Biophysical Journal Volume 78 March 2000 1420–1430

Molecular and Mesoscopic Properties of Hydrophilic Polymer-Grafted Phospholipids Mixed with Phosphatidylcholine in Aqueous Dispersion: Interaction of Dipalmitoyl *N*-Poly(Ethylene Glycol)Phosphatidylethanolamine with Dipalmitoylphosphatidylcholine Studied by Spectrophotometry and Spin-Label Electron Spin Resonance

Salvatore Belsito,* Rosa Bartucci,* Giuseppina Montesano,* Derek Marsh,† and Luigi Sportelli*
*Dipartimento di Fisica and Unità INFM, Università della Calabria, I-87036 Arcavacata di Rende (CS), Italy, and †Abteilung Spektroskopie, Max-Planck-Institut für biophysikalische Chemie, D-37070 Gottingen, Germany

ABSTRACT Spin-label electron spin resonance (ESR) spectroscopy, together with optical density measurements, has been used to investigate, at both the molecular and supramolecular levels, the interactions of *N*-poly(ethylene glycol)-phosphatidylethanolamines (PEG-PE) with phosphatidylcholine (PC) in aqueous dispersions. PEG-PEs are micelle-forming hydrophilic polymer-grafted lipids that are used extensively for steric stabilization of PC liposomes to increase their lifetimes in the blood circulation. All lipids had dipalmitoyl (C16:0) chains, and the polymer polar group of the PEG-PE lipids had a mean molecular mass of either 350 or 2000 Da. PC/PEG-PE mixtures were investigated over the entire range of relative compositions. Spin-label ESR was used quantitatively to investigate bilayer-micelle conversion with increasing PEG-PE content by measurements at temperatures for which the bilayer membrane component of the mixture was in the gel phase. Both saturation transfer ESR and optical density measurements were used to obtain information on the dependence of lipid aggregate size on PEG-PE content. It is found that the stable state of lipid aggregation is strongly dependent not only on PEG-PE content but also on the size of the hydrophilic polar group. These biophysical properties may be used for optimized design of sterically stabilized liposomes.

INTRODUCTION

Liposomes containing diacyl lipids with sterically bulky hydrophilic polar heads are of particular interest both for basic biomembrane research and for biotechnological applications (Lasic, 1993). For example, liposomes obtained by swelling a variety of bilayer-forming lipids that contain a given proportion of hydrophilic polymer-lipids (i.e., lipids with water-soluble polymers covalently attached at the polar head) in water act as very effective drug encapsulation and delivery systems (Blume and Cevc, 1990; Lasic, 1993; Lasic and Martin, 1995). In particular, it has been established that phosphatidylcholine (PC) host bilayer matrices containing phosphatidylethanolamine that has been derivatized by attachment of poly(ethylene-glycol) polymers (PEG-PEs, with PEG molecular masses of 2000 and 5000 Da) have a blood circulation time from one to two orders of magnitude longer (from a few hours to days) than conventional, unprotected phospholipid liposomes (Blume and Cevc, 1990; Klibanov et al., 1990; Allen et al., 1991; Papahadjopoulos et al., 1991). The extended lifetimes in vivo arise from the steric barrier provided by the grafted polymers that stabilizes the lipid bilayer against attack by diverse elements of the immune system (Lasic et al., 1991a; Blume and Cevc, 1993; Torchilin et al., 1994).

From a biophysical perspective, lipid/PEG-lipid/water dispersions have been studied both experimentally (Lasic et al., 1991b; Hristova et al., 1995; Kenworthy et al., 1995; Bedu-Addo et al., 1996; Baekmark et al., 1997; Edwards et al. 1997; Belsito et al., 1998; Szleifer et al., 1998) and theoretically (Hristova and Needham, 1995; Hristova et al., 1995; Szleifer et al., 1998). An essential feature for a successful stealth liposome delivery system is the optimization of the composition of the liposomes, in particular with regard to the release of their content. In this light it is useful to study the molecular interactions that exist, and the dynamics of the aggregates that coassemble, when micelle-forming lipids, such as PEG-lipids, are dispersed together with bilayer-forming lipids in an aqueous environment. For the present work, we have studied fully hydrated binary mixtures of ungrafted and polymer-lipids with identical acyl chain compositions, namely dipalmitoylphosphatidylcholine (DPPC) mixed with dipalmitoylphosphatidylethanolamine (DPPE) bearing poly(ethylene glycol)s of either low or intermediate average molecular masses (PEG:350 or PEG:2000, respectively) at the polar head, over the entire composition range of the two lipids from 0 to 100 mol%. This has been done by using spectrophotometry at fixed wavelength, and both conventional and saturation transfer electron spin resonance spectroscopies (ESR and STESR) of spin-labeled phosphatidylcholine having the nitroxide moiety at the C-5 or at the C-16 positions in the *sn*-2 acyl chain (5- and 16-PCSL).

Received for publication 4 August 1999 and in final form 11 November 1999.

Address reprint requests to Dr. L. Sportelli, Dipartimento di Fisica, Università della Calabria, I-87036 Arcavacata di Rende (CS), Italy. Tel.: +39-0984-493131; Fax: +39-0984-493187; E-mail: sportelli@fis.unical.it.

0006-3495/00/03/1420/11 $2.00

It is shown here that spin label ESR spectroscopy is particularly helpful not only for investigating the rotational dynamics of the lipid chains on different time scales (Thomas et al., 1976; Marsh, 1981, 1989; Hemminga and de Jager, 1989) but also for studying the micelle formation that inevitably occurs in mixtures of polymer-lipids with bilayer-forming phospholipids (Hristova and Needham, 1995; Hristova et al., 1995). Because at low temperature the lamellar bilayer lipid components are in the gel phase, whereas the micellar components of the lipid mixture are in a fluid phase, these two environments are readily resolved and quantified in the conventional spin-label ESR spectra. In this way, it is found that the stable phases, lamellar or micellar, of the mixed-lipid systems are affected critically, in a polymer chain length-dependent manner, by the content of PEG-lipid in the dispersions. These results have direct relevance both for the stability of these liposomal formulations in serum, and for the ability of the liposomes to release their contents in a controlled fashion on interaction with cells.

MATERIALS AND METHODS

Materials

The synthetic lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was from Sigma (St. Louis, MO). High-purity PEG-lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol) with PEG of average molecular mass 350 or 2000 Da (PEG:350-DPPE and PEG:2000-DPPE, respectively), and the spin-labeled lipids 1-palmitoyl-2-(*n*-(4, 4-dimethyl-oxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL with $n = 5, 16$) were from Avanti Polar Lipids (Birmingham, AL). 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.

Spin-label ESR measurements

Samples for ESR measurement were prepared by dissolving the required amounts of DPPC and PEG-DPPE, together with 1% by weight of the spin-labeled lipid (*n*-PCSL), in chloroform. The solvent was evaporated in a nitrogen gas stream and then kept under vacuum overnight. The dried lipid samples were hydrated fully with PBS at pH 7.5 (final lipid concentration 25 mM), by heating at 60°C and periodically vortexing for 40 min. The hydrated lipid dispersions were then sealed in 1 mm (i.d.) 100-μl glass capillaries and then incubated for 24 h at 10°C before ESR measurements. ESR spectra were recorded on a 9-GHz Bruker (Karlsruhe, Germany) spectrometer (model ER 200D-SRC) and digitized with the spectrometer's built-in microcomputer with OS-9 compatible ESP1600 spectral acquisition and handling software. Sample capillaries were inserted in a standard 4-mm (i.d.) quartz ESR tube containing light silicone oil for increased thermal stability and were centered in a TE_{102} rectangular ESR cavity (ER 4201; Bruker). Measurements were performed at thermal equilibrium starting from low temperature. Sample temperature was controlled with a Bruker ER 4111VT variable-temperature control unit (accuracy $\pm$ 0.5°C). Conventional first-harmonic in-phase absorption ESR spectra were recorded at a microwave power of 10 mW with 1 $G_{p\text{-}p}$ magnetic field modulation amplitude and a frequency of 100 kHz of the magnetic field modulation used 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 $G_{p\text{-}p}$. 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 the standardized protocol given by Fajer and Marsh (1982) and Hemminga et al. (1984).

Spectrophotometric measurements

Aqueous dispersions of DPPC/PEG-DPPE for the spectrophotometric measurements were prepared as described above, but with omission of the spin label, at a final lipid concentration of 1 mg/ml. The dried lipid films were first hydrated in PBS at pH 7.5, then transferred to a 3-ml quartz cell with 1-cm optical path, and finally incubated overnight at 10°C before measurement.

Optical density measurements at 400 nm were made with a Jasco 7850 spectrophotometer equipped with a Peltier thermostatted 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 were carried out with the built-in microcomputer accessory of the spectrophotometer.

The data presented in this paper are single measurements, but the reproducibility of the results has been tested by repeating the experiments.

RESULTS

Spectrophotometry

Fig. 1 *A* shows the optical density at 400 nm (OD_{400}) for DPPC/PEG:350-DPPE dispersions, measured as a function of the content of PEG:350-DPPE, at 10 and 50°C. At 10°C, the optical density first drops rapidly with increasing PEG:350-DPPE content up to ~7–10 mol%, then decreases gradually to close to zero from 40 mol% onward. Almost the same dependence on composition is observed at 50°C, except that the OD_{400} values are somewhat lower than the corresponding ones at 10°C. It is interesting to note that the difference between the optical densities at low and high temperatures (i.e., $OD_{400}(T = 10°C) - OD_{400}(T = 50°C)$) decreases with increasing PEG:350-DPPE content and becomes approximately zero at 50 mol% of PEG:350-DPPE.

For hydrated mixtures of DPPC with PEG:2000-DPPE, the dependence of the optical density at 10°C and 50°C on the content of PEG:2000-DPPE shown in Fig. 1 *B* differs somewhat from that observed with the mixtures containing PEG:350-DPPE. Indeed, at both temperatures, the initial decrease in optical density is steeper and then takes place continually with increasing content of PEG:2000-DPPE, reaching a limiting low value at ~20–30 mol% PEG:2000-DPPE. Moreover, the difference in optical densities between low and high temperatures is reduced, as compared to mixtures containing PEG:350-DPPE, and already attains a value close to zero at 30 mol% PEG:2000-DPPE.

A straightforward interpretation of these changes in optical density with increasing content of PEG-lipid in the dispersions is that the initial rapid drop corresponds to a decrease in overall size of the lipid aggregates. At least part of the initial decrease may be attributed to disaggregation of

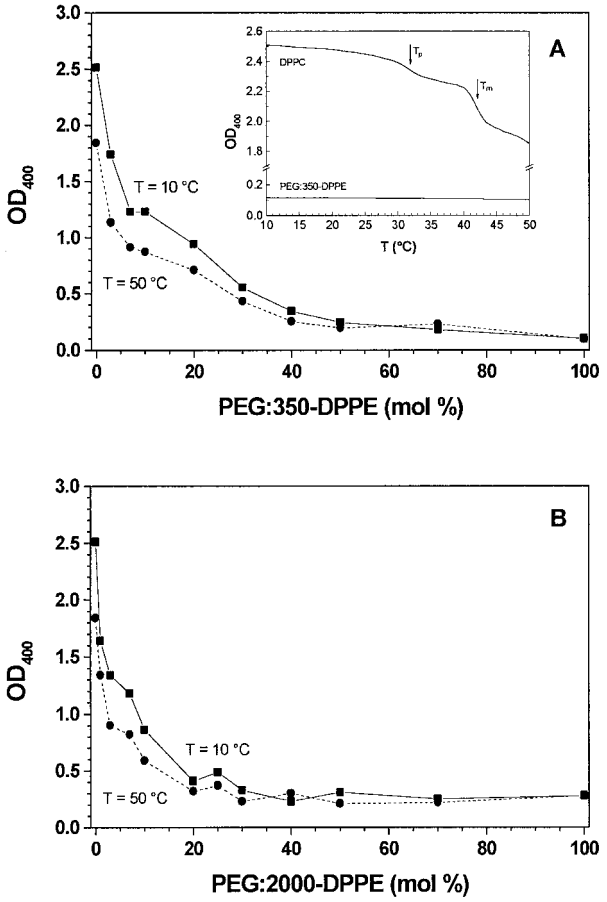

FIGURE 1 Dependence of the optical density at 400 nm on the PEG-DPPE lipid content of aqueous dispersions of DPPC/PEG-DPPE mixtures. DPPC/PEG:350-DPPE mixtures (*A*) and DPPC/PEG:2000-DPPE mixtures (*B*) at 10°C (■) and 50°C (●). The errors are smaller than the symbols. The inset shows the temperature dependence of the optical density for DPPC (*upper curve*) and PEG:350-DPPE (*line*) aqueous dispersions. The arrows indicate the temperatures T_p and T_m at which the pretransition and main transition, respectively, are detected in DPPC dispersions.

the liposomes. Finally, the closed bilayer vesicles of reduced size convert to micelles, when the OD_{400} becomes very low. A further criterion for micelle formation is the identity of the optical densities at low and high temperatures. For micelles, there is no longer a change in optical density that results from the change in refractive index of the lipid that is associated with the chain-melting transition in lipid bilayers (see Fig. 1, *inset*). In terms of the different behavior of mixtures containing PEG:350-DPPE and PEG:2000-DPPE, it appears that the longer polymer-lipid is more effective in reducing the size of the lipid aggregates and achieves micellization more readily than does the shorter polymer-lipid.

Conventional electron spin resonance

Results are reported for three different temperatures, 10°C, 30°C, and 50°C, which correspond to the gel, intermediate, and fluid phases, respectively, of dipalmitoyl phosphatidylcholine bilayers (Mabrey and Sturtevant, 1976).

Gel phase

Typical conventional ESR spectra of 5- and 16-PCSL spin label positional isomers in mixtures of DPPC with different contents of the short polymer-lipid PEG:350-DPPE, at 10°C, are given in Fig. 2, *A* and *B*, respectively. The spectrum of 5-PCSL in dispersions of DPPC is close to the rigid limit of sensitivity to rotational motion on the conventional nitroxide ESR time scale. It is indicative of the slow segmental rotational motion that is typical for phospholipid

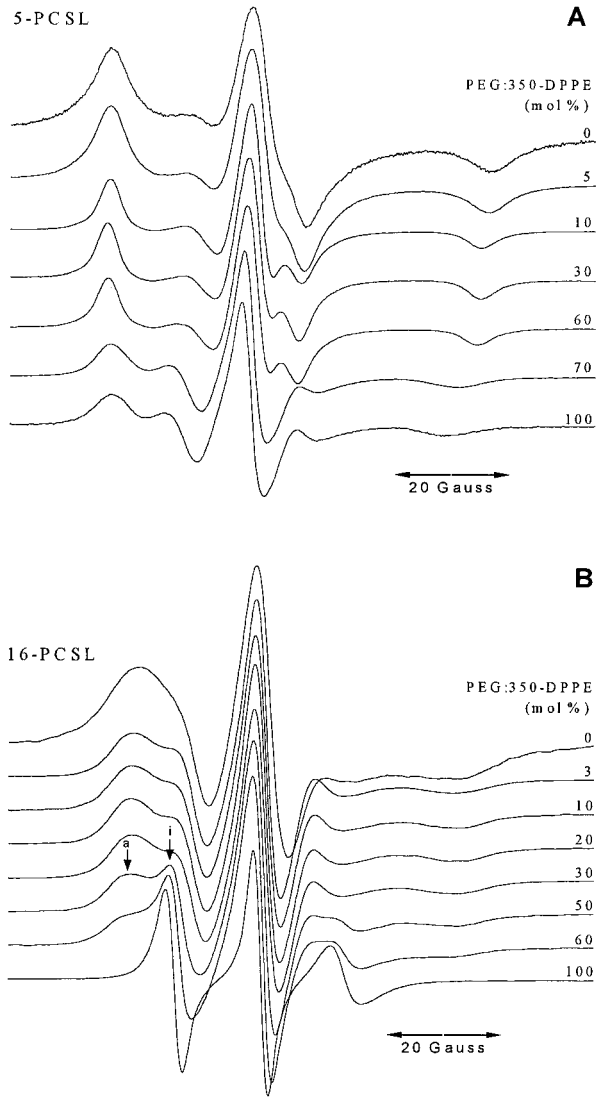

FIGURE 2 Conventional ESR spectra at 10°C of 5-PCSL (*A*) and 16-PCSL (*B*) spin labels in aqueous dispersions of DPPC/PEG:350-DPPE mixtures at the mole fractions indicated on the figure. Total scan width = 100 gauss. The arrows indicate the more anisotropic (a) and the more isotropic (U) components in the composite spectra of 16-PCSL.

chains in the gel phase (see, e.g., Bartucci et al., 1993). Addition of up to 5 mol% of PEG:350-DPPE in the DPPC dispersions does not influence the ESR spectra of 5-PCSL appreciably, relative to that of DPPC alone. Further increases in the PEG:350-DPPE content of lipid mixtures up to 60 mol% produce small changes in the ESR spectra, which nonetheless remain characteristic of spin labels undergoing slow motion in lamellar gel phase. At 70 mol% the spectrum has a partially motionally averaged, axial lineshape, and finally at 100 mol% it shows spectral features of intermediate mobility characteristic of a fluid environment at low temperature in dispersions of DPPE-PEG:350 alone.

The spectrum of 16-PCSL in dispersions of DPPC (Fig. 2 *B*) shows a lower degree of anisotropy and a considerable motional broadening relative to that of 5-PCSL in the same system. This spectral difference between the two extreme positions of chain labeling is diagnostic of a noninterdigitated phospholipid lamellar gel phase (see, e.g., Bartucci et al., 1993). At the other extreme, the spectrum of 16-PCSL in dispersions of PEG:350-DPPE consists of a near-isotropic triplet, with differential broadening of the three hyperfine manifolds and little residual anisotropy. This type of spectrum for the 16-PCSL probe is characteristic of a liquid-crystalline environment that, in this case (cf. the optical density results above), must correspond to polymer-lipid micelles. Progressive mixing of PEG:350-DPPE with DPPC, up to 30–40 mol% of the polymer-lipid, leads to a limited motional restriction of the 16-PCSL, and the spectra are still characteristic of the lipid gel phase. At 50 and 60 mol% of PEG:350-DPPE the spectra of 16-PCSL clearly consist of two components at 10°C. One component (designated i in Fig. 2 *B*) corresponds to the quasi-isotropic environment that is characteristic of the micelles of PEG:350-DPPE at this temperature. The second broader component (designated a in Fig. 2 *B*) represents the residual gel-phase environment that is characteristic of the mixtures with a lower content of the PEG-lipid. Most significantly, the relative proportion of the micelle-like component increases with increasing content of PEG-lipid (compare the spectra for 50 and 60 mol%).

ESR spectra of 5- and 16-PCSL at 10°C were also recorded in DPPC/PEG:2000-DPPE dispersions (spectra not shown). Increasing the content of the longer PEG:2000-DPPE polymer-lipid has the same qualitative effect as that observed with the shorter PEG:350-DPPE. However, the changes in the spectral lineshapes occur at much lower concentrations of the longer polymer-lipid than is the case for the shorter one. Specifically, little effect on the typical lamellar gel-phase lineshape in the ESR spectra of 5-PCSL at 10°C in DPPC/PEG:2000-DPPE mixtures are seen on adding up to 7 mol% of the longer polymer-lipid. Further admixture up to 25–30 mol% of PEG:2000-DPPE monotonically decreases the spectral anisotropy. Beyond 40 mol% of PEG:2000-DPPE, the ESR spectra steadily approach the fast motional regime of conventional spin label ESR spectroscopy. As concerns the spectra of 16-PCSL in DPPC/PEG:2000-DPPE mixtures, they have the same gel-phase lineshape as that in DPPC alone up to 7 mol% of the polymer-lipid. With a further increase of the content of PEG:2000-DPPE in the dispersions, progressively increasing proportions of the quasi-isotropic spectral component are found throughout the range from 15 to 40 mol% of PEG:2000-DPPE. At 40 mol%, the spectrum consists almost entirely of this latter component.

The implications of these conventional ESR results are clear. The longer PEG:2000-DPPE polymer-lipid is much more effective in inducing micelle formation in DPPC bilayers than is the shorter-chain PEG:350-DPPE analog.

Intermediate phase

The ESR spectra at 35°C of 5-PCSL in DPPC/PEG:350-DPPE mixtures at different molar ratios are reported in Fig. 3 *A*. The spectra of 5-PCSL, both in DPPC and in PEG:350-DPPE dispersions, show a considerable reduction of the spectral anisotropy, relative to those recorded for the same systems at 10°C (compare Fig. 2 *A*). For dispersions of DPPC alone, this temperature corresponds to the intermediate or rippled gel phase. Consequently, the spectra of DPPC alone are asymmetrically broadened. This corresponds to two unresolved components of slightly different mobility that is a characteristic of the intermediate phase (Tsuchida and Hatta, 1988). With increased content of PEG:350-DPPE in the mixtures to 40 mol% and above, a second spectral component of spin-labeled lipids with much higher rotational mobility is very clearly resolved in the spectra of 5-PCSL. Comparison with the spectrum from dispersions of PEG:350-DPPE alone shows that this second component corresponds to a population of lipid micelles.

The coexistence of the micellar and lamellar spectral components is also seen clearly in the conventional ESR spectra at 30°C of the 16-PCSL spin probe in DPPC dispersions with a high content of PEG:350-DPPE reported in Fig. 3 *B*. For 16-PCSL, the best resolution of the two spectral components is obtained at a lower temperature of 30°C. The calorimetric pretransition of fully hydrated DPPC occurs at a temperature higher than 30°C (Mabrey and Sturtevant, 1976). Taken together with the results for 16-PCSL at 10°C, this further confirms that the second spectral component corresponds to a population of micelles, rather than to lipids with increased rotational mobility in a lamellar phase.

ESR spectra of the 5-PCSL at 35°C and 16-PCSL at 30°C in DPPC/PEG:2000-DPPE mixtures containing the longer polymer-lipid are given in Fig. 3, *C* and *D*, respectively. A systematic pattern of micelle-bilayer coexistence is observed that is similar to that found with the shorter polymer-lipid. For the lipid with a longer grafted PEG chain, however, the micellar-like component is already evident at much lower contents of PEG-lipid (from 10 mol% upward). This

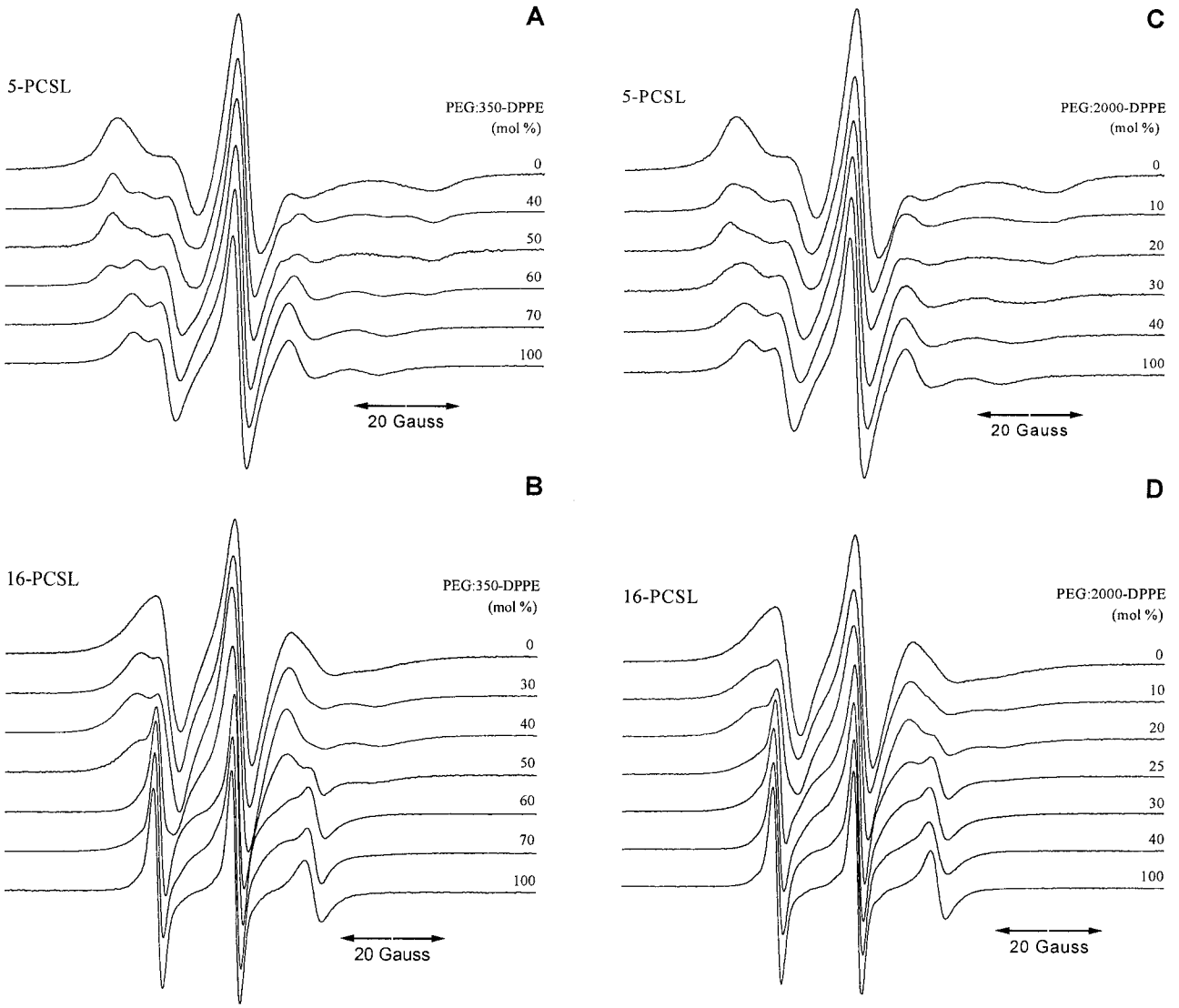

FIGURE 3 Conventional ESR spectra of 5-PCSL in aqueous dispersions of DPPC/PEG:350-DPPE mixtures at 35°C (*A*); 16-PCSL in aqueous dispersions of DPPC/PEG:350-DPPE mixtures at 30°C (*B*); 5-PCSL in aqueous dispersions of DPPC/PEG:2000-DPPE mixture at 35°C (*C*); and 16-PCSL in aqueous dispersions of DPPC/PEG:2000-DPPE mixtures at 30°C (*D*). The mole fractions of PEG-lipid are indicated. Total scan width = 100 gauss.

is in agreement with the comparative results obtained with the two polymer-lipids at lower temperatures.

Taking advantage of the good resolution of the two spectral components in this temperature regime, the relative populations of the *n*-PCSL spin labels in the micellar and bilayer environments were quantified by using difference spectroscopy as described by Marsh (1982). Subtraction of the micellar (lamellar) component from the two-component spectra of the mixtures, by using the single-component spectrum from dispersions of the PEG-lipid (DPPC) alone, yielded the fraction *f*(micelle) (*f*(lamellar)) of spin labels in the micellar (lamellar) phase. The fraction of micellar component, *f*(micelle), is given as a function of the composition of the DPPC/PEG-lipid mixtures in Fig. 4. In cases where reliable subtraction is possible with the spectra of 5-PCSL, the results agree with those obtained with 16-PCSL, even though the temperatures of measurement differ. Corresponding spectral subtractions performed on the 16-PCSL spectra at 10°C (cf. Fig. 2 *B*) were only possible over a much more restricted range of composition, because of the suboptimal resolution of the two components. Nevertheless these gave values of *f*(micelle) in good agreement with those obtained at 30°C, i.e., the temperature giving the best resolution of the two components. This clearly demonstrates that the mobile spectral component corresponds to micelles, rather than to domains with different chain mobilities in an extended wholly lamellar phase. It should be noted that micelles assayed operationally by this method include bilayer fragments that are sufficiently small so as not to undergo the cooperative chain packing characteristic of

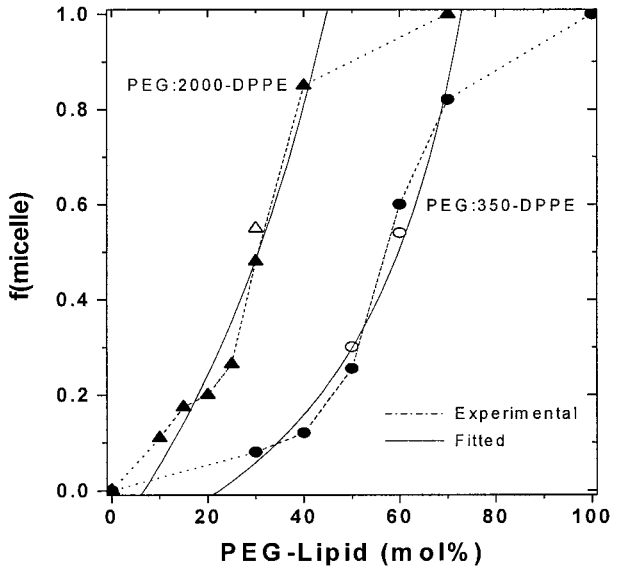

FIGURE 4 Fraction, *f*(micelle), of more fluid 16-PCSL spin labels as a function of PEG-lipid content in aqueous dispersions of DPPC/PEG:350-DPPE mixtures (*solid circles*) and of DPPC/PEG:2000-DPPE mixtures (*solid triangles*) obtained from spectral subtractions at 30°C. Data from 5-PCSL at 35°C are given by the open symbols. Solid lines are nonlinear least-squares fits with Eqs. 2 and 3 over the transition range.

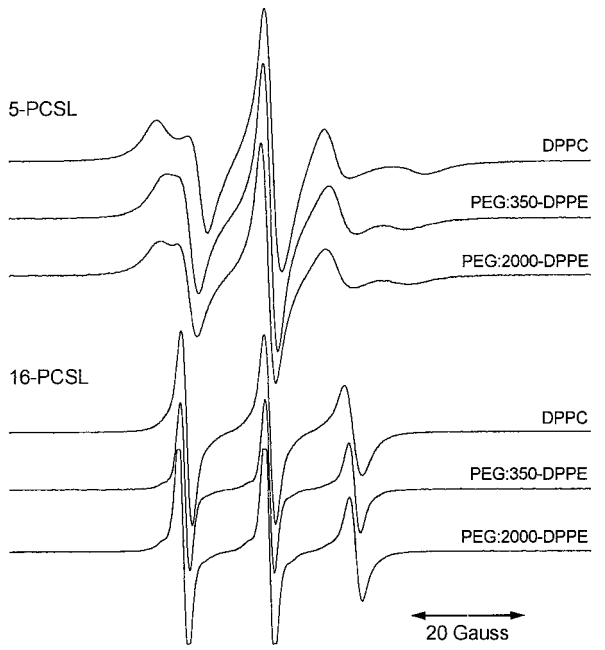

FIGURE 5 Conventional ESR spectra at 50°C of 5-PCSL (*upper spectra*) and of 16-PCSL (*lower spectra*) in aqueous dispersions of DPPC, PEG:350-DPPE, and PEG:2000-DPPE. Total scan width = 100 gauss.

lipid gel phases. Larger fragments that support cooperative chain immobilization are here classified as lamellar.

The earlier onset of micelle formation in mixtures with the longer polymer-lipid, i.e., at lower concentrations of PEG:2000-DPPE relative to PEG:350-DPPE, is very clear from Fig. 4. In both cases, there is an initial slow increase in micelle formation with PEG-lipid content. This is followed by a relatively steep increase in micelle population, with half-point *f*(micelle) = 0.5 at ~30 mol% PEG:2000-DPPE and 57 mol% PEG:350-DPPE, respectively. After this pseudo-cooperative transition, the bilayer-micelle conversion gradually achieves completion at 100 mol% of the PEG-lipid. It should be noted that the values of *f*(micelle) refer to the population of phosphatidylcholine spin labels. From the phase rule, it is expected that the micellar population of the PEG-lipid (which drives micelle formation) will be higher than that of the phosphatidylcholine component, at any given composition of the lipid mixtures (cf. Hristova and Needham, 1995). This point is considered explicitly in the Discussion and the Appendix.

Fluid phase

Representative ESR spectra of 5-PCSL and of 16-PCSL at 50°C in hydrated dispersions of DPPC, PEG:350-DPPE, and PEG:2000-DPPE are given in Fig. 5. The spectra of 5-PCSL are all axial, anisotropically averaged powder patterns that are typical of flexible lipid chains in a fluid liquid-crystalline environment. The extent of motional averaging is, however, much larger for the micellar states of the PEG-lipids than for the fluid lamellar state of DPPC. This indicates an increased amplitude of chain segmental mobility in the polymer-lipid that reflects, at least in part, the tendency of the polymer-grafted lipids to form micelles and to decrease the lipid chain packing density on going from bilayers to micelles.

The spectra of 16-PCSL are all isotropic ^{14}N hyperfine triplets. The linewidths are narrower, however, for the PEG-lipids than for DPPC, indicating a more rapid segmental chain rotation in the micellar environment. The much larger degree of anisotropic averaging for the 16 position, relative to the 5 position, of chain labeling represents the chain flexibility gradient that is inherent to fluid lyotropic liquid crystalline environments (Bartucci et al., 1993).

Note that the ESR spectra of the 5-PCSL and 16-PCSL spin labels in DPPC/PEG:350-DPPE and DPPC/PEG:2000-DPPE dispersions do not consist of two resolved components in the fluid phase, at any composition of the lipid mixtures. This is because, unlike the situation in the gel phase, the difference in chain motional anisotropy between the lamellar and micellar fluid phases is insufficient for resolution of the two overlapping spectral components.

Saturation transfer ESR

Because, at 10°C, the rotational rate of 5-PCSL in DPPC/PEG-DPPE dispersions is close to the limits of motional sensitivity of conventional spin label ESR spectroscopy, the

rotational dynamics at this temperature can be defined more precisely by use of the STESR spectroscopy (Thomas et al., 1976; Marsh, 1981; Hemminga and de Jager, 1989). STESR spectra of 5-PCSL at 10°C in dispersions of DPPC/PEG:350-DPPE mixtures at selected mole ratios are given in the inset of Fig. 6. The spectral lineshapes indicate that the rotational motion of the spin-labeled lipid chains lies in the saturation transfer ESR regime for DPPC/PEG:350-DPPE mixtures up to 60 mol% of PEG-lipid. Beyond this concentration, the spectra contain components from motion on the slow conventional spin-label ESR time scale (see Fig. 2 *A*) and therefore are not appropriate for quantitative analysis by STESR.

The STESR spectra from 5-PCSL in DPPC/PEG:350-DPPE dispersions at 10°C were analyzed by measuring the diagnostic lineheight ratios, *R* (i.e., L''/L, C'/C, and H''/H in the low-, central-, and high-field hyperfine manifolds, respectively. The dependence on lipid composition of the lineheight ratios is given in Fig. 6. The most striking feature of this dependence is the rapid initial decrease of all lineheight ratios on addition of the PEG-lipid to gel-phase DPPC. The decrease in the C'/C ratio may indicate an increase in rate of rotation about the long molecular axis of the lipids (Marsh, 1980). However, the conventional ESR spectra in Fig. 2 *A* suggest that there is no increase in chain segmental rotation rates, which would be reflected in the L''/L and H''/H ratios. Therefore the initial decrease in the latter most probably reflects a decrease in size of the lamellar aggregates with the addition of the PEG-lipid. In Table 1 are given the effective rotational correlation times, τ_R^{eff}. They have been deduced from standard calibrations of the lineheight ratios for isotropic motion taken from Horváth and Marsh (1988), Marsh (1992), and Marsh and Horváth (1992), using the expression

$$\tau_R^{eff} = k/(R_o - R) - b \qquad (1)$$

where R_o is the rigid-limit value of *R*, and the calibration constants, *k* and *b*, are given in Marsh (1999). For the short correlation time regime (for micelles), calibrations were taken from Thomas et al. (1976) and Fajer and Marsh (1982). As can be seen in Table 1, comparable values are obtained from the low- and high-field ratios, but shorter effective correlation times are obtained from the central ratio. The latter most probably reflects preferential faster rotation about the long axis of the lipid molecules (Marsh, 1980). The values of τ_R^{eff} deduced from L''/L and H''/H decrease up to 5–10 mol% of PEG:350-DPPE. Beyond this, they vary nonsystematically (cf. Fig. 6), which may indicate changing anisotropy in the motion, as possibly suggested by the varying lineshape in the H'' diagnostic region of the spectrum.

The STESR spectra of 5-PCSL in DPPC/PEG:2000-DPPE mixtures at 10°C (not shown) reveal the absence of submicrosecond motions only over a much smaller range of polymer-lipid contents. A decrease in τ_R^{eff} occurs only up to 7 mol% of PEG:2000-DPPE (see Table 1), again suggesting rapid induction of small lamellar aggregates with the addition of PEG-lipid.

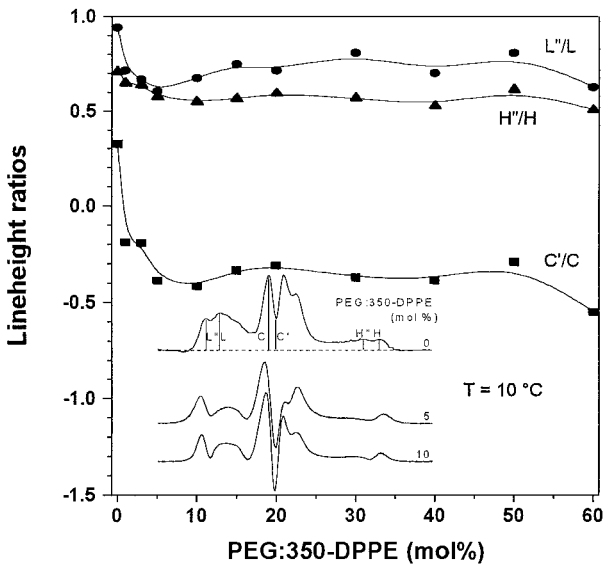

FIGURE 6 Dependence on PEG:350-DPPE content of the diagnostic lineheight ratios, L''/L (●), C'/C (■), and H''/H (▲), in the low-, central-, and high-field regions, respectively, of the STESR spectra of 5-PCSL at 10°C in aqueous dispersions of DPPC/PEG:350-DPPE mixtures. The errors range from ±4 to ±7% for L''/L, from ±3 to ±6% for C'/C, and from ±15 to ±25% for H''/H. *Inset*: Selected STESR spectra of 5-PCSL at 10°C in aqueous dispersions of DPPC/PEG:350-DPPE mixtures. The lineheights L'', L; C', C; and H'', H in the low-, central-, and high-field regions of the spectrum are indicated. Total scan width = 100 gauss.

TABLE 1 Effective rotational correlation times, τ_R^{eff}, derived 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 DPPC/PEG:350-DPPE and of DPPC/PEG:2000-DPPE at different molar ratios at 10°C

PEG-DPPE (mol%)	τ_R^{eff} (L''/L) (μs)	τ_R^{agg} (μs)	τ_R^{eff} (C'/C) (μs)	τ_R^{eff} (H''/H) (μs)	τ_R^{agg} (μs)
0	56	—	10	69	—
PEG:350-DPPE					
1	32	72	2.4	132	—
3	28	54	2.4	60	470
5	23	39	0.9	46	134
10	28	57	0.7	41	103
15	34	88	1.3	44	120
20	31	71	1.4	48	163
40	30	66	0.9	38	84
PEG:2000-DPPE					
3	28	55	2.4	54	251
7	22	36	0.2	34	68
10	29	59	0.5	45	125

Estimates of the rotational correlation time, τ_R^{agg}, of the lipid aggregates according to Eq. 4 are also given (see text).

DISCUSSION

Optical density

The optical density measurements give qualitative information on the changes in size of the lipid structures and the formation of micelles, which are, on the whole, in agreement with previous studies on related PEG-grafted lipid systems. For the corresponding DSPC/PEG:2000-DSPE mixtures of lipids with matched distearoyl (DS-) chains, the dependence of the optical density on lipid composition is very similar to that found here for the DPPC/PEG:2000-DPPE mixtures with dipalmitoyl chains and the same average PEG polymer length (Kenworthy et al., 1995). From polarized light microscopy, the latter authors ascribed the decrease of the absorbance of the lipid dispersions to a gradual conversion from multilamellar vesicles to small unilamellar vesicles and to micelles. From the similarity in behavior of the optical densities, it can be assumed that this is also the case here for the DPPC/PEG:2000-DPPE lipid mixtures. For DPPC/PEG:1000-DPPE and DPPC/PEG:3000-DPPE hydrated lipid dispersions, it was found that the turbidity of the suspensions decreased first gradually, then more abruptly, and finally the suspensions became completely transparent at higher content of the PEG-lipid (Bedu-Addo et al., 1996). Coexisting smaller multilamellar and unilamellar vesicles were found by optical microscopy for dispersions containing 7.5 mol% of PEG:2000-DPPE in egg yolk phosphatidylcholine (Lasic et al., 1991b).

In contrast to the system studied here with dipalmitoyl chains, rather different results have been obtained from optical density measurements on the shorter PEG:350 polymer-lipid in DSPC/PEG:350-DSPE mixtures with longer distearoyl lipid chains (Kenworthy et al., 1995). In the latter case, it was found that the optical density of the mixed lipid dispersions increased progressively with increasing content of the PEG-lipid, which was attributed to the existence of multilamellar vesicles over the entire range of lipid mixtures. This points to a very sensitive dependence of the optical density behavior on lipid chain length for PEG-lipids with relatively short polymer polar groups (compare with Fig. 1 *A*). The degree of polymerization of PEG:350 is approximately 7, corresponding to 14 methylene units plus seven ether oxygen links, as compared with 36 C-units and two acyl oxygen links in the chains of a distearoyl phospholipid. Because these sizes are comparable, it is expected that the behavior of the PEG:350-lipids could be critically modulated by the lipid chain length, as is observed. This is not expected, however, for the much longer PEG:2000-lipids, which again is in agreement with experimental observation (see previous paragraph).

In addition to steric repulsion and hydration forces between the polymer headgroups, longer range electrostatic bilayer-bilayer repulsion likely also contributes to the reduction in size of the lipid aggregates by the negatively charged PEG-lipids. Comparison of results obtained with PEG:350-DPPE and PEG:2000-DPPE, which bear the same charge, indicate the predominance of the headgroup-size-dependent effects. Electrostatic effects are, of course, strongly dependent on the ionic strength of the suspending buffer.

Rotational dynamics of the lipid chains

The conventional ESR spectral lineshapes of the chain-labeled lipids, at a temperature corresponding to the gel phase of DPPC and a temperature corresponding to the fluid phase, contain important information on the segmental mobility of the lipid chains in the various lipid mixtures. There are very marked differences in the effects of the two polymer-lipids on the local chain mobility at 10°C. The PEG-lipid with the longer polymer chain is more effective in disrupting the tight packing of the lipid chains in the gel phase than is the shorter polymer-lipid. The anisotropy of the slow-motion spectra of 5-PCSL is reduced appreciably over the range up to 25 mol% of PEG:2000-DPPE, for which the degree of micellization remains relatively low (cf. Fig. 4). In contrast, the chain mobility remains characteristic of a slightly perturbed gel phase over the range up to 60 mol% of PEG:350-DPPE, despite the fact that the optical density indicates a considerable decrease in particle size (see Fig. 1 *A*).

In the fluid phase, admixture of the PEG-lipids has a marked effect on the angular amplitude of the lipid-chain motion. The direction of the change is that expected from the difference between DPPC bilayers and PEG-lipid micelles but takes place well before appreciable micelle formation. However, the extent of the effects is not strongly dependent on the size of the polymer headgroup.

Bilayer-micelle conversion

Hristova and Needham (1995) have modeled the transition from bilayers to micelles, as a function of the total PEG-lipid content, by calculating the minimum free energies of both structures. Very significant general thermodynamic features emerge from these calculations that allow fitting of the results given in Fig. 4, in a manner consistent with only two free parameters, as outlined below.

Over the range from the start of the bilayer to micelle transition at total mole fraction of PEG-lipid X_{PEG}^{tr} to the completion at total mole fraction X_{PEG}^{comp}, the compositions of the coexisting micellar and bilayer phases remain constant, at the values specified by the respective onset and completion points (Hristova and Needham, 1995). This is in agreement with the Gibbs phase rule. Consequently, the mole ratio of PEG-lipid to phosphatidylcholine is equal to X_{PEG}^{tr} in the bilayer phase and to X_{PEG}^{comp} in the micellar phase, throughout the transition. The total fraction of lipid (i.e.,

PEG-lipid + PC) in the micellar phase is then given by the lever rule (see Appendix):

$$f_{tot}(\text{micelle}) = \frac{X_{PEG} - X_{PEG}^{tr}}{X_{PEG}^{comp} - X_{PEG}^{tr}} \quad (2)$$

Therefore the degree of conversion to the micellar phase is linear in the total mole fraction X_{PEG} of PEG-lipid, over the entire transition region. This is exactly the result found in the theoretical calculations of Hristova and Needham (1995) and is required by the phase rule.

To interpret the spin label results of Fig. 4, we require the fraction f_{PC}(micelle) of the phosphatidylcholine component in the micellar phase. It can be shown that this is given by (see Appendix)

$$f_{PC}(\text{micelle}) = \frac{1 - X_{PEG}^{comp}}{1 - X_{PEG}} f_{tot}(\text{micelle}) \quad (3)$$

Combination of Eqs. 2 and 3 therefore allows fitting of the dependence of the fraction of micellar PC spin label on the total mole fraction X_{PEG} of PEG-lipid. The two fitting parameters are the onset and completion points, X_{PEG}^{tr} and X_{PEG}^{comp}, respectively, of the bilayer to micelle transition. The fits to the data for PEG:350-DPPE and for PEG:2000-DPPE are given by the solid lines in Fig. 4. It is seen that the model is reasonably able to represent the experimental data. The values of the fitting parameters are $X_{PEG}^{tr} = 7 \pm 3$ (22 ± 7) mol% and $X_{PEG}^{comp} = 45 \pm 5$ (73 ± 6) mol% for PEG:2000-DPPE (PEG:350-DPPE), respectively. The values for the longer polymer-lipid are smaller than those for the shorter polymer-lipid, which is consistent with considerations of the molecular shape (see, e.g., Israelachvili, 1985; Hristova et al., 1995). The value of X_{PEG}^{tr} for PEG:2000-DPPE is in agreement with the explicit calculation by Hristova and Needham (1995), but that of X_{PEG}^{comp} is considerably higher than in the same calculation. The higher completion point is more in agreement with the experimental measurements by Hristova et al. (1995). In contrast, experimental estimates for mixtures of phosphatidylcholine with PEG-lipids of unmatched lipid acyl chain length (DPPC and PEG:2000-DSPE) yielded degrees of micellization greater than those predicted by the above theoretical calculations (Baekmark et al., 1997). These experiments used differential scanning calorimetry and could conceivably underestimate the bilayer population.

Saturation transfer ESR

In large multilamellar vesicles of DPPC alone, the STESR spectra are dominated by the molecular rotational mobility of the 5-PCSL phospholipid probe (Marsh, 1980). As PEG-lipids are mixed with DPPC, changes in the effective rotational correlation time measured by STESR may occur because of changes in the local molecular mobility (characterized by τ_R^{mol}) and because of a decrease in size of the lipid aggregates. The effective rotational rate (i.e., $1/\tau_R^{eff}$) is then the sum of the rates of molecular rotation (i.e., $1/\tau_R^{mol}$) and of the overall rotation of the lipid aggregates:

$$(\tau_R^{eff})^{-1} = (\tau_R^{mol})^{-1} + (\tau_R^{agg})^{-1} \quad (4)$$

where τ_R^{agg} is the rotational correlation time of the lipid aggregates. An estimate of τ_R^{agg} for the DPPC/PEG-lipid mixtures can be obtained by assuming that τ_R^{mol} remains the same as in DPPC alone. This is likely to be a reasonable approximation for low contents of PEG-lipids, especially if τ_R^{eff} is taken from the L''/L or H''/H lineheight ratios, which are insensitive to rotation around the long molecular axis.

Applying this assumption to the data derived from the L''/L and H''/H diagnostic STESR ratios gives the effective values of τ_R^{agg} that are listed in Table 1. On this basis, the rotational mobility of the lipid aggregates increases rather rapidly with increasing content of PEG-lipid. At PEG:350-DPPE lipid contents in the region of 1–15 mol%, the effective correlation times for overall rotation of the aggregates are in the region of 70 and 120 μs, deduced from L''/L and H''/H, respectively. Somewhat shorter values are obtained for comparable contents of PEG:2000-DPPE.

The correlation time for overall rotation of the lipid aggregates about their symmetry axis is given by a Debye-type expression:

$$\tau_R^{agg} = \eta V_{agg}/(k_B T) \quad (5)$$

where η is the aqueous viscosity, k_B is Boltzmann's constant, and T is the absolute temperature. This expression is correct to within an asymmetry factor that depends on the shape of the aggregates. For the rotational correlation times quoted above, Eq. 5 corresponds to effective diameters in the region of 100 nm. The volume of the lipid aggregates can be expressed, quite generally, in terms of the aggregation number, n_{agg}. The resulting dependence of the overall correlation time on the mole fraction, X_{PEG}, of PEG-lipid is given by

$$\tau_R^{agg} = \frac{\eta}{k_B T}(v_l^o + v_{PEG} X_{PEG}) n_{agg} \quad (6)$$

where v_l^o (≈ 1.1 nm^3) is the core volume of the phospholipid molecule without polymer attached to the headgroup, and v_{PEG} is the effective volume of the PEG-lipid polymer headgroup. For a flexible polymer, the latter depends on the surface concentration of PEG-lipids. In the low concentration regime, the PEG polymer forms isolated "mushroom" structures of volume v_{PEG} (mushroom) $= v_{OE} n_{OE}^{9/5}$, where v_{OE} is the volume of an oxyethylene monomer unit [-$(CH_2)_2$O-] and n_{OE} (= 7, 46 for PEG:350, 2000, respectively) is the degree of polymerization of the PEG headgroup (de Gennes, 1980). In the higher concentration regime, the PEG polymer forms a continuous brush on the surface with v_{PEG} (brush) $= v_{OE} n_{OE} X_{PEG}^{-2/3}$, which is depen-

dent on the mole fraction, X_{PEG}, of PEG-lipid (de Gennes, 1980).

This is, of course, an oversimplification of the effects of a flexible polymer on overall rotational diffusion. However, the different scaling laws in the two polymer concentration regimes do explain, in part, the biphasic dependence of the rotational correlation time on the mole fraction of PEG-lipid that is implicit in Fig. 6. In the low-concentration regime τ_R^{agg} is directly proportional to X_{PEG}, whereas in the high-concentration regime it increases only as $X_{PEG}^{1/3}$ (see Eq. 6), for a fixed aggregation number. Added to this, the decrease in aggregation number with progressive micelle formation competes against this slow increase with the added bulk of the polymer headgroup (see Eq. 6), resulting in the relatively constant values of τ_R^{agg} that are obtained after the initial drop with increasing PEG-lipid content. The rapid initial drop with the increasing mole fraction X_{PEG} of polymer-lipid suggests that the rather open "mushroom" configuration of the surface polymer makes little contribution to the rotational mobility of the lipid vesicles, which is dominated by the decreasing size, i.e., a rapid reduction in n_{agg}.

In the case of cylindrical micelles, for which there is direct structural evidence in PC/PEG-lipid systems (Edwards et al., 1997), both the aggregation number and rotational correlation time decrease directly with decreasing length l of the aggregates. For spherical micelles, on the other hand, the aggregation number is expected to remain approximately fixed.

At least qualitatively, these results are consistent with direct structural studies on similar PEG-lipid/PC systems. By using cryotransmission electron microscopy, Edwards et al. (1997) observed the formation of open bilayer discs in PC/PE-lipid mixtures, before the transition to mixed micelles, which were found to have a thread-like rather than globular shape in the absence of cholesterol. In addition, Lasic et al. (1991b) have reported a reduction in the hydrodynamic radius of lamellar dispersions of egg yolk phosphatidylcholine mixed with 7.5 mol% of PEG:2000-DPPE. From quasielastic light scattering measurements on hydrated egg PC/PEG:2000-DPPE mixtures, Szleifer et al. (1998) observed that the average diameter of the aggregates decreased with insertion of the polymer-lipid, lying in the range 420–550 nm for PEG-lipid contents of 0.2–10 mol%. By using measurements of dynamic light scattering, Bedu-Addo et al. (1996) showed a reduction in aggregate size of DPPC/PEG:1000,3000-DPPE dispersions from 3000 to 2000 nm on going from 0 to 5 mol% of PEG-lipid, and then further to 40 nm on increasing the polymer-lipid content to 17 mol%.

CONCLUSIONS

Conventional and saturation transfer ESR spectroscopies, together with spectrophotometry, were used to study the lyotropic phase behavior of DPPC/PEG-DPPE dispersions at full hydration. We have concentrated on PEG-lipid headgroups of two rather different molecular masses, i.e., 350 and 2000 Da.

1. The local segmental mobility of the lipid chains is sensitive to the content of PEG-lipid in the low concentration regime. Chain mobility is increased with increasing PEG-lipid concentration in both gel and fluid PC bilayers. Dependence on polymer headgroup size is marked in the gel phase but smaller in the fluid phase.

2. Conversion from bilayer vesicles to micelles could be quantitated from the resolved two-component ESR spectra and is shown to be consistent with a thermodynamic description of the bilayer-micelle transition. The onset and completion points determined for the transition depend on polymer-lipid headgroup size, in a way that is consistent with shape concepts of lipid polymorphism.

3. Saturation transfer ESR measurements of the effective rotational diffusion rates of the lipid aggregates demonstrate the sensitive dependence in the "mushroom" regime at low concentrations of PEG-lipid, and a much weaker dependence in the "brush" regime with increasing content of PEG-lipid.

APPENDIX: BILAYER-MICELLE TRANSITION THERMODYNAMICS

For a mixture of polymer-lipid and phosphatidylcholine (i.e., $C = 2$, with water in excess), in the bilayer-micelle coexistence region (i.e., $P = 2$), the number of degrees of freedom given by the Gibbs phase rule is $F = C - p + 1 = 1$, at constant pressure. Therefore, at a fixed temperature, the compositions of the coexisting bilayer and micellar phases are fixed, independent of the total composition (see, e.g., Cevc and Marsh, 1987). Varying the total composition results in bilayer-micelle conversion. The composition of the bilayer phase is specified by the end of the tie line at the beginning of the transition (viz., mole fraction of PEG-lipid = X_{PEG}^{tr}). The composition of the micellar phase is specified by the end of the tie line at the completion of the transition (viz., mole fraction of PEG-lipid = X_{PEG}^{comp}).

If f_{tot}(micelle) is the fraction of total lipid in the micellar phase, the total mole fraction of PEG-lipid, X_{PEG}, in the mixture is given in terms of the compositions of the individual phases by conservation of mass:

$$X_{PEG} = [1 - f_{tot}(\text{micelle})]X_{PEG}^{tr} + f_{tot}(\text{micelle})X_{PEG}^{com\,p} \quad \text{(A1)}$$

The degree of conversion to micelles is therefore given by

$$f_{tot}(\text{micelle}) = \frac{X_{PEG} - X_{PEG}^{tr}}{X_{PEG}^{com\,p} - X_{PEG}^{tr}} \quad \text{(A2)}$$

i.e., by the lever rule.

Within the micellar phase, the mole fraction of phosphatidylcholine is simply $1 - X_{PEG}^{comp}$. For the whole sample, the mole fraction of phosphatidylcholine present in the micellar phase is therefore $(1 - X_{PEG}^{comp}).f_{tot}$(micelle), viz., when referred to both phases, where the total mole fraction of phosphatidylcholine is $1 - X_{PEG}$). Hence, the fraction of phosphatidylcholine that is in the micellar phase is given by

$$f_{PC}(\text{micelle}) = \frac{1 - X_{PEG}^{com\,p}}{1 - X_{PEG}} f_{tot}(\text{micelle}) \quad \text{(A3)}$$

This is the relation that is required for interpreting the results on micelle formation registered by spin-labeled phosphatidylcholine.

This work, financially supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) and by the Istituto Nazionale per la Fisica della Materia (INFM), is part of the Ph.D. thesis of SB. SB and GM thank MURST and European Community, respectively, for the award of Ph.D. fellowships.

REFERENCES

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

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

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

Bedu-Addo, F. K., P. Tang, Y. Xu, and L. Huang. 1996. 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.* 13:710–717.

Belsito, S., R. Bartucci, and L. Sportelli. 1998. Sterically stabilized liposomes of DPPC/DPPE-PEG:2000. A spin label and spectrophotometric study. *Biophys. Chem.* 75:33–43.

Blume, G., and G. Cevc. 1990. Liposomes for the sustained drug release in vivo. *Biochim. Biophys. Acta.* 1029:91–97.

Blume, G., and G. Cevc. 1993. Molecular mechanism of the lipid vesicles longevity in vivo. *Biochim. Biophys. Acta.* 1146:157–168.

Cevc, G., and D. Marsh. 1987. Phospholipid Bilayers. Physical Principles and Models. Wiley-Interscience, New York.

de Gennes, P. G. 1980. Conformations of polymers attached to an interface. *Macromolecules.* 13:1069–1075.

Edwards, K., M. Jonsson, G. Karlsson, and M. Silvander. 1997. Effect of polyethyleneglycol-phospholipids on aggregate structure in preparation of small unilamellar liposomes. *Biophys. J.* 73:258–266.

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

Hemminga, M. A., and P. A. de Jager. 1989. Saturation transfer spectroscopy of spin labels: techniques and interpretation of spectra. *In* Spin Labeling. Theory and Applications. L. J. Berliner and J. Reuben, editors. Plenum Press, New York. 131–178.

Hemminga, M. A., P. A. de Jager, D. Marsh, and P. Fajer. 1984. Standard conditions for the measurement of saturation transfer ESR spectra. *J. Magn. Reson.* 59:160–163.

Horváth, L. I., and D. Marsh. 1988. Improved numerical evaluation of saturation transfer electron spin resonance spectra. *J. Magn. Reson.* 80:314–317.

Hristova, K., A. Kenworthy, and T. J. McIntosh. 1995. Effect of bilayer composition on the phase behavior of liposomal suspensions containing poly(ethylene glycol)-lipids. *Macromolecules.* 28:7693–7699.

Hristova, K., and D. Needham. 1995. Phase behavior of a lipid/polymer-lipid mixture in aqueous medium. *Macromolecules.* 28:991–1002.

Israelachvili, J. N. 1985. Intermolecular and Surface Forces. Academic Press, London.

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

Klibanov, A. L., K. Maruyama, V. P. Torchilin, and L. Huang. 1990. Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 268:235–237.

Lasic, D. D. 1993. Liposomes: From Physics to Applications. Elsevier, Amsterdam, London, New York, Tokyo.

Lasic, D. D., and F. Martin. 1995. Stealth Liposomes. CRC Press, Boca Raton, London, Tokyo.

Lasic, D. D., F. J. Martin, A. Gabizon, S. K. Huang, and D. Papahadjopoulos. 1991a. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. *Biochim. Biophys. Acta.* 1070:187–192.

Lasic, D. D., M. C. Woodle, F. J. Martin, and T. Valentincic. 1991b. Phase behavior of "stealth-lipid"-lecithin mixtures. *Period. Biol.* 93:287–290.

Mabrey, S., and J. M. Sturtevant. 1976. Investigation of phase transitions of lipids and lipid mixtures by high-sensitivity differential scanning calorimetry. *Proc. Natl. Acad. Sci. USA.* 73:3862–3866.

Marsh, D. 1980. Molecular motion in phospholipid bilayers in the gel phase: long axis rotation. *Biochemistry.* 19:1632–1637.

Marsh, D. 1981. Electron spin resonance: spin labels. *In* Membrane Spectroscopy. E. Grell, editor. Springer-Verlag, Berlin. 51–142.

Marsh, D. 1982. Electron spin resonance: spin label probes. *Tech. Life Sci. Biochem.* B4/II:B426/1-B426/44.

Marsh, D. 1989. Experimental methods in spin label spectral analysis. *In* Spin Labeling. Theory and Applications. L. J. Berliner and J. Reuben, editors. Plenum Press, New York. 255–303.

Marsh, D. 1992. Exchange and dipolar spin-spin interactions and rotational diffusion in saturation transfer ESR spectroscopy. *Appl. Magn. Reson.* 3:53–65.

Marsh, D. 1999. Spin label ESR spectroscopy and FTIR spectroscopy for structural/dynamic measurements on ion channels. *Methods Enzymol.* 294:59–92.

Marsh, D., and L. I. Horváth. 1992. A simple analytical treatment of the sensitivity of saturation transfer EPR spectra to slow rotational diffusion. *J. Magn. Reson.* 99:323–331.

Papahadjopoulos, D., T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K.-D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. 1991. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA.* 88:11460–11464.

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

Thomas, D. D., L. R. Dalton, and J. S. Hyde. 1976. Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance. *J. Chem. Phys.* 65:3006–3024.

Torchilin, V. P., V. P. Omelyanenko, M. I. Papisov, A. A. Bogdanov, Jr., V. S. Trubetskoy, J. N. Herron, and C. A. Gentry. 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta.* 1195:11–20.

Tsuchida, K., and I. Hatta. 1988. ESR studies of the ripple phase in multilamellar phospholipid bilayers. *Biochim. Biophys. Acta.* 945: 73–80.