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Lipid chain length effect on the phase behaviour of PCs/PEG:2000-PEs mixtures. A spin label electron spin resonance and spectrophotometric study

S. Belsito, R. Bartucci, L. Sportelli*

Dipartimento di Fisica and Unità INFM, Università della Calabria, Ponte P. Bucci, Cubo 31 C, I-87030 Arcavacata di Rende (CS), Italy

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Abstract

Spin-label electron spin resonance (ESR) spectroscopy and spectrophotometry at fixed wavelength are used to study fully hydrated aqueous dispersions of phosphatidylcholines (PCs) with poly(ethylene glycol:2000)–phosphatidylethanolamines (PEG:2000-PEs). PEG:2000-PE is a micelle-forming polymer–lipid that is extensively used for increasing the lifetime of PC liposomes in the blood circulation through a steric stabilisation effect. The PC lipids and the PEG:2000-PE polymer–lipids have the same acyl chain length of either dimiristoyl (DM) or distearoyl (DS) chains. DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE mixtures were investigated over the entire range of relative compositions (0–100 mol%). In both dispersions, the low-temperature conventional spin label ESR spectra and the temperature dependence of the absorbance at 400 nm give an indication of the conversion from lamellae to micelles with increasing PEG:2000-PEs content. The physical state of the lipid assemblies, lamellar or micellar, is dependent not only on PEG:2000-PEs content, but also on the length of hydrocarbon chain of the lipid matrix. Micellisation is attained more readily in dispersions with longer hydrocarbon chains (i.e. in DSPC/PEG:2000-DSPE mixtures) than in those with shorter acyl chains (i.e. in DMPC/PEG:2000-DMPE mixtures). Saturation transfer ESR (ST-ESR) and absorbance measurements reflect the disaggregation of the bilayers and a reduction in the size of the lipid aggregates by PEG:2000-PEs at low content.

Keywords: Liposomes; Sterically stabilised liposomes; Polymer–lipids; Spin label; Electron spin resonance; Spectrophotometry

* Corresponding author. Tel.: +39-0984-496076; fax: +39-0984-494401.
E-mail address: sportelli@fis.unical.it (L. Sportelli).

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1. Introduction

In the last years a novel class of liposomes, known as sterically stabilised liposomes (SSLs), or Stealth® liposomes, used as drug carriers in vivo, has been developed [1–4]. SSLs are obtained by swelling common diacyl bilayer-forming lipids and small percentages of micelle-forming polymer–lipids, i.e. lipids derivatised on the polar heads with water-soluble polymers, in water. The most used polymer–lipids are the phosphatidylethanolamines (PEs), which have the hydrophilic polymer *N*-poly(ethylene glycol) (PEG) of average molecular weight of 2000 and 5000 Da covalently attached on the polar heads. The increased longevity of the SSLs from hours to days [3–7] relative to that of common liposomes is ascribed to the steric barrier provided by the polymeric coating [8–10].

In order to optimise the composition of the Stealth® liposomes, a variety of lipid/PEG–lipid dispersions have been studied, both experimentally [11–20] and theoretically [18,21,22].

The aim of the present work is to investigate the effect of the acyl chain length on the thermotropic and lyotropic phase behaviour and on the segmental lipid-chain dynamics of polymer-grafted dispersions. To ensure good mixing, we used phosphatidylcholines (PCs) and poly(ethylene glycol:2000)–phosphatidylethanolamines (PEG:2000-PEs) with matched acyl chain length. In particular, we use dimyristoyl- and distearoyl-PC and the corresponding PEG:2000-PE, the hydrocarbon chains of which consist of 14 (DM) and 18 (DS) carbon atoms, respectively. DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE dispersions at full hydration are investigated over the entire range of relative compositions (0–100 mol%). We employ conventional electron spin resonance (ESR) and saturation transfer ESR (ST-ESR) spectroscopy of spin-labelled phosphatidylcholines having the nitroxide moiety at the C-5 or at the C-14/C-16 positions in the *sn*-2 acyl chain (*n*-PCSL, $n = 5$, 14 and 16). These techniques are suitable for determining the rotational dynamics of lipid chains in amphiphilic aggregates on a broad time-scale range from ns to μs [23–26]. In addition, spectrophotometry at a fixed wavelength of 400 nm is used to study the phase behaviour and to obtain qualitative information about molecular properties of the mixed lipid dispersions [27,28].

The magnetic and spectrophotometric data indicate that the incorporation of increasing polymer–lipid content in the PCs host matrix progressively favours the conversion from liposomes to micelles. Moreover, the longer the length of the acyl chain of the host bilayer, the lower is amount of the polymer–lipid that can be incorporated into the bilayer before micellisation starts. In both DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE dispersions, disaggregation of the liposomes and a reduction in the size of the lamellar structures at low PEG–lipid content occur.

2. Materials and methods

2.1. Chemicals

The synthetic lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were from Sigma (St. Louis, MO). High-purity (> 99%) PEG–lipids 1,2-dimirystoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol) with PEG of average molecular weight 2000 Da (PEG:2000-DMPE, PEG:2000-DSPE, respectively), and the spin-labelled lipids 1-palmitoyl-2-[n-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phospho-choline (*n*-PCSL with $n = 5$, 16) were from Avanti Polar Lipids (Birmingham, AL), whereas, 1-palmitoyl-2-[14-(4,4-dimethyl-ox-azolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phospho-choline (14-PCSL) was synthesised as described by Marsh and Watts [29]. The reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH 7.5 were from Merck (Darmstadt, Germany). All materials were used as purchased, with no further purification. Distilled water was used throughout.

2.2. Lipid dispersion preparation

Aqueous dispersions for the spectrophotometric measurements were prepared by dissolving the required amounts of DMPC (DSPC) and PEG:2000-DMPE (PEG:2000-DSPE) in chloroform. The solvent was evaporated with a nitrogen gas stream and the samples were then kept under vacuum overnight. The dried lipid samples were fully hydrated with PBS at pH 7.5 (final lipid concentration of 1 mg/ml) by heating and vortexing at 40°C (65°C) and then transferred to a 3-ml quartz cell with a 1-cm optical path, and incubated overnight at 4°C before measurement.

Samples for ESR measurements were prepared as described above, except that 1% by weight of the spin-labelled lipid (*n*-PCSL) was dissolved in chloroform together with the other lipids. The hydrated lipid dispersions at a final concentration of 50 mM were sealed in a 1-mm (i.d.) 100-μl glass capillaries and then incubated for 24 h at 4°C before ESR measurements.

2.3. Optical and ESR measurements

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

ESR spectra were recorded with a 9-GHz Bruker (Karlsruhe, Germany) spectrometer, model ER 200D-SRC, and digitised with the spectrometer's built-in microcomputer using OS-9-compatible ESP1600 spectral acquisition and handling software. Sample capillaries were inserted into a standard 4-mm (i.d.) quartz ESR tube containing light silicone oil to increase thermal stability, and were centred in a TE_{102} rectangular ESR cavity (ER 4201, Bruker). Measurements were performed at thermal equilibrium by controlling sample temperature with a Bruker ER 4111VT variable-temperature control unit (accuracy ± 0.5°C).

Conventional, first-harmonic, in-phase, absorption ESR spectra were recorded at 10 mW of microwave power with a magnetic field modulation frequency of 100 kHz and a modulation amplitude of 1 G_{p-p} for phase-sensitive detection.

Saturation transfer ESR spectra were recorded in the second-harmonic, 90° out-of-phase, absorption mode with a modulation frequency of 50 kHz and a modulation amplitude of 5 G_{p-p}. The microwave power was set for each sample to give an average microwave field over the sample of $H_1 = 0.25$ G, according to standard protocols [30,31].

3. Results and discussion

3.1. Spectrophotometric measurements

The absorbance changes at 400 nm, ABS_{400}, vs. temperature for aqueous dispersions of DMPC/PEG:2000-DMPE at different molar ratios are shown in Fig. 1a. From the transition curve of DMPC in buffer, the pre-transition and main transition are observed at $T_p \cong 16.1$ and $T_m \cong 24.8$°C, respectively, as expected for fully hydrated multilamellar dispersions of DMPC in buffer (Fig. 1 and Table 1) ([32], and references therein). The addition of increasing concentrations of PEG:2000-DMPE firstly decreases the pre-transition temperature up to 7 mol%, and then abolishes the pre-transition itself (Fig. 1a and Table 1). Moreover, no appreciable modifications are evident for the main transition temperature up to 30 mol%. At 40 mol%, the main transition is no longer detected, and from 50 mol%, the absorbance remains approximately constant at a very low value throughout the whole temperature range, as occurs in micellar dispersions of PEG:2000-DMPE (Fig. 1a and Table 1).

The thermal profiles of ABS_{400} for aqueous DSPC/PEG:2000-DSPE mixtures at selected molar ratio are reported in Fig. 1b. For the DSPC/buffer dispersion, the pre- and main transitions temperatures are at $T_p \cong 50.1$ and $T_m \cong 54.7$°C, respectively (Fig. 1b and Table 1), in agreement with literature data [32]. The pre-transition is already abolished with the addition of the

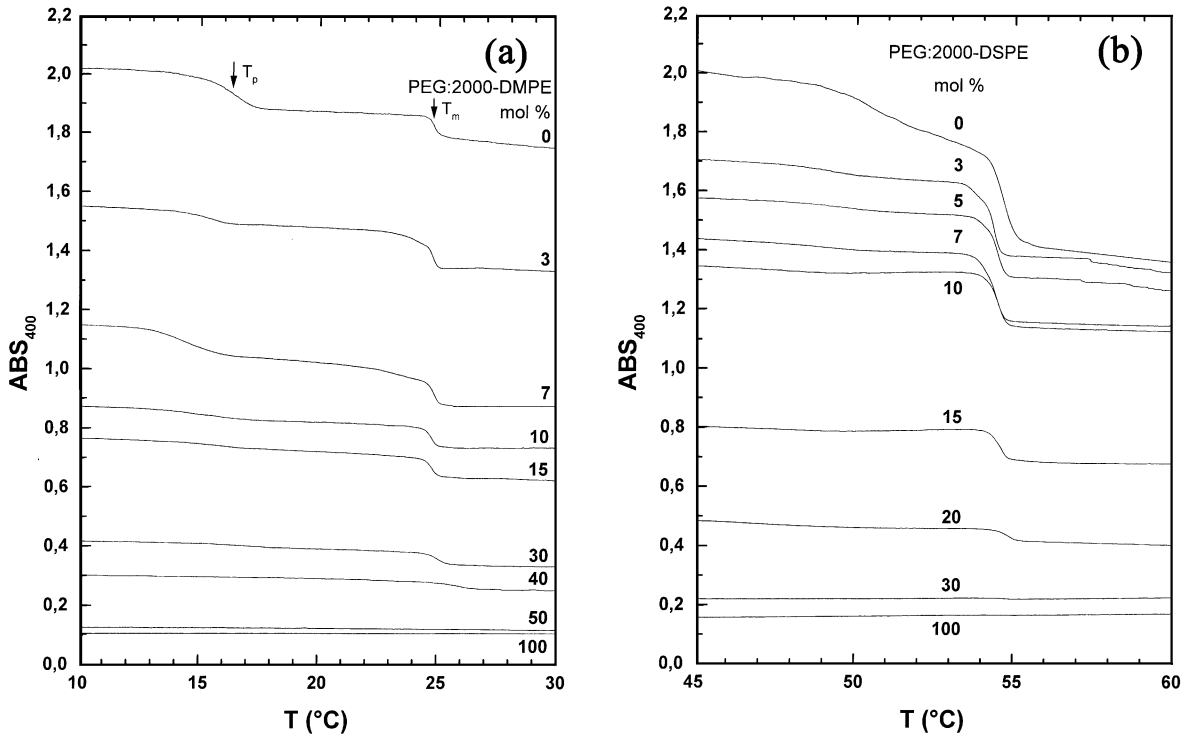

Fig. 1. Temperature dependence of the absorbance at 400 nm for aqueous dispersions of (a) DMPC/PEG:2000-DMPE and (b) DSPC/PEG:2000-DSPE at different mole fractions of the PEG–lipids. The arrows indicate the temperatures T_p and T_m at which the pre-transition and the main transitions are detected, respectively, in DMPC dispersions.

lowest polymer–lipid content. The progressive addition of PEG:2000-DSPE content up to 20 mol% leaves the main transition temperatures almost unaffected, whereas the amplitude of the transition progressively decreases (Fig. 1b and Table 1). Finally, beyond 30 mol% of PEG:2000-DSPE, the

Table 1
Temperatures of the pre-transition, T_p, and of the main transition, T_m, in aqueous mixtures of DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE at different mole fractions, as determined by spectrophotometry

Molar ratio	PEG:2000-DMPE		PEG:2000-DSPE	
(mol%)	T_p (°C)	T_m (°C)	T_p (°C)	T_m (°C)
0	16.1	24.8	50.1	54.7
3	15.1	24.8	–	54.3
5	14.8	24.3	–	54.4
7	14.5	24.9	–	54.4
10	–	24.8	–	54.4
15	–	24.8	–	54.5
20	–	24.8	–	54.7
30	–	25.0	–	–
40	–	–	–	–
100	–	–	–	–

The error in T_p and T_m is ± 0.1°C.

main transition disappears and the absorbance has a very low, constant value over the whole temperature range, as is obtained with aqueous dispersions of PEG:2000-DSPE.

An interesting feature of Fig. 1a,b is that the progressive addition of PEG–lipids in bilayers of PCs significantly decreased the absorbance value at any temperature. This is better demonstrated in Fig. 2, where ABS_{400} vs. polymer–lipid concentration is plotted at 10°C for DMPC/PEG:2000-DMPE and at 20°C for DSPC/PEG:2000-DSPE mixtures.

For DMPC/PEG:2000-DMPE dispersions, the absorbance first decreases rapidly from ~2.0 to ~0.4 on passing from 0 to 20 mol%, then there is a plateau level up to 40 mol%, and finally the absorbance drops close to zero for 50 mol% onward. A different trend is observed with mixtures of longer acyl chains. The absorbance for DSPC/DSPE-PEG:2000 dispersions, indeed, first drops slowly from ~2.1 to ~1.35 from 0 to 10 mol%, than decreases rapidly to ~0.2 at 30 mol%, and finally beyond this content, it levels off to nearly zero.

Overall, the spectrophotometric results are in keeping with literature data on a variety of lipid/PEG–lipid mixtures [11,13,14,17,19,20]. At low polymer–lipid content, the values observed for the absorbance and the presence of the chain melting transitions (see Figs. 1 and 2 and Table 1) are consistent with the presence of lamellar aggregates in buffer that scatter significantly at 400 nm. Moreover, the initial reduction in the absorbance in PCs/PEG:2000-PEs dispersions is due to the disaggregation and reduction of the number of lamellae by the PEG–lipids. This effect is due not only to the steric repulsion and hydration forces between the polymer headgroups, but also to the electrostatic bilayer–bilayer repulsion by the negatively charged PEG–lipids [11,13,14,17,19,20]. Lamellar aggregates of reduced size, such as large and small unilamellar vesicles other than multilamellar vesicles, are likely to be present in the dispersions at low PEG–lipid content, as suggested by the disappearance of the pretransition in some lipid/PEG–lipid mixtures (Fig. 1 and Table 1) [32].

At high polymer–lipid content, the optical clarity achieved by the lipid/polymer–lipid mixtures and the absence of the endothermic phase transitions in the thermal profile of the dispersions (Figs. 1 and 2 and Table 1) indicate the conver-

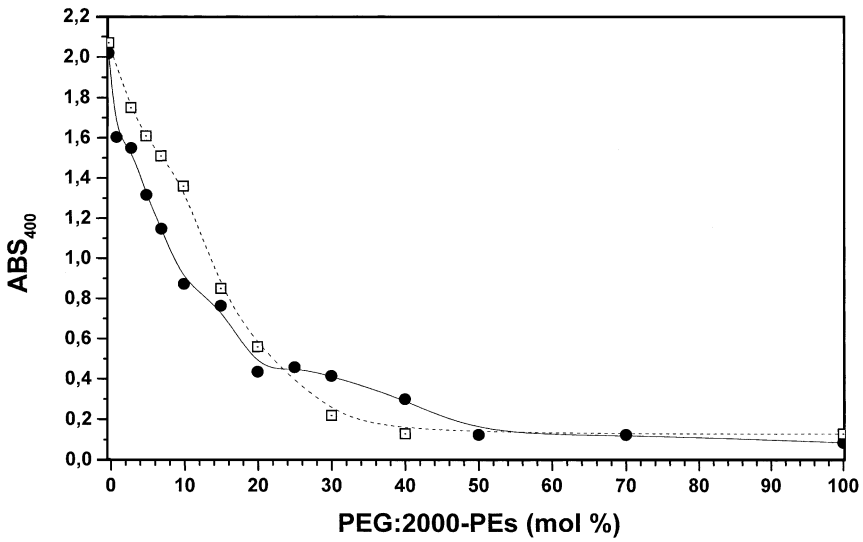

Fig. 2. Absorbance at 400 nm vs. PEG–lipid content for DMPC/PEG:2000-DMPE (●) and DSPC/PEG:2000-DSPE (□) aqueous dispersions at 10 and 20°C, respectively. The errors are smaller than the symbols.

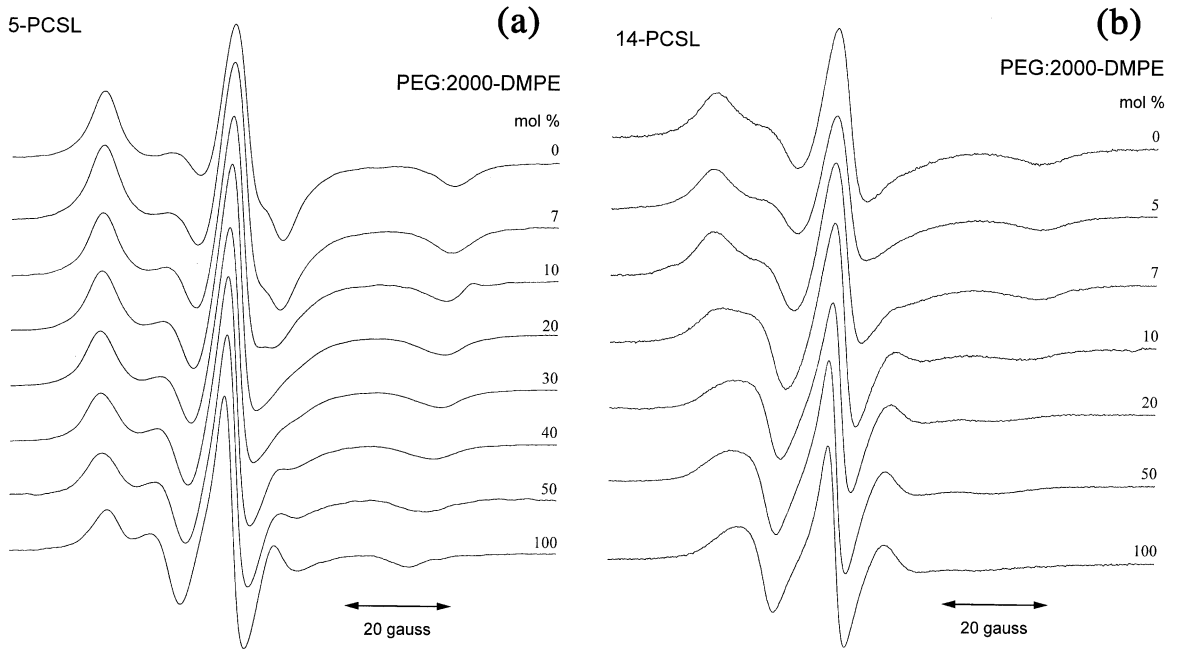

Fig. 3. Conventional ESR spectra at 10°C of (a) 5-PCSL and (b) 14-PCSL in aqueous mixtures of DMPC with different PEG:2000-DMPE content. Total scan width, 100 G.

sion from small, closed, bilayer vesicles to micelles.

Comparing the spectrophotometric results obtained with DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE, it appears that the dispersions with longer hydrocarbon chain achieve micellisation more readily than the dispersions with shorter acyl chains (see Fig. 1 and trends of ABS at high polymer–lipid content in Fig. 2).

3.2. Conventional spin label electron spin resonance measurements

3.2.1. DMPC / PEG:2000-DMPE aqueous dispersions

Conventional ESR spectra at 10°C of 5-PCSL in mixtures of DMPC with different concentrations of PEG:2000-DMPE are reported in Fig. 3a. This spin-labelled lipid is suitable for investigating the region around the glycerol backbone/beginning of the acyl chains of the amphiphilic aggregates.

The spectrum of 5-PCSL in DMPC/buffer dispersions is a powder pattern close to the rigid limit of sensitivity to rotational motion on the conventional nitroxide ESR time-scale. It indicates that the spin labels in the phospholipid lamellar gel phase undergo slow segmental rotational motion [24,33]. The spectral lineshapes do not change on inserting up to 7 mol% of PEG:2000-DMPE in the host lipid matrix of DMPC, and a slight reduction in the outermost peak separation is observed at 10 mol%. From 20 mol% onward, the spectral anisotropy gradually decreases. Indeed, the outermost peak separations move in, the perpendicular regions become better resolved, and the linewidths are broadened. At 40 mol%, and even more so at 50 mol%, the spectra have a partially motionally averaged axial lineshape. Finally, the spectrum of 5-PCSL in PEG:2000-DMPE dispersions shows spectral features of intermediate mobility, characteristic of a fluid environment that is typical of micellar assemblies of PEG–lipids at low temperature [19,20].

Conventional ESR measurements on DMPC/

PEG:2000-DMPE/buffer dispersions have also been performed using 14-PCSL, which has the nitroxide group located around the methyl chain end of PC molecules.

The spectra at 10°C of 14-PCSL in aqueous mixtures of DMPC and different concentrations of PEG:2000-DMPE are shown in Fig. 3b. The spectrum at 10°C of 14-PCSL in DMPC/buffer dispersions shows a lower degree of anisotropy relative to that of 5-PCSL in the same system. The spectral difference between the two positions of chain labelling is diagnostic of non-interdigitated phospholipid lamellar gel phase [33]. The addition of a low polymer–lipid content of up to 5–7 mol% leaves the spectral anisotropy almost unaffected, confirming good mixing of the lipid and polymer–lipids assembled in lamellar aggregates in the gel phase. The anisotropy progressively decreases on increasing the molar fraction of PEG:2000-DMPE incorporated with DMPC (see spectra from 10 to 50 mol% in Fig. 3b), and quasi-isotropic spectra, similar to that obtained for micellar dispersions of PEG:2000-DMPE alone, are evident from 50 mol% onward.

3.2.2. DSPC / PEG:2000-DSPE aqueous dispersions

ESR measurements were also carried out on DSPC/PEG:2000-DSPE dispersions using phosphatidylcholine spin-labelled both near the polar/apolar interface (5-PCSL) and near the terminal methyl end of the hydrocarbon chain (16-PCSL).

The spectra at 10°C of 5- and 16-PCSL in DSPC/PEG:2000-DSPE dispersions at selected molar ratios are reported in Fig. 4a,b, respectively. As in the case of pure DMPC, the spectrum of 5-PCSL in DSPC/buffer dispersions (Fig. 4a) at 10°C is a powder pattern, characteristic of spin labels in phospholipid lamellar gel phase. The spectra of 5-PCSL in mixtures of DSPC and

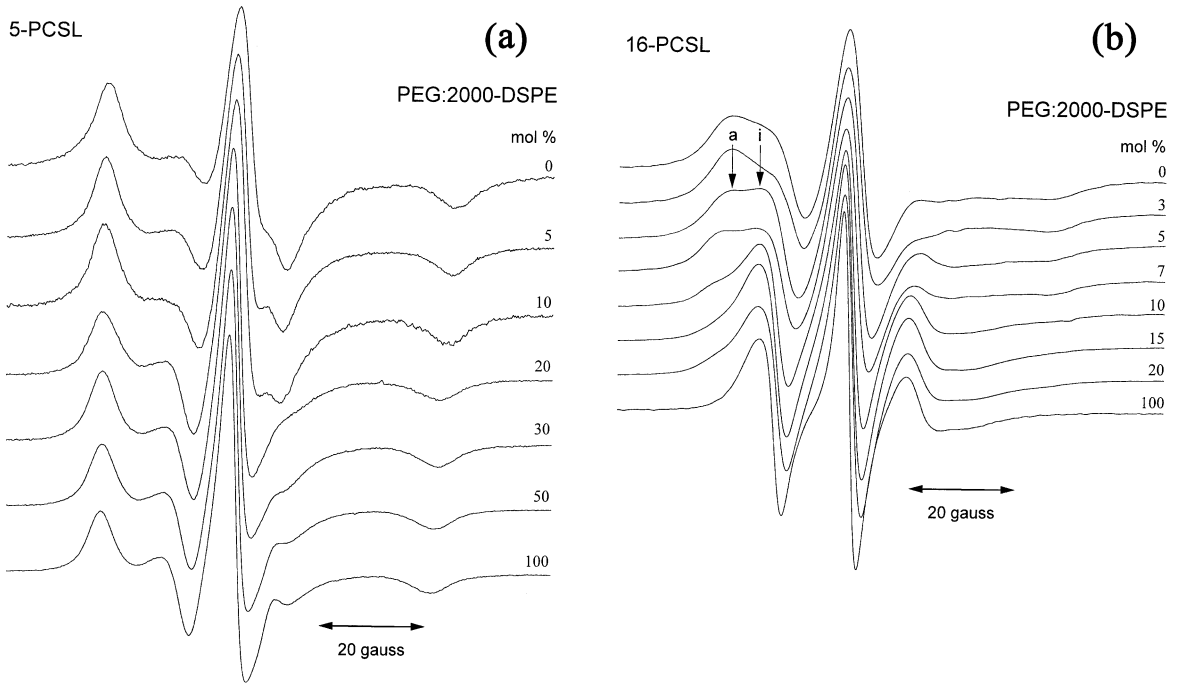

Fig. 4. Conventional ESR spectra at 10°C of (a) 5-PCSL and (b) 16-PCSL in aqueous mixtures of DSPC with different DSPE-PEG:2000 content. The arrows in (b) indicate the more anisotropic 'a' and isotropic 'i' components in the composite spectra of 16-PCSL. Total scan width, 100 G.

PEG:2000-DSPE up to 10 mol% show variations in the central region and a slight decrease in the outer hyperfine splitting. A considerable, progressive decrease of the spectral anisotropy is evident from 20 mol% onward, and already from 30 mol%, the spectral lineshapes are very similar to that of the spectrum of 5-PCSL in PEG:2000-DSPE dispersions at 10°C.

The spectrum of 16-PCSL in lamellar gel phase of DSPC dispersions (Fig. 4b) at 10°C shows a lower degree of anisotropy and a considerable motional broadening relative to that of 5-PCSL in the same dispersion [33]. At the other extreme, the spectrum at 10°C of 16-PCSL in micellar dispersions of the PEG:2000-DSPE polymer–lipid is a near-isotropic triplet with differentially broadened ^{14}N hyperfine manifolds, characteristic of a more mobile environment. Addition of PEG:2000–lipid up to 3 mol% in the host lipid matrix of DSPC causes a slight distortion of the spectral lamellar lineshape. When the concentration of the polymer–lipid is further increased, however, the ESR spectra of 16-PCSL consist of a superposition of two spectral components. One component (designated 'a' in Fig. 4b), with larger outer hyperfine splitting, corresponds to spin-labelled lipids in a lamellar gel phase environment that is characteristic of the mixtures with low PEG–lipid content. The second, sharper spectral component (designated 'i' in Fig. 4b) corresponds to the quasi-isotropic environment of the micellar phase that is formed by the polymer–lipid alone at this temperature. The relative proportion of the micelle-like component increases with increasing PEG:2000-DSPE content over the range 5–20 mol%. For concentrations of polymer–lipid beyond this range, the spectra consist of a single component with lineshapes that resemble that of the spin label in PEG:2000-DSPE micelles alone.

The low-temperature conventional ESR measurements in PCs/PEG:2000-PEs dispersions, using different positional isomers of *n*-PCSL, indicate that a conversion from bilayers to micelles occurs in such mixtures when increasing the polymer–lipid content. This relies on the fact that at 10°C, the labels in dispersions of PCs with low PEG:2000-PEs concentration are in the slow motion regime and give rise to spectra with large anisotropy, similar to those of lamellar aggregates of PCs alone. At the same low temperature, more isotropic spectra, similar to those of micellar aggregates of PEG-2000-PE alone, are instead obtained in mixtures with higher polymer–lipid content. At intermediate PEG–lipid concentrations, in some favourable circumstances, the lamellar and micellar components are clearly resolved in the ESR spectra (see Fig. 4b), indicating the coexistence of bilayers and micelles. Two-component ESR spectra are also observed in the gel phase of DPPC/PEGs-DPPE dispersions with PEG of either low (i.e. 350 Da) or intermediate (i.e. 2000 and 5000 Da) size [19,20]. Coexistence regions of bilayer and micellar phases are also obtained with several biophysical techniques in polymer-grafted dispersions of very different lipid and polymer–lipid compositions [11–16].

Comparing the spectra of 5-PCSL and those of 14- and 16-PCSL in both polymer–lipid dispersions, it appears that micelle formation occurs earlier in DSPC/PEG:2000-DSPE than in DMPC/PEG:2000-DMPE dispersions. In other words, lower amounts of PEG:2000-DSPE can be incorporated into DSPC than of PEG:2000-DMPE in DMPC before the bilayers are destabilised and converted into micelles.

A similar dependence of the lamellar-to-micellar conversion on the lipid chain length is obtained in mixtures of either DPPC or DSPC with the corresponding DPPE and DSPE derivatised with PEG:5000 [14]. Indeed, mixed micelle formation and bilayer solubilisation were delayed in DPPC/PEG:5000-DPPE as compared to DSPC/PEG:5000-DSPE dispersions.

3.3. ST-ESR measurements

At low temperature, the rotational rate of 5-PCSL in mixtures of PCs and low PEG:2000-PEs content is close to the limit of motional sensitivity of the conventional ESR time-scale. Therefore, further insight into the rotational dynamics of the lipid/polymer–lipid dispersions in the lamellar state are obtained by performing saturation transfer ESR measurements [23–26].

Selected ST-ESR spectra at 10°C of 5-PCSL in

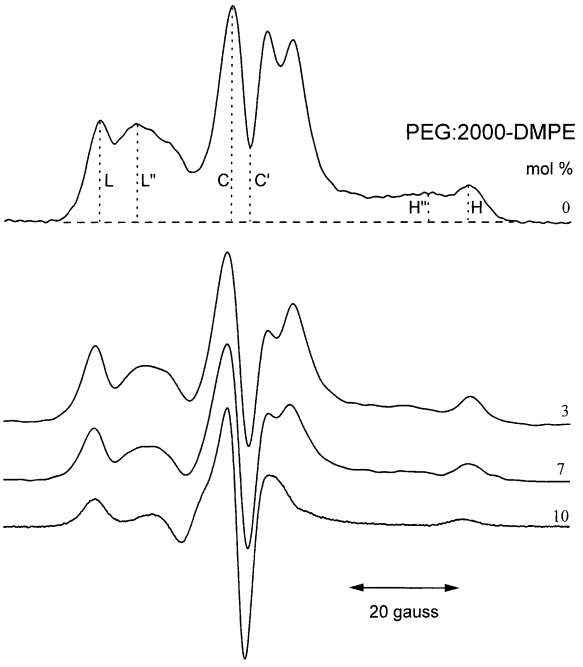

Fig. 5. ST-ESR spectra at 10°C for 5-PCSL in DMPC/PEG:2000-DMPE dispersions at different PEG–lipid content. The lineheights L'', L, C', C, H'' and H in the low-, central- and high-field region of the spectrum are indicated. Total scan width, 100 G.

DMPC/PEG:2000-DMPE at different molar ratios are reported in Fig. 5. The spectral lineshapes indicate that the rotational motion of the spin-labelled lipid chains lies in the saturation transfer ESR regime for up to ≈ 7 mol% of PEG:2000-DMPE. Beyond this concentration, the spectra contain components from motion on the slow, conventional spin-label ESR time-scale, and therefore are not appropriate for analysis by ST-ESR (see spectrum at 10 mol% in Fig. 5).

As is evident in Fig. 5, the addition of low PEG:2000-DMPE content in DMPC dispersions, unlike the situation for conventional spectra, induces spectral modifications, both in the central region, and (to a lesser extent) in the extreme resonances of the spectra. Indeed, a rapid decrease in the lineheight ratios in the low-, L''/L, central-, C'/C, and high-field, H''/H, hyperfine manifolds occurs on inserting the lowest PEG–lipid content. The decrease in the C'/C ratio indicates an increase in the rate of rotation about the long molecular axis of the lipids [24,33,34]. The decrease in L''/L and H''/H likely reflects a decrease in size of the lamellar structures on addition of the PEG–lipid, since the conventional ESR spectra in Fig. 3a suggest that there is no increase in chain segmental rotation rates, which would be reflected in L- and H-ratios [19].

The ST-ESR spectra of 5-PCSL in DSPC/PEG:2000-DSPE dispersions up to 5 mol% (not shown) display the same spectral features as those of 5-PCSL in dispersions of DMPC/PEG:2000-DMPE with shorter hydrocarbon chain length.

The effective rotational correlation times, τ_R^{eff}, in terms of the diagnostic lineheight ratios, are given for DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE in Table 2. They have been deduced from standard calibrations of the lineheight ratios for isotropic motion using the expression [35–38]:

$$\tau_R^{eff} = \frac{k}{P_0 - P} - b \quad (1)$$

where P is the lineheight ratio (i.e. L''/L or C'/C or H''/H), P_0 is the rigid limit value of P,

Table 2
Effective rotational correlation times, τ_R^{eff}, evaluated from the low- (L''/L), central- (C'/C), and high- (H''/H) field diagnostic line-height ratios in the ST-ESR spectra of 5-PCSL in mixtures of DMPC/PEG:2000-DMPE and of DSPC/PEG:2000-DSPE at different molar ratios at 10°C

Molar ratio (mol%)	τ_R^{eff} (μs)		
	(L''/L)	(C'/C)	(H''/H)
PEG:2000-DMPE			
0	57	10	83
1	35	2.4	70
3	33	2.3	58
5	30	1.3	50
7	25	0.8	46
PEG:2000-DSPE			
0	60	14	87
1	37	2.7	73
3	28	1	44
5	22	0.2	34

and k and b are the calibration constants given in [36,38].

As is evident in Table 2, all the effective rotational correlation times decrease with increasing polymer–lipid content. This accounts for the induction of more loosely packed, smaller lamellar aggregates, in which the labels have more freedom of motion. Moreover, at any fixed composition of the mixtures, comparably higher values are obtained from the low- and high-field ratios, but shorter effective rotational correlation times are obtained from the central ratios. This indicates anisotropic motion with faster rotation about the long axis of the lipid molecules and slower off-axial motion, as normally occurs in lamellar assemblies of phospholipids at low temperature [24,33,34].

It is interesting to note that both ST-ESR and absorbance measurements reflect disaggregation of the bilayers and a reduction in the size of lamellar aggregates by PEG:2000-PEs at low content. Moreover, the different concentration range of the polymer–lipids for which the labels are in the motional regime of ST-ESR validity once more confirms the tendency for micellisation to occur earlier in DSPC/PEG:2000-DSPE than in DMPC/PEG:2000-DMPE dispersions.

4. Conclusions

Conventional and saturation transfer spin-label ESR spectroscopy and spectrophotometry have been used to study fully hydrated DMPC/PEG:2000-DMPE and DSPC/PEG:2000-DSPE mixtures over the entire range of relative compositions. The data indicate that increasing the PEG:2000–lipid content in both dispersions causes the PC liposomes to disaggregate and to first pack into lamellar structures of reduced size, and then in mixed micelles. Moreover, PC bilayers with longer hydrocarbon chains achieve micellisation at lower PEG–lipid concentrations than those with shorter chains. On the basis of the conventional and ST-ESR data, it is likely that the onset of micelle formation is $\approx 7/10$ mol% in DMPC/PEG:2000-DMPE, whereas it is approximately 5 mol% in DSPC/PEG:2000-DSPE dispersions. Spectrophotometry and spin-label conventional ESR results indicate that micellisation is complete at ≈ 40 and ≈ 30 mol% of the polymer–lipids in DMPC/PEG:2000-DMPE and DMPC/PEG:2000-DMPE dispersions, respectively. The dependence of the bilayer-to-micelle transition on the length of the hydrocarbon chain is the same if we also consider previous spin label ESR results in dispersions of DPPC/PEG:2000-DPPE [19].

It seems very likely that the conversion from the lamellar to the micellar phase in PEG:2000-grafted dispersions of PCs is regulated by the tensile strength of the phospholipid matrix. The tighter, lateral acyl-chain packing of the gel phase bilayers of DSPC leads the PEG:2000-DSPE polymer–lipid to experience a restricted diffusional motion within the bilayer, resulting in a more effective formation of mixed micelles. The loosened, lateral acyl-chain packing and the higher fluidity of the DMPC bilayer matrix, instead, favour the mixing of PEG:2000-DMPE with DMPC and delay micelle formation.

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References

[1] D.D. Lasic, Liposomes: From Physics to Applications, Elsevier, Amsterdam–London–New York–Tokyo, 1993.
[2] J.M.J. Marjan, T.M. Allen, Long circulating liposomes: past, present and future, Biotechnol. Adv. 14 (2) (1996) 151–175.
[3] G. Blume, G. Cevc, Liposomes for the sustained drug release in vivo, Biochim. Biophys. Acta 1029 (1990) 91–97.
[4] D.D. Lasic, F. Martin, Stealth Liposome, CRC Press, Boca Raton, London, Tokyo, 1995.
[5] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes, FEBS Lett. 268 (1990) 235–237.

[6] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, Biochim. Biophys. Acta 1066 (1991) 29–36.

[7] D. Papahadjopoulos, T.M. Allen, A. Gabizon et al., Sterically stabilised liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl. Acad. Sci. USA 88 (1991) 11460–11464.

[8] D.D. Lasic, F.J. Martin, A. Gabizon, S.K. Huang, D. Papahadjopoulos, Sterically stabilised liposomes: a hypothesis on the molecular origin of the extended circulation times, Biochim. Biophys. Acta 1070 (1991) 187–192.

[9] G. Blume, G. Cevc, Molecular mechanism of the lipid vesicles longevity in vivo, Biochim. Biophys. Acta 1146 (1993) 157–168.

[10] V.P. Torchilin, V.P. Omelyanenko, M.I. Papisov et al., Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity, Biochim. Biophys. Acta 1195 (1994) 11–20.

[11] D.D. Lasic, M.C. Woodle, F.J. Martin, T. Valentincic, Phase behavior of 'stealth-lipid'–lecithin mixtures, Period. Biol. 93 (1991) 287–290.

[12] K. Hristova, A. Kenworthy, T.J. McIntosh, Effect of bilayer composition on the phase behaviour of liposomal suspension containing poly(ethylene glycol)–lipids, Macromolecules 28 (1995) 7693–7699.

[13] A.K. Kenworthy, S.A. Simon, T.J. McIntosh, Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol), Biophys. J. 68 (1995) 1903–1920.

[14] F.K. Bedu-Addo, P. Tang, Y. Xu, L. Huang, Effects of polyethyleneglycol chain length and phospholipid acyl chain composition on the interaction of polyethyleneglycol–phospholipid conjugate with phospholipid. Implications in liposomal drug delivery, Pharm. Res. 13 (1996) 710–717.

[15] T.R. Baekmark, S. Pedersen, K. Jorgensen, O.G. Mouritsen, The effects of ethylene oxide-containing lipopolymers and tri-block copolymers on lipid bilayers of dipalmitoylphosphatidylcholine, Biophys. J. 73 (1997) 1479–1491.

[16] K. Edwards, M. Jonsson, G. Karlsson, M. Silvander, Effect of polyethyleneglycol–phospholipids on aggregate structure in preparation of small unilamellar liposomes, Biophys. J. 73 (1997) 258–266.

[17] S. Belsito, R. Bartucci, L. Sportelli, Sterically stabilised liposomes of DPPC/DPPE-PEG: a spin label ESR and spectrophotometric study, Biophys. Chem. 75 (1998) (2000) 33–43.

[18] I. Szleifer, O.V. Gerasimov, D.H. Thompson, Spontaneous liposome formation induced by grafted poly(ethylene oxide) layers: theoretical prediction and experimental verification, Proc. Natl. Acad. Sci. USA 95 (1998) 1032–1037.

[19] S. Belsito, R. Bartucci, G. Montesano, D. Marsh, L. Sportelli, Molecular and mesoscopic properties of hydrophilic polymer-grafted phospholipids mixed with phosphatidylcholine in aqueous dispersion. Interaction of dipalmitoyl *N*-poly(ethylene glycol) phosphatidylethanolamine with dipalmitoyl phosphatidylcholine studied by spectrophotometry and spin-label electron spin resonance, Biophys. J. 78 (2000) 1420–1430.

[20] G. Montesano, R. Bartucci, S. Belsito, D. Marsh, L. Sportelli, Lipid membrane expansion and micelle formation by polymer-grafted lipids: scaling with polymer-length studied by electron spin resonance, Biophys. J. 80 (2001) 1372–1383.

[21] K. Hristova, D. Needham, The influence of polymer-grafted lipids on the physical properties of lipid bilayers: a theoretical study, J. Colloid Interface Sci. 168 (1994) 302–314.

[22] K. Hristova, D. Needham, Phase behaviour of a lipid/polymer–lipid mixture in aqueous medium, Macromolecules 38 (1995) 991–1002.

[23] D.D. Thomas, L.R. Dalton, J.S. Hyde, Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance, J. Chem. Phys. 65 (1976) 3006–3024.

[24] D. Marsh, Electron spin resonances: spin labelsin: E. Grell (Ed.), Membrane Spectroscopy, Springer-Verlag, Berlin, 1981, p. 51.

[25] D. Marsh, Experimental methods in spin label spectral analysisin: L.J. Berliner, J. Reuben (Eds.), Spin Labelling. Theory and Applications, Plenum Press, New York, 1989, p. 255.

[26] M.A Hemminga, P.A. de Jager, Saturation transfer spectroscopy of spin labels: techniques and interpretation of spectrain: L.J. Berliner, J. Reuben (Eds.), Spin Labelling. Theory and Applications, Plenum Press, New York, 1989, p. 131.

[27] P.N. Yi, R.C. Macdonald, Temperature dependence of optical properties of aqueous dispersions of phosphatidylcholine, Chem. Phys. Lipids 11 (1973) 114–134.

[28] E.S. Rowe, Thermodynamic reversibility of phase transitions. Specific effects of alcohols on phosphatidylcholines, Biochim. Biophys. Acta 813 (1985) 321–330.

[29] D. Marsh, A. Watts, Spin labelling and lipid–protein interactions in membranesin: P.C. Jost, O.H. Griffth (Eds.), Lipid–Protein Interaction, 2, John Wiley & Sons, New York, 1982, p. 53.

[30] P. Fajer, D. Marsh, Microwave and modulation field inhomogeneities and the effect of cavity Q in saturation transfer ESR spectra. Dependence on sample size, J. Magn. Reson. 49 (1982) 212–224.

[31] M.A. Hemminga, P.A. de Jager, D. Marsh, P. Fajer, Standard conditions for the measurement of saturation transfer ESR spectra, J. Magn. Reson. 59 (1984) 160–163.

[32] J.R. Silvius, Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteinsin: P.C. Jost, O.H. Griffith (Eds.), Lipid–Protein Interaction, 2, John Wiley & Sons, New York, 1982, p. 239.

[33] R. Bartucci, T. Páli, D. Marsh, Lipid chain motion in an interdigitated gel phase: conventional and saturation transfer ESR of spin-labelled lipids in dipalmitoylphosphatidylcholine–glycerol dispersions, Biochemistry 32 (1993) 274–281.

[34] D. Marsh, Molecular motion in phospholipid bilayers in the gel phase: long axis rotation, Biochemistry 19 (1980) 1632–1637.

[35] L.I. Horvath, D. Marsh, Improved numerical evaluation of saturation transfer electron spin resonance spectra, J. Magn. Reson. 80 (1988) 314–317.

[36] D. Marsh, Exchange and dipolar spin-spin interactions and rotational diffusion in saturation transfer ESR spectroscopy, Appl. Magn. Reson. 3 (1992) 53–65.

[37] D. Marsh, L.I. Horvath, A simple analytical treatment of the sensitivity of saturation transfer EPR spectra to slow rotational diffusion, J. Magn. Reson. 99 (1992) 323–331.

[38] D. Marsh, Spin label ESR spectroscopy and FTIR spectroscopy for structural/dynamics measurements on ion channels, Methods Enzymol. 249 (1999) 59–92.