipids, PEG molecular weights 2000 and 5000 Da) have a blood circulation time from one to two orders of

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magnitude longer (from a few hours to days) relative to conventional, unprotected liposomes [5–7]. They are called either 'Stealth' or 'Sterically Stabilized Liposomes' (SSLs). The extended lifetime in vivo seems to arise from the steric barrier provided by the grafted polymers that stabilizes the lipid bilayer against attacks by diverse elements of the immune system in vivo [4,8–10].

From a fundamental perspective, the lipid/PEG-lipid/water dispersions have been characterized structurally by X-ray diffraction [11–14], calorimetrically by differential scanning calorimetry (DSC) [10,13,15,16] and morphologically by spectrophotometry [13], light scattering [16,17], electron [16,18] and light microscopy [13,17]. Additionally, other different experimental methods, including electrophoretic mobility [19], surface forces apparatus [20], electron micropipette manipulations [11], nuclear magnetic resonance (NMR) [13,16], photon correlation spectroscopy and permeability measurements [21] have been employed to characterize the physical properties of the polymer-grafted membranes. Correspondingly, theoretical studies [12,14,18,22,23], which coherently merge membrane and polymer physics, model the influence of the polymer-bearing lipids on the structural and interactive properties of the bilayers and regard the self-assembly, the polymorphic phase behavior and the stability of the polymer-liposomes. From these studies two points emerge. First, on mixing bilayer forming lipids (such as DPPC) and micelle forming lipids (such as PEG-lipids), there exists a maximum concentration of the polymer-lipids that can be incorporated in the bilayer before it is solubilized and converted in the micelle [11–13,15,16,22,23]. The liotropic transition from lamellar to micellar phase depends on different parameters, such as PEG-chain length, acyl chain length and degree of unsaturation of the PEG-phospholipid conjugate, and the acyl chain composition of the host bilayer matrix. Second, the physical configuration of grafted polymer chains plays an important role in determining the phase behavior of the polymer-grafted membranes. According to de Gennes [24], when the density of grafting of the polymers is low, there will be little interaction between neighbouring chains, which extend in water in a dilute non-overlapping rounded 'mushroom'. Instead, at high grafting level, the chains interact laterally to form an extended 'brush'. Several parameters should therefore be controlled in order to obtain sterically stabilized liposomes able to encapsulate and to deliver water-soluble drugs.

In this paper we focus our attention on SSLs obtained by dispersing at full hydration in buffer dipalmitoylphosphatidyl-choline (DPPC) with different concentrations up to 7 mol% of dipalmitoylphosphatidyl-ethanolamine (DPPE) bearing on the polar head poly(ethylene glycol) of average molecular weight 2000 (PEG:2000). We use DPPC and DPPE-PEG:2000 (for their molecular structures see Fig. 1a,b) as the same chain composition ensure good mixing. Moreover, the structure and thermal properties of DPPC liposomes are well understood [25] and poly(ethylene glycols) grafted onto phosphatidylethanolamine are the best studied polymer-lipids [3,4]. In addition, it has been found that the blood circulation times for DPPC/DPPE-PEG:2000 are similar to those of other PEG-liposomes [2]. We added up to 7 mol% of DPPE-PEG:2000 in the host lipid matrix of DPPC, as at these concentrations, stable lamellar phases are obtained as indicated by X-ray [12] and DSC measurements [15,16] on the same systems.

By spin label electron spin resonance (ESR) spectroscopy we directly compare the dynamics of multilamellar vesicles (MLVs) of DPPC/DPPE-PEG:2000/buffer dispersions to those of DPPC alone. The conventional ESR spectra from spin-labelled biomolecules are sensitive to rotational motions on the time-scale of 10^{-11}–10^{-8} s. The technique is, therefore, particularly appropriate for studying the lipid chain motions in membranes, which are found to lie in the nanosecond range [26–28]. We use the spin label TEMPO-stearate, that locates the nitroxide moiety in the interfacial region (Fig. 1c), and the spin-labelled phosphatidylcholine at the C-5 (5-PCSL) (Fig. 1d) and at the C-16 (16-PCSL) (Fig. 1e) positions of the acyl chain in order to study the response to perturbations in the headgroup region and the segmental motion of the lipid chains, respectively.

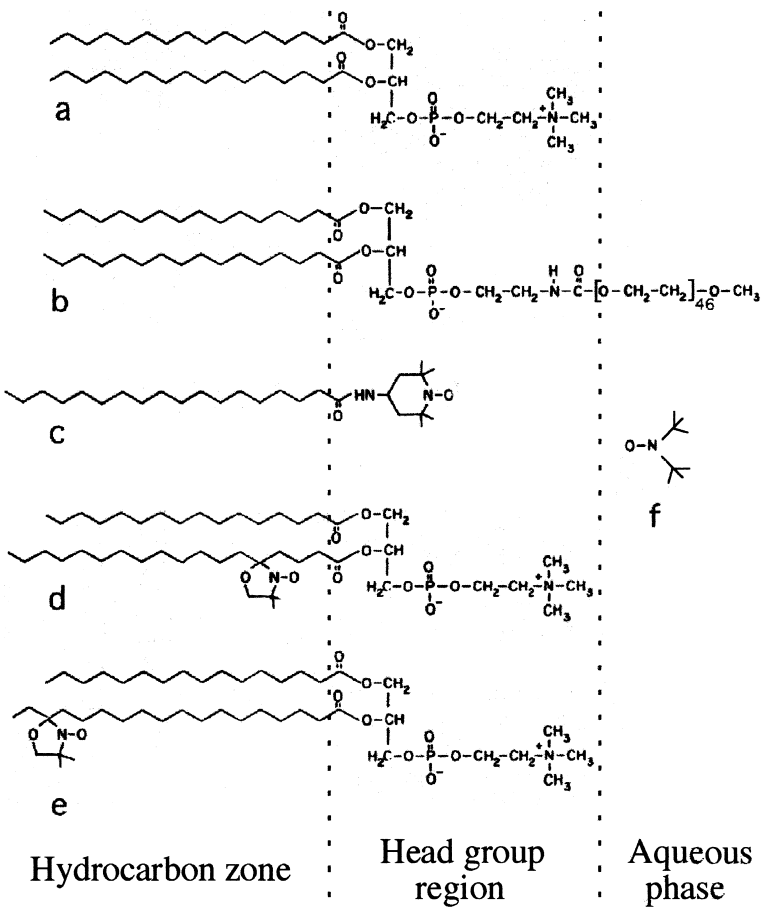

Fig. 1. Structures of the lipid, polymer-lipid and spin label molecules used in this study: (a) zwitterionic lipid DPPC; (b) modified lipid DPPE-PEG:2000 (negatively charged); (c) spin label TEMPO-stearate; spin-labelled phosphatidylcholine at (d) C-5 position in the sn-2 chain (5-PCSL) and at (e) C-16 position in the sn-2 chain (16-PCSL); (f) hydrophobic spin probe DTBN. The different bilayer regions are also indicated.

We find that relative to DPPC dispersions, the grafted bilayers have a loosened cohesion in the interfacial region and a tight molecular packing density in the hydrocarbon zone. The temperature dependence both of the partition coefficient (P_c) of the spin probe DTBN (Fig. 1f) between the aqueous and the fluid hydrocarbon regions of the bilayers and of the optical density (OD) at 400 nm have been used to investigate the endothermic phase behavior of multilamellar dispersions [29,30]. The progressive addition of the polymer-lipid in the DPPC dispersions has a slight effect on the thermotropic phase behavior of the aqueous dispersions, the influence being more evident on the pre-transition.

2. Experimental

2.1. Materials

The synthetic lipid 1,2-palmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was from Sigma. The PEG-lipid 1,2-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine-*n*-[poly(ethylene-glycol)2000] (DPPE-PEG:2000) and the spin-labelled lipids 1-palmitoyl-2-(5-doxyl stearoyl)-*sn*-glycero-3-phos-

phatidylcholine (5-PCSL) and 1-palmitoyl-2-(16-doxyl stearoyl)-*sn*-glycero-3-phosphatidylcholine (16-PCSL) were from Avanti Polar Lipids. The spin label 2,2,6,6-tetramethylpiperidin-1-oxyl-4-yl octadecanoate (TEMPO-stearate) was from Molecular Probes Inc. and the spin probe di-*tert*-butyl-nitroxide (DTBN) was from Aldrich Chemical Company. The reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH = 7.5 were from Merck. All materials were used as purchased with no further purification. Distilled water was used throughout.

2.2. Spin label ESR measurements

Multilamellar vesicles dispersions have been prepared dissolving in chloroform DPPC and DPPE-PEG:2000 at the concentrations of 0, 1, 3 and 7 mol%. The desired spin label was added in the lipid dispersions at the molar ratio 1:100. After solvent evaporation with a nitrogen gas stream, the dried samples have been fully hydrated (final lipid concentration 25 mM) with PBS at pH = 7.5, heating and vortexing at 60°C. For samples tested with DTBN, the spin probe was added in the hydration buffer. The dispersions were finally sealed off in 1 mm (i.d.), 100-μl glass capillaries and incubated overnight at 10°C before the running of ESR spectra.

ESR spectra were recorded with a 9-GHz, X-band Bruker spectrometer, model ER 200D-SRC, and digitized with the spectrometer's built-in microcomputer using OS-9 compatible ESP1600 spectral acquisition and handling software. Sample capillaries were inserted in a standard 4-mm (i.d.) ESR quartz tube containing light silicone oil for enhancing samples thermal stability and centered in a TE_{102} rectangular ESR cavity (ER 4201, Bruker). Measurements were performed at thermal equilibrium starting from a low temperature. Sample temperature was controlled with an ER 4111VT variable temperature control system (accuracy $\pm$ 0.5°C). The spin label ESR spectra were acquired at 10 mW microwave power using 1 G_{p-p} as the amplitude of the magnetic field modulation signal and 100 kHz as the magnetic field modulation frequency for phase sensitive detection. They were displayed as first derivative in-phase absorption signal.

2.3. Spectrophotometric measurements

Aqueous multilamellar dispersions of DPPC/DPPE-PEG:2000 for the spectrophotometric measurements were prepared as described above without the spin labels. The lipid films were first hydrated in PBS at pH 7.5 and then transferred to a 3-ml quartz cell, 1-cm optical path and incubated overnight at 10°C before measuring. The final lipid concentration was 1 mg/ml.

Optical measurements at 400 nm were performed with a Jasco 7850 spectrophotometer equipped with a Peltier type thermostated cell holder model EHC-441 and a temperature programmer (model TPU-436) (accuracy $\pm$ 0.1°C). A heating rate of 1°C/min was used. Data acquisition and manipulation was carried out with the spectrophotometer's built-in microcomputer.

3. Results

3.1. ESR measurements

3.1.1. Measurements with TEMPO-stearate

The ESR spectra of TEMPO-stearate in MLVs dispersions of DPPC with 0 and 7 mol% of DPPE-PEG:2000 at 10, 30 and 50°C are reported in Fig. 2a–c, respectively. The nitroxide moiety of this spin label is positioned in the phospholipids headgroup region (Fig. 1c) so that the bilayer surface regions are investigated. It is interesting to note that TEMPO-stearate has a different rotational symmetry compared to the spin-labelled phospholipids used. Indeed, its nitroxide *x*-axis coincides with the long molecular axis, whereas in 5- and 16-PCSL the *z*-axis is directed along the long molecular axis (Fig. 1c–e). The spectrum of TEMPO-stearate in DPPC dispersions at 10°C (upper spectrum in Fig. 2a) is a three-line derivative single component spectrum characteristic of some anisotropic motion in the intermediate regime on the conventional ESR timescale. It shows a line broadening likely originating from dipole–dipole interaction of the labels that are

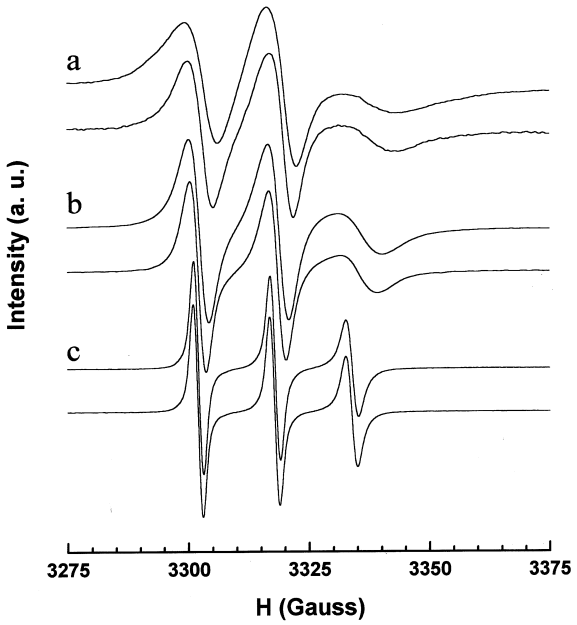

Fig. 2. ESR spectra of TEMPO-stearate in aqueous dispersions of multilamellar vesicles of DPPC with 0 (upper spectra) and 7 (lower spectra) mol% of DPPE-PEG:2000 at (a) $T = 10$°C; (b) $T = 30$°C; and (c) $T = 50$°C.

not homogeneously distributed at low temperature in the gel phase. On increasing the temperature still in the gel phase, a reduction of the spectral anisotropy of TEMPO-stearate in the DPPC dispersions is observed. Indeed, from the upper spectrum in Fig. 2b and Table 1 it can be seen that the extreme resonances move-in and the peak-to-peak separation of the low, ΔH^{L}_{p-p}, and central, ΔH^{C}_{p-p}, field resonance decreases of approx. 3.3 and approx. 2.5 gauss, respectively, on going from 10 to 30°C. This indicates that as the temperature increases, the freedom of motion of the spin label increases.

Relative to the spectra of TEMPO-stearate in the DPPC dispersions in buffer, the spectral anisotropy decreases with the addition of DPPE-PEG:2000 in the lipid mixtures at any temperature in the gel phase. Indeed, comparing the spectra in Fig. 2a, can be seen that the hyperfine splitting between the two outer extreme peaks decreases of approx. 1.5 gauss and the low and central field linewidths reductions of 1.6 and 1.3 gauss, respectively (Table 1) with the addition of 7 mol% of polymer-lipid in the DPPC host bilayers at 10°C. On increasing the temperature in the gel phase, the spectral anisotropy is always lower in the polymer-grafted membranes, although the differences in the spectral anisotropy between the two systems decreases. In fact, it can be seen from Fig. 2b and Table 1 that at 30°C in the gel phase, the spectrum of the label in the polymer-grafted membranes displays a lower anisotropy and the linewidths decrease by approx. 0.2 gauss. The ESR results, therefore, indicate that the label experiences more motion at the MLVs surface region in the SSLs compared to the unmodified DPPC dispersions.

At 50°C, any difference in the spectral anisotropy of TEMPO-stearate in DPPC/buffer and in DPPC/DPPE-PEG:2000/buffer dispersions (Fig. 2c) disappears. In both systems the labels are principally in the fast motion regime and the spectra are characteristic of bilayers in the fluid phase.

Table 1
Polymer-lipid concentration dependence of the low, ΔH^{L}_{p-p}, and central, ΔH^{C}_{p-p}, field peek-to-peek separation linewidth values from ESR spectra of TEMPO-stearate in multilamellar vesicles of DPPC at different temperatures

DPPC + DPPE-PEG:2000 (mol%)	$T = 10$°C		$T = 30$°C		$T = 50$°C	
	ΔH^{L}_{p-p} (gauss)[a]	ΔH^{C}_{p-p} (gauss)[a]	ΔH^{L}_{p-p} (gauss)[a]	ΔH^{C}_{p-p} (gauss)[a]	ΔH^{L}_{p-p} (gauss)[a]	ΔH^{C}_{p-p} (gauss)[a]
0	6.7	6.1	3.4	3.6	1.8	1.8
1	6.5	5.8	3.4	3.6	1.8	1.8
3	5.4	5.1	3.2	3.4	1.8	1.8
7	5.1	4.8	3.2	3.4	1.8	1.8

[a] The error is ±0.1 gauss.

3.1.2. Measurements with 5-PCSL

The ESR spectra of 5-PCSL in DPPC dispersions both in the absence and in the presence of 7 mol% of DPPE-PEG:2000 at 10, 30 and 50°C are reported in Fig. 3a–c, respectively. Using this spin-labelled lipid the glycerol backbone/beginning of the acyl chains of the bilayer regions is investigated (see Fig. 1d). Moreover, with the acyl chain labels, the amplitude and the rate of the segmental motion (trans-gauche isomerism) are mainly monitored.

At 10°C the spectra of 5-PCSL in MLVs of both DPPC dispersed in buffer and DPPC plus 7 mol% of DPPE-PEG:2000 approximate to a powder pattern, characteristic of a rather rigid phase with an outermost peak separation $2A_{zz}$ of approx. 64.5 gauss, as expected for phospholipid membranes in the gel phase [26,27,29]. However, the gel state temperature variation of the spectral anisotropy of DPPC dispersions is different from that in the presence of the polymer-lipid. Indeed, it can be seen from the spectra in Fig. 3b that, at 30°C still in the gel phase, the label in MLVs of DPPC displays a considerable reduction of its anisotropic motion that leads the two extreme resonance peaks to move-in, the linewidth to become broader, and to a better resolution of the perpendicular region of the spectrum. In contrast, the spectral anisotropy of 5-PCSL in the mixture of DPPC and 7 mol% of DPPE-PEG:2000 changes relatively little with an increase in temperature. The spectrum at 30°C retains a high degree of motional restriction giving rise to a hyperfine splitting separation of 61.6 gauss, i.e. 2.6 gauss higher than the corresponding value in DPPC dispersions in buffer.

The spectra of 5-PCSL in aqueous dispersions of DPPC and DPPC plus 7 mol% of DPPE-PEG:2000 recorded at 50°C are shown in Fig. 3c. In both systems they display the same considerably lower anisotropy that is characteristic of a fluid liquid crystalline phase.

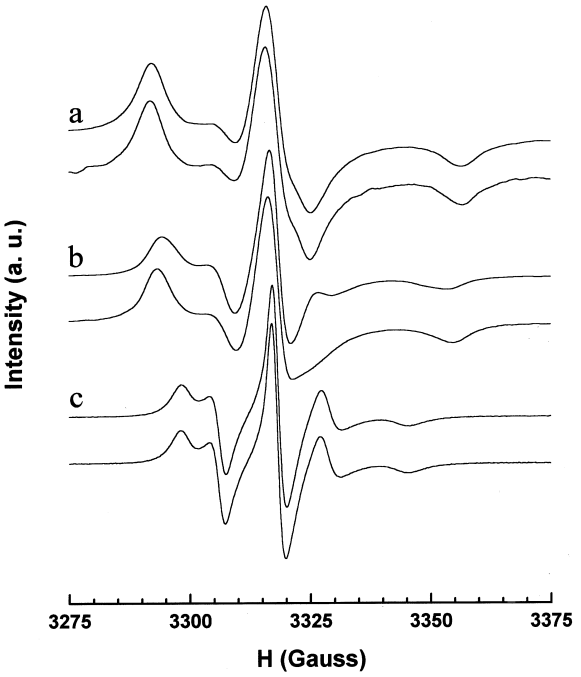

Fig. 3. ESR spectra of the spin label 5-PCSL in aqueous dispersions of multilamellar vesicles of DPPC with 0 (upper spectra) and 7 mol% of DPPE-PEG:2000 (lower spectra) at (a) $T = 10$°C; (b) $T = 30$°C; and (c) $T = 50$°C.

3.1.3. Measurements with 16-PCSL

The ESR spectra at 10, 33 and 50°C of 16-PCSL in aqueous dispersions of MLVs of DPPC with 0 and 7 mol% DPPE-PEG:2000 are reported in Fig. 4a–c, respectively. With this spin-labelled lipid the deep interior of the hydrocarbon region is investigated (see Fig. 1e). At 10°C, the spectra of 16-PCSL both in DPPC/buffer and in DPPC/DPPE-PEG:2000/buffer systems show a degree of anisotropy in which the two components, parallel and perpendicular of the hyperfine tensor, can be measured (Fig. 4a). They are representative of the lamellar L_{β}, gel phase. The spectra at 50°C (Fig. 4c) are, instead, isotropic triplets with different lineheight and a hyperfine coupling constant, A_0, of 14.2 gauss. They are representative of the lamellar L_{α} liquid crystalline phase. At the intermediate temperature of 33°C (see Fig. 4b) the spectrum of 16-PCSL in the MLVs of DPPC in buffer is the superposition of an ordered motionally restricted component and of an isotropic one (see arrows in Fig. 4b). Similar spectral features have already been observed by ESR in DPPC multilamellar dispersions using the stearic acid bearing the nitroxide moiety at the 16th carbon atom (16-NSA) [31]. They have been

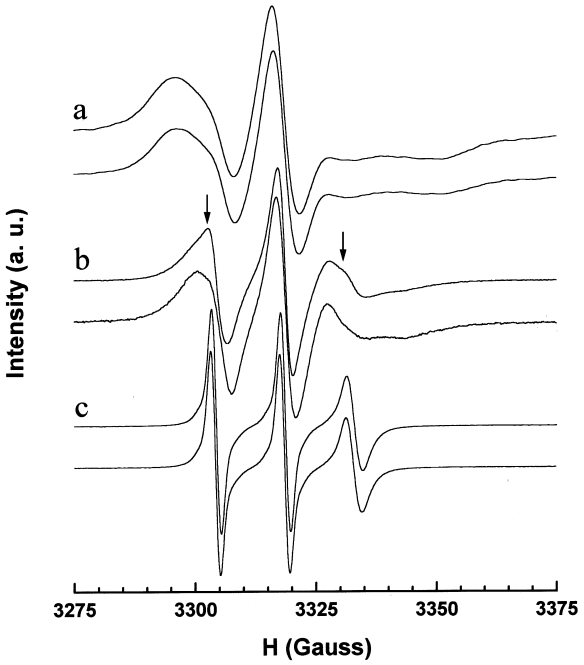

Fig. 4. ESR spectra of the spin label 16-PCSL in aqueous dispersions of multilamellar vesicles of DPPC with 0 (upper spectra) and 7 mol% of DPPE-PEG:2000 (lower spectra) at (a) $T = 10$°C; (b) $T = 33$°C; and (c) $T = 50$°C.

explained with the existence of a non-homogeneous structure in the ripple $P_{\beta'}$ phase, i.e. the coexistence of ordered regions present in the $L_{\beta'}$ phase (to which corresponds the anisotropic component) and disordered regions present in the L_{α} phase (responsible for the isotropic component). The lineshape of the spectrum of 16-PCSL in the mixture of DPPC with 7 mol% of DPPE-PEG:2000 at 33°C also consists of two components, with different motional properties but it is dominated by the larger anisotropic component. This suggests that the presence of the polymer-lipid in the DPPC dispersions induces motional restriction of 16-PCSL in the gel phase, as already observed for 5-PCSL.

Comparing the gel phase spectra of 5- and 16-PCSL of the same systems (Figs. 3 and 4), the anisotropy decreases as the doxyl group is moved towards the hydrocarbon end in both DPPC and DPPC mixed with different amounts of DPPE-PEG:2000, as normally occurs in phospholipid membranes. It is interesting to note, however, that in the polymer-grafted membranes the gel state temperature variation of the anisotropy of both labels is relatively little compared to that of DPPC.

3.1.4. Measurements with DTBN

When the small hydrophobic spin probe DTBN (see Fig. 1f) is dissolved in a lipid dispersion, it partitions between the aqueous and fluid hydrocarbon lipid phases (see chapters 12 and 13 in [26,27,29]). The corresponding ESR spectrum is the superposition of two isotropic triplets arising from DTBN in the two environments. As membranes have greater viscosity and smaller polarity than water, there are small differences in the isotropic hyperfine coupling constant, A_0, and in the isotropic g-factor, of the ESR spectra. This leads to a partial spectral resolution so that the resonance line at high magnetic field ($m_I = -1$) is clearly resolved at 9 GHz (see inset of Fig. 5). Moving toward high fields, first the label signal in the lipid region (H_L) is seen and then the signal in the aqueous phase (H_W). An estimation of the fraction of DTBN in the fluid hydrocarbon region of the bilayer can be obtained by evaluating the partition coefficient, $P_c = H_L/(H_L + H_W)$, neglecting the correction of the contributions of both the ^{13}C satellite resonance and the line width. Plots of P_c vs. temperature are used to detect bilayers phase transitions (see chapters 12 and 13 in [26,29]).

The temperature dependence of the partition coefficient, P_c, of DTBN in DPPC dispersions both in the absence and in the presence of 1, 3 and 7 mol% of DPPE-PEG:2000 is reported in Fig. 5. As can be seen, in the DPPC/buffer system the partitioning of DTBN occurs around the pre-transition at a T_p of approx. 33.5°C and a considerable enhancement is clearly seen at the main transition at T_m of approx. 41.5°C (Table 2). Moreover, the characteristics of these phase transitions, i.e. temperature, amplitude and width, are in agreement with the experimental method and mesophase used [29,32]. On increasing the concentration of DPPE-PEG:2000 in the DPPC dispersions, the partition coefficient values do not change significantly relative to those corresponding to the DPPC/buffer system in the gel phase.

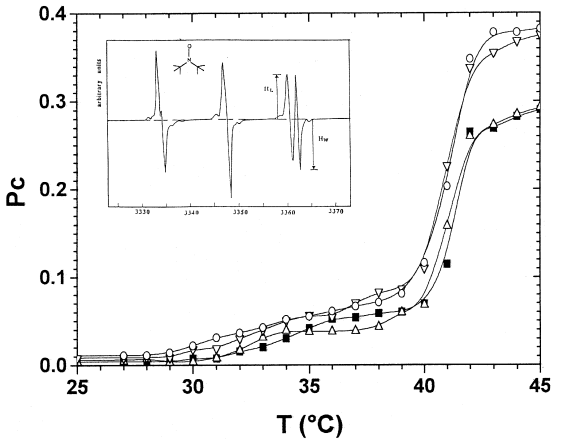

Fig. 5. Temperature dependence of the partition coefficient, P_c, of DTBN in DPPC dispersions with 0 (■), 1 (△), 3 (▽) and 7 (○) mol.% of DPPE-PEG:2000. Inset: representative ESR spectrum of DTBN in aqueous dispersions of multilamellar vesicles of DPPC at 45°C.

Moreover, T_m remains almost unchanged, whereas the pre-transition is downward shifted to 31.5°C at 7 mol% of the polymer-lipid (Table 2). It is interesting to note that the partition coefficient changes significantly in the fluid phase ($T > T_m$) being higher in the brush regime. It is reported that the permeability of extruded vesicles prepared from mixtures of DPPC, phosphatidylinositol and DPPE-PEG:2000 toward radioactive D-glucose increases in the range of the transition of the grafted polymer from the mushroom into the brush conformation [21].

3.2. Spectrophotometric measurements

The absorbance change at 400 nm vs. temperature, for multilamellar dispersions in a buffer of DPPC and DPPC plus 7 mol% of DPPE-PEG:2000 are reported in Fig. 6. The pre- and the main transitions at T_p of approx. 34.8°C and T_m of approx. 41.8°C, respectively, are clearly seen in the transition curve for DPPC/buffer system (Table 2). The characteristics of these transitions are in agreement with those reported in the literature using spectrophotometry [30] and other direct methods ([32] and references therein). The presence of 7 mol% of the polymer-lipid in the dispersions slightly affects the DPPC endothermic phase transitions characteristics, the effects being more evident on the pre-transition. Indeed, from the melting curve in Fig. 6 it can be seen that the pre-transition temperature decreases to approx. 33°C, whereas T_m decreases to approx. 40.7°C (Table 2).

Table 2
Temperatures of the pre- T_p, and main phase transition, T_m, of multilamellar vesicles of DPPC with 0, 1, 3 and 7 mol% of DPPE-PEG:2000 as determined by ESR using DTBN and by spectrophotometry

DPPC + DPPE-PEG:2000 (mol%)	DTBN		A_{400}	
	T_p (°C)[a]	T_m (°C)[a]	T_p(°C)[b]	T_m(°C)[b]
0	33.5	41.5	34.8	41.8
1	32.5	41.0	34.7	41.3
3	32.5	41.0	34.7	41.5
7	31.5	41.0	32.9	40.7

[a] The error is ±0.5°C.
[b] The error is ±0.1°C.

The most striking feature of Fig. 6 is that the progressive addition of the polymer-lipid significantly decreases the value of the absorbance at any temperature. As it is shown in the inset of Fig. 6, at 20°C the absorbance of the dispersions reduces progressively as the concentration of DPPE-PEG:2000 is increased and drops to 0.9 at 7 mol% of the polymer-lipid. The absorbance at 520 nm for DSPC/DSPE-PEG:2000 mixtures measured as a function of mol% of PEG-lipid showed a drop with the addition of the lowest concentration of PEG-lipids [13]. This effect has been ascribed to a decreased size of the vesicles. In the same study, light microscopy on 85:15 DSPC:DSPE-PEG:2000 suspensions showed birefringent spherical particles at 20 and 65°C with the appearence typical of multilamellar vesicles both in the gel and in the liquid crystalline phase, respectively. The suspensions become less and less birefringent as the percentage of polymer-lipid incorporated into the DSPC bilayers was progressively increased indicating the existence of either small unilamellar vesicles or micelles. The decrease of the mean size of extruded vesicles of DPPC (85 mol%) plus phosphatidylinositol (9 mol%) and DPPE-PEG:2000 (6 mol%) at temperatures higher than the main transition temper-

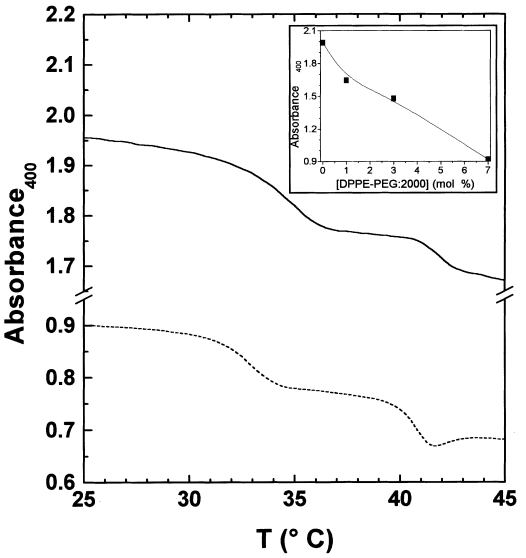

Fig. 6. Temperature dependence of the absorbance at 400 nm of aqueous dispersions of multilamellar vesicles of DPPC with 0 (full line) and 7 mol% of DPPE-PEG:2000 (broken line). Inset: absorbance at 400 nm of DPPC/DPPE-PEG:2000/buffer dispersions as a function of the concentration of the polymer-lipid.

ature of the major lipid component has also been evidenced by photon correlation spectroscopy [21]. Lasic et al. [17] reported a reduction of the hydrodynamic radius of MLVs dispersions of egg yolk PC with 7.5 mol% of DPPE-PEG:2000 and the appearence in optical microscopy of smaller multilamellar vesicles and unilamellar vesicles.

4. Discussion

The spin label ESR and spectrophotometric results reported in this study indicate that fully hydrated multilamellar vesicles of DPPC incorporating low concentrations of DPPE-PEG:2000 have the dynamical and thermotropic features of conventional bilayer lamellar phases. Indeed, at different temperatures we obtained both ESR spectra whose anisotropy is peculiar of lamellar phases [26,27] and thermal profiles exhibiting the two endothermic transitions at T_p and T_m, as normally occurs in saturated symmetrical chain PCs [25]. This is in keeping with X-ray data that indicates that up to 8 mol% of DPPE-PEG:2000 can be incorporated in DPPC without disrupting the bilayer structure [12]. Moreover, Bedu-Addo and Huang [15] and Bedu-Addo et al. [16] reported that lamellar phases with components exhibiting miscibility are obtained from the mixture up to 7 mol% of short chain PEG-phospholipid conjugates (PEG of mol. wt. 1000 and 3000 Da) and phosphatidylcholine. We provided experimental evidence that the thermotropic phase behaviour and, even more, the segmental lipid chain dynamics of SSLs of DPPC/DPPE-PEG:2000 dispersions differ in some extent from those of DPPC.

As low concentrations of DPPE (approx. 10 mol%) in the mixture with DPPC do not affect the structural, dynamical and calorimetric properties of DPPC [33], the effects that we observe should be related to the DPPE-PEG:2000 molecule and, as already pointed out, to the polymer chain.

As concerns the segmental lipid chain dynamics, our spin label ESR results indicate a moderate influence on increasing the concentration of DPPE-PEG:2000 in the interfacial zone and a more marked one in the hydrocarbon region of the DPPC dispersions. The slightly reduced (increased) spectral anisotropy in the spectra of TEMPO-stearate (5- and 16-PCSL) in the gel phase indicates that the presence of the polymer-lipid enhances the freedom of the motion in the head group region and notably reduces that in the hydrocarbon zone in the gel phase. In particular, although a gradient of increased mobility on proceeding down the hydrocarbon chain is obtained both in DPPC and in DPPC/DPPE-PEG:2000 dispersions, a restricted chain mobility in the gel phase is observed in the polymer-grafted membranes. Accordingly, the motion of 5-PCSL is restricted and 16-PCSL does not possess the freedom of motion that is possible at the bilayer midplane and that is evident in DPPC dispersions. The spin label ESR results, therefore, clearly indicate that the SSLs obtained by mixing DPPC and up to 7 mol% of DPPE-PEG:2000 have a loosened packing density in the interfacial region and an improved cohesion in the hydrophobic zone in the gel phase relative to normal bilayers of DPPC.

These results can be explained in the framework of the 'lateral pressure model' proposed by Hristova and Needham [22,23] to predict the concentration of the polymer-lipid that can be incorporated into the bilayer before it is converted into a micelle. The model is based on the lateral equilibrium between the polymer steric repulsive forces and the cohesive forces (van der Waals, hydrophobic, etc.) within the bilayers provided that the concentration of the polymer-lipid is low. The repulsive steric forces between the polymer chains when the polymer-lipid are mixed with lipid will be felt as an isotropic tension in the bilayer. Just as in micropipette experiments [11], where applied tension leads to area change, the polymer will cause area expansion. To prevent the bilayer from rupturing, the lateral pressure must be balanced by the cohesive forces between the lipid molecules. This is just what our measurements indicate. If the incorporation of DPPE-PEG:2000 in MLVs dispersions of DPPC leads to an increased area/polarhead and allows for tighter chain packing, it is very likely that TEMPO-stearate experiences more mobility in the interfacial region and, in turn, more motion in the gel phase, whereas 5- and 16-PCSL are motionally hindered. Moreover, zeta potential data [34] on the neutral liposomes of PCs suggested a close correlation between the conformation of the headgroup and the packing state of the lipid molecules. This correlation can be affected by various parameters, such as temperature, ionic strength and so on. It is very likely that in SSLs the presence on the polar region of a bulky and hydrophilic polymeric long chain changes the orientation of the polar heads (increasing headgroup mobility) and enhances the chain packing density (leading to motional restriction) just as indicated by our spin label ESR measurements.

The effects of DPPE-PEG:2000 on the lipid chain dynamics of DPPC are more evident in the gel phase, where the area/polarhead is lower, and are not appreciable in the liquid crystalline phase.

As concerns the thermotropic phase behaviour of DPPC/DPPE-PEG:2000 dispersions it slightly differs from that of DPPC (see Figs. 5 and 6, Table 2). Several studies report that the thermal behaviour of lipid/polymer-lipid/water dispersions differs from that of the host lipid matrix when the concentration of the polymer-lipid approachs that which destabilizes the bilayer [13,15,16]. Kenworthy et al. [13] reported that T_m and T_p of the DSPC/DSPE-PEG:2000 suspensions were not markedly changed from their control values by the incorporation of up to 50 mol% of PEG:2000 from Liposome Technology or 5 mol% of PEG:2000 from Avanti Polar Lipids. Moreover, the more evident influence of PEG-lipid on the pre-transition suggests that the structure of the bilayer at the interface is perturbed by the presence of the bulky polymer-lipid headgroups. In addition, the effects of increasing the concentration of PEG-lipid in DPPC bilayers on the thermal behaviour of the dispersions can be interpreted, at least qualitatively, considering that PEG-lipid is negatively charged, the polar head is more bulky and it allows for the formation of a less stable hydrogen bond network. These factors are known to lower the transition temperatures [35].

Edwards et al. [18] have carried out a cryo-transmission electron microscopy study about the effects of polyethyleneglycol-phospholipids (PEG:2000 linked either to DPPE or to DSPE) at different concentrations on the aggregate structure of small unilamellar liposomes with varying bilayer composition (e.g. egg yolk lecithin, DSPC and DPPC with cholesterol). They reported that the transition to micelle may occur via intermediate structures, such as open bilayer discs. These open or perforated bilayer aggregates severely decrease, or even abolish, the encapsulation efficiency of the liposomal preparation. Although the techniques used in the present study are not suitable to determine whether such morphological modifications occur in our multilamellar vesicles, the spectrophotometric measurements show a decrease in the average number of lipid lamellae with increasing DPPE-PEG:2000 concentration in the bilayers. Moreover, our ESR data on extruded large unilamellar vesicles of DPPC/DPPE-PEG:2000/buffer dispersions (measurements in progress) also indicate that PEG-lipids make the MLVs more LUVs-like. The effect of

the reduction in size is more evident at the highest concentrations used. It is probably due to both steric and electrostatic bilayer–bilayer repulsion from the charged PEG-lipid. On the other hand, as reported in the literature, the structural, calorimetric and morphological modifications are evident at the concentrations of the polymer-lipid that pertub the bilayers, i.e. ≥ 7 mol% in the case of DPPC/DPPE-PEG:2000 dispersions [12,13,15–17].

In summary, our results confirm that stable bilayer phases are obtained including a small percentage (up to 7 mol%) of the polymer-modified lipid DPPE-PEG:2000 in the gel phase of DPPC bilayers. We provided evidence that the presence of the polymer-lipid in the DPPC matrix affects the segmental lipid chain motions and the thermotropic phase behaviour in a way involving the molecular organization of the polar headgroup region and the packing of the alkyl chains.

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