Polarity Profiles in Oriented and Dispersed Phosphatidylcholine Bilayers Are Different: An Electron Spin Resonance Study

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ABSTRACT A novel method was utilized to accurately measure the *z*- component of the nuclear hyperfine interaction tensor, A_{zz}, of a chain-labeled lipid, 16PC, and a headgroup-labeled lipid, dipalmitoylphosphatidyl-tempocholine (DPPTC), in macroscopically oriented dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) membranes, which were compared with the A_{zz} values of the two labels in dispersions of the same lipids in the gel phase. We found that the A_{zz} values of 16PC (DPPTC) in the oriented DPPC and DMPC bilayers are ~1 Gauss smaller (greater) than in the corresponding dispersions. These results indicate that the headgroup region is more polar in macroscopically oriented bilayers than in dispersions, whereas in the chain region, the order in polarity is reversed. This is consistent with previous results on partial molar volumes in the liquid-crystal phase. Differences in the morphology of the macroscopically oriented and dispersed bilayers, which might be responsible, are discussed. Nonlinear least-squares fits of the electron spin resonance spectra of DPPTC in DPPC show that there is a substantial orienting potential in the headgroup region of dispersions that is lipid phase dependent. However, in oriented membrane samples hydrated in 100% relative humidity, this orienting potential is very weak.

INTRODUCTION

Macroscopically oriented lipid multilayers (multilamellar stacks) and lipid dispersions (multilamellar vesicles or liposomes) are two kinds of model membranes that have been used widely in studies of structure and dynamics of lipid bilayers, and of lipid/protein interactions. Although their basic structure is the same (viz. multibilayers), the molecular packing in the two systems is different. Therefore, the molecular interactions in the two model membranes could be expected to be different, which should be manifested in the different structural and mechanical properties. For example, we observed that the ordering of acyl chains in macroscopically aligned bilayers hydrated in a 100% relative humidity (RH) is higher than that in dispersions in the presence of excess water (Ge et al., 1994) and that the effects of the peptide gramicidin A' on the chain ordering are different for aligned membranes and dispersions (Ge and Freed, 1993; Tanaka and Freed, 1985). Furthermore, electron spin resonance (ESR) studies revealed that there is a phase transition of oriented dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) membranes at temperatures well above their gel-to-liquid crystalline phase transitions (Tanaka and Freed, 1984; Ge et al., 1994). However, to the best of our knowledge, such a phase change has not been reported for dispersion samples.

White et al. (1987) found that the volume of the hydrated headgroup in the oriented lipid membranes is greater than in dispersions, implying that the headgroups in the oriented membranes might be more hydrated than in dispersions. Evens and Waugh (1977) predicted that oriented membranes in the presence of excess water would be essentially free of membrane tension. However, in liposomes the curvature is always greater for the inner bilayer than for the outer bilayer; thus the surface tensions in the headgroup/water interfaces in the inner and outer bilayers must be different. As a result, the liposomes might never be in a state free of tension.

To better understand the nature of differences in physicochemical and chemico-mechanical properties for these model membranes, it is necessary to explore the molecular packing and molecular interactions in both headgroup and acyl chain regions in these systems. We notice that nonbilayer lipid assemblies (inverted hexagonal and cubic phases) and macroscopically oriented bilayers share a common structural feature, e.g., the edges of these samples are open, i.e., there is no confinement on the edges of the lipid monolayers, and thus the water layers in macroscopically oriented bilayers and the water columns in nonbilayer structures are interfaced with the surroundings. We hope that the present study also sheds light on the relation between the geometry of lipid/water assemblies and molecular interactions, irrespective of their phase structures. It is well known that the degree of hydration of lipid is closely related to the lipid phase behavior, and it has dramatic effects on the structural, dynamical, and mechanical properties of lipid membranes. Thus the aim of this study is to investigate the differences in the hydration properties between the oriented and "dispersed" bilayers.

The ESR technique has proved to be a powerful tool for the study of lipid hydration. Specifically, A_{zz}, the *z*-component of the nitrogen nuclear hyperfine interaction tensor (A tensor) of nitroxide spin labels, is very sensitive to the polarity of the surroundings (Griffith et al., 1974; Earle et al., 1994; Ge and Freed, 1993), which can provide valuable

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information on the degree of hydration of the immediate environment around the spin labels. We developed a novel method of measuring the A_{zz} of a headgroup-labeled and a chain-labeled PC dissolved in oriented DPPC and DMPC membranes. The values obtained were compared with those measured from the corresponding lipid dispersions. The comparison shows that the polarity of the headgroup region is greater in the oriented membranes than in the dispersions, whereas the polarity of the acyl chain region is in the reverse order. Moreover, by fitting the spectra from the headgroup spin-labeled DPPTC, we found that there is a substantial orienting potential on the polar interface of DPPC dispersions, which is dependent on the phase of the lipid bilayers. However, this potential was found to be very weak in the aligned DPPC samples.

MATERIALS AND METHODS

Materials

The lipids dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) and the spin labels, which included a chain-labeled lipid, 16PC, and a headgroup-labeled lipid, dipalmitoylphosphatidyl tempo (2,2,6,6,-tetramethyl-1-oxy) choline (DPPTC), were purchased from Avanti Lipids (Alabaster, AL) and were used without further purification.

Sample preparations

The lipid dispersions were prepared as follows. Measured amounts of chloroform stock solutions of lipid (containing 3 mg of dry lipid) and spin label (0.5 mol% of the lipid) were mixed together in a vial. The solvent was evaporated by nitrogen gas flow. To ensure complete removal of the solvent, the lipid was evacuated for an hour. After the addition of 2 ml deionized water to the vial, the lipid was scratched off the wall with a glass rod. The dispersion was stirred for 2–3 min and left at room temperature in the dark for 2 h to allow for hydration of the lipid. To get lipid pellets, the dispersion was spun in a desk-top centrifuge for about a half hour.

The ISDU (isopotential spin-dry ultracentrifugation)-aligned lipid membranes were prepared as described by Ge et al. (1994). This involves sedimentation of the membrane fragments (in the gel phase) onto a gravitational isopotential surface with simultaneous evaporation of the solvent in a vacuum ultracentrifuge. After ultracentrifugation, the aligned membranes were sandwiched between two glass plates and tied with a thin Teflon thread. The sample was hydrated either in 100% relative humidity (RH), as we did previously, or in excess water. In the latter case, the sample (the sandwiched membranes) was dipped in deionized water for 1 day. After it was removed from the water, the sample was placed on a Teflon tape, and three to four drops of deionized water were added directly to the sample to ensure the presence of excess water. The sample was then wrapped with the Teflon tape and immediately sealed with epoxy between Mylar sheets. (Only the ISDU-aligned DPPC bilayers could be hydrated in excess water. The ISDU-aligned DMPC bilayers, when dipped in water, will become fluid; these were crushed by the glass coverslips.) The procedure for preparing pressure-annealed membranes is given by Shin and Freed (1989). In brief, membranes in the liquid crystalline phase, which are sandwiched between two glass slide plates, are aligned by rubbing the surfacees of the glass plates. A comparison between the ISDU and pressure-annealing techniques has been made by Ge et al. (1994). They found that both techniques yield macroscopically well-aligned multilamellar stacks. The order parameters and rotational diffusion rates of spin labels dissolved in the bilayers aligned by the two methods are quite similar. Because of a lower water content in the pressure-annealed samples in their comparison, they found that the pressure-annealed samples yielded order parameters that were slightly larger than those in IDSU-aligned samples.

ESR spectroscopy

ESR spectra were obtained on a Bruker ER-200 spectrometer at a frequency of 9.55 GHz under standard conditions. The field sweeps were calibrated with a Bruker ER 035M NMR gaussmeter. The microwave frequency was monitored with a frequency counter.

Determination of A_{zz} from oriented membranes

We have developed a method for obtaining an effective powder spectrum from macroscopically oriented membranes, from which the g and A tensor components can be determined accurately. As is well known, the rigid limit spectrum from a spin label dissolved in lipid dispersions is just a powder spectrum. The A_{zz} of the spin label is just one-half the outer-peak separation of the rigid limit spectrum. However, for a spin label dissolved in a macroscopically oriented membrane that has been frozen, A_{zz} cannot be determined by simply measuring the outer-peak separations of rigid limit spectra at any director tilt angle, Ψ (the angle between the normal to the sample plate and the magnetic field). This is because the outer-peak separations of these spectra depend not only on the tilt angle, as shown in Fig. 1, but also on the degree of ordering of the spin label in the frozen lipid bilayers, which is usually unknown. In fact, Fig. 1 shows that the dependence of the outer-peak separation on the tilt angle is different for ISDU-aligned and pressure-annealed DPPC membranes. In the absence of a convenient way to reliably determine the A_{zz} of a nitroxide spin label in aligned lipid membranes, the A_{zz} of the same spin label frozen in the corresponding lipid dispersions was used in the past to simulate spectra of the spin label in oriented membranes.

We have now developed a convenient and reliable method, which shows that, in general, it is not correct to use the A_{zz} determined from frozen lipid dispersions for the oriented membranes. We now describe this method. First, with the aligned membrane frozen to a temperature of -135°C, we varied the tilt angle of the sample between 0° and 90° in steps of 3°, and at each tilt angle an ESR spectrum was taken. The magnetic field and the microwave frequency during each scan were monitored by the gaussmeter and frequency counter respectively. Next, we summed all of the spectra that were collected. (This was done after each was normalized and corrected for any field or frequency shift or baseline drifts. Actually, we found that any shift of magnetic field was always less than 0.1 G, which is small enough that no correction was needed.) The sum is weighted by

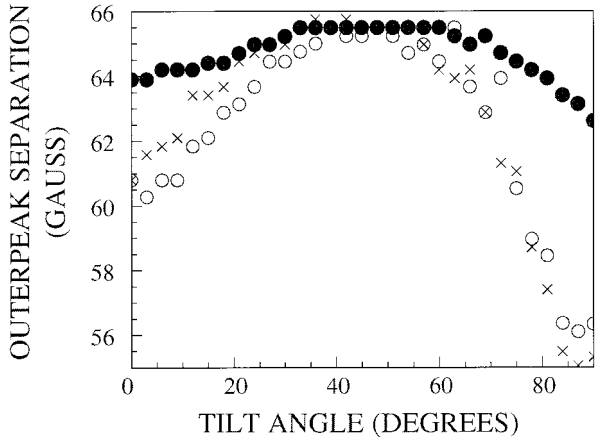

FIGURE 1 Variation in the outer-peak separations of the angle-dependent rigid limit ESR spectra of 16PC in ISDU-aligned DPPC (●), in pressure-annealed DPPC (○), and in pressure-annealed DMPC (×) bilayers with tilt angle.

sin Ψ, where Ψ is the tilt angle of each spectrum. In effect, this "powder" spectrum is constructed as though it were generated from spin labels dissolved in a dispersion consisting of randomly distributed bilayer segments, each of which is microscopically oriented. In Fig. 2 are typical spectra of 16PC in pressure-annealed DPPC bilayers for tilt angles 0°, 45°, and 90° at the temperature of −135°C from which the powder spectrum was constructed. It is then possible to "read" $2A_{zz}$ directly from the outer peak separations of the "powder" spectrum. We have simulated some of these effective powder spectra to obtain the full g and A tensors. These were performed by the spectral simulation method described next, wherein the rotational parameters are chosen to be slow enough to correspond to the rigid limit.

ESR spectral simulations

Because the spin label, DPPTC, was used for the first time in the study of structure and dynamics of headgroups, its ESR spectra in oriented membranes and dispersions of DPPC were simulated by using the nonlinear least-squares fitting program developed in this laboratory (Schneider and Freed, 1989; Budil et al., 1996). To carry out a full analysis of an ESR spectrum to study the rotational dynamics and the orientational ordering of the spin label, the following coordinate systems have to be defined first. As shown in Fig. 3, the tempo nitroxide radical replaces one of the methyl groups in the trimethyl ammonium (TMA) group. The first axis system (x''', y''', z''') is the magnetic frame, on which the g and A tensors are defined. The x''' axis points along the N-O bond, the z''' axis is parallel to the 2pz axis of the nitrogen atom, and the y''' axis is perpendicular to others, forming a right-handed coordinate system. Assuming that the nitroxide radical has a twisted boat conformation similar to that of tempone (Hwang et al., 1975), and considering that the radical moiety would undergo axial rotation around the N-C_4 bond and the TMA group would rotate around the N-$C[\beta]$ bond (see Fig. 3), the second axis system, the molecular diffusion frame (x', y', z'), is defined as follows. The z' axis is parallel to both the N-O and the N-C_4 bonds, which are collinear, i.e., the z' axis is parallel to the x''' axis. If the twisted boat form of the piperidine ring is approximately viewed as a plane, then the y' is defined as the normal to the plane, which is parallel to the z''' axis. Obviously, the x' axis would coincide with the y''' axis. Therefore this is an x''' ordering nitroxide radical, if we consider the z' axis as the main molecular symmetry axis for the radical moiety. The

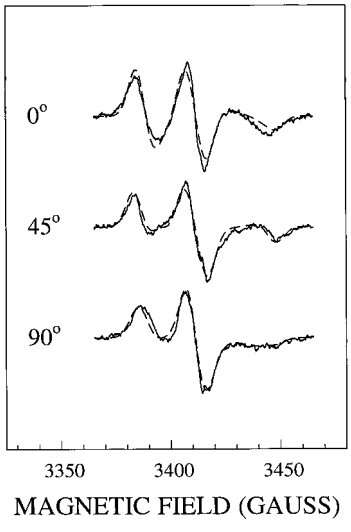

FIGURE 2 Experimental (——) and simulated (– – –) tilt-angle-dependent rigid limit ESR spectra of 16-PC in pressure-annealed DPPC bilayers. The values in the figure are the tilt angles. The g and A tensor components used were obtained by fitting the effective powder spectrum: $g_{xx} = 2.0089$, $g_{yy} = 2.0058$, $g_{zz} = 2.0021$, $A_{xx} = A_{yy} = 4.8$ G, $A_{zz} = 32.0$ G.

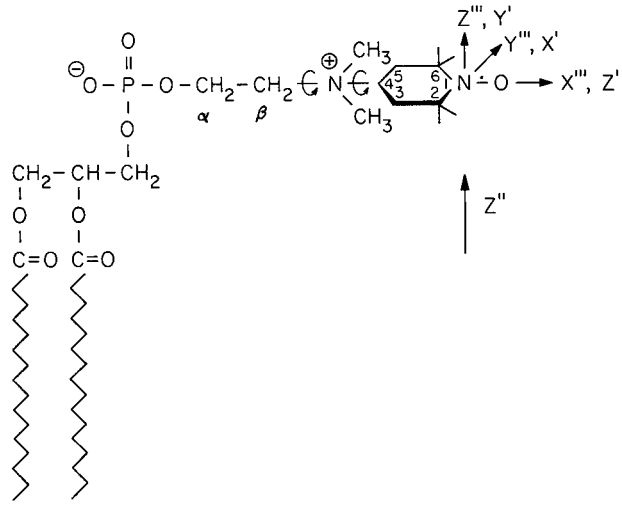

FIGURE 3 The structure of phosphoryl-tempo-choline in DPPTC and the various axis systems defined for the spectral simulations (see text).

third reference frame is the bilayer surface orienting potential frame, (x'', y'', z'').

As will be shown below, in DPPC dispersions, the TMA group experiences a potential, which in the gel phase tends to align the TMA group perpendicular to the bilayer surface, whereas in the liquid crystalline (LC) phase it tends to align the TMA group so that it lies on the bilayer surface. Because the surface orienting potential at any point on the surface of a bilayer must be axially symmetrical about the normal to that point, the z'' axis is taken to be the local normal at each point on the surface, whereas the local x'' and y'' axes are tangential to the bilayer surface, but are otherwise arbitrarily chosen. The fourth reference frame is the laboratory frame (x, y, z), with its z axis being defined by the static magnetic field. Because all TMA groups are ordered on the bilayer surface (microscopically ordered), while the orientation of the main ordering axis, the z'' axis, for each TMA group is random (macroscopically disordered), the MOMD (microscopic order and macroscopic disorder) model (Meirovitch et al., 1984) was used to simulate the ESR spectrum of DPPTC in DPPC dispersions. It should be pointed out that the surface-orienting potential on the inner and outer layer surfaces could be different; therefore the surface-orienting potential we calculated (see below) should be regarded as an average over the two monolayers.

The microscopic or local orientational ordering of the spin label is characterized by the order parameter, S, which is defined as

$$S \equiv \langle D^2_{00} \rangle = \left\langle \frac{1}{2}(3\cos^2\theta - 1) \right\rangle = \frac{\int d\Omega \exp(-U(\Omega)/kT) D^2_{00}(\Omega)}{\int d\Omega \exp(-U(\Omega)/kT)} \quad (1)$$

where k is Boltzmann's constant, T is the temperature, D^2_{00} is a Wigner rotational matrix element, and $U(\Omega)$ is the surface-orienting potential ($\Omega \equiv (\theta, \phi)$), which may be written as

$$-U(\theta, \phi)/kT = \frac{\epsilon^2_0}{2}(3\cos^2\theta - 1) + \sqrt{\frac{3}{2}}\epsilon^2_2 \sin^2\theta \cos 2\phi \quad (2)$$

ϵ^2_0, ϵ^2_2 are the dimensionless potential coefficients, and θ and ϕ represent the polar and azimuthal angles of the z'' axis of the surface-orienting potential (the normal to the bilayer surface) in the magnetic axis system, which specify the orientation of the nitroxide radical moiety with respect to the bilayer surface. Another order parameter, $S_2 = \langle D^2_{0+2} + D^2_{0-2} \rangle$, is defined in a similar manner as S (Shin and Freed, 1989). It represents the deviation

from cylindrical symmetry of the molecular alignment relative to the bilayer surface (i.e., the molecular biaxiality).

The dynamics of the spin label is characterized by $R_\perp$ and $R_\parallel$, which are the principal components of the rotational diffusion tensor. They represent the rotational rates of the nitroxide radical moiety around the axes perpendicular and parallel to the main symmetry axis of the radical z' axis, respectively.

The x''', y''', z''' magnetic frame is the same for the end-chain spin label, 16PC. However, for this spin label z' is taken as parallel to the fully extended direction of the acyl chains, and it corrsponds to the case of z''' ordering (Tanaka and Freed, 1984; Ge and Freed, 1993; Ge et al., 1994). In the simulation of spectra from 16PC in frozen aligned bilayers, we specify the angle of tilt between the z''' and z' axes, which we shall refer to as "ordering tilt." Readers who are interested in the details of the simulation methods and the algorithm adopted in the nonlinear least-squares fitting program are referred to previous work (Meirovitch et al., 1982; Schneider and Freed, 1989; Budil et al., 1996; Ge and Freed, 1993).

RESULTS

The effective powder spectrum of DPPTC in ISDU-aligned DPPC membranes and its simulation are plotted in Fig. 4 *A*. The rigid limit spectrum of 16PC in DPPC dispersions in the presence of excess water, and the effective powder spectrum of 16PC in oriented membranes by the ISDU and the pressure-annealing methods, are plotted in Fig. 4, *B*, *C*, and *D*, respectively. The values of A_{zz} of 16PC and DPPTC in dispersions and macroscopically oriented membranes of DPPC and DMPC are listed in Tables 1 (16PC) and 2 (DPPTC), respectively. They were determined by measuring the outer-peak separations of the rigid limit spectra (for dispersions) or of the effective powder spectra (for oriented membranes). The A_{zz} values for DPPC clearly show the following features: 1) The A_{zz} values for 16PC in oriented (ISDU-aligned and pressure-annealed) membranes, 32.0–32.4 G, are more than 1 G smaller than in the dispersions, 33.7 G; whereas the A_{zz} of DPPTC in the ISDU-aligned membranes, 36.0 G, is more than 1 G greater than in the dispersions, 34.8 G. 2) The A_{zz} values of both spin labels in the ISDU-aligned membranes do not depend on whether they are hydrated in excess water or in 100% RH within experimental error. Qualitatively, the DMPC data exhibit the same features as the DPPC data. That is, the A_{zz} of 16PC in ISDU-aligned DMPC membranes, i.e., 33.1 G, is 0.7 G smaller than in the dispersions, i.e., 33.8 G, and the A_{zz} of DPPTC in the ISDU-aligned DMPC membranes, i.e., 35.6 G, is 0.8 G greater than in the dispersions, i.e., 34.8 G.

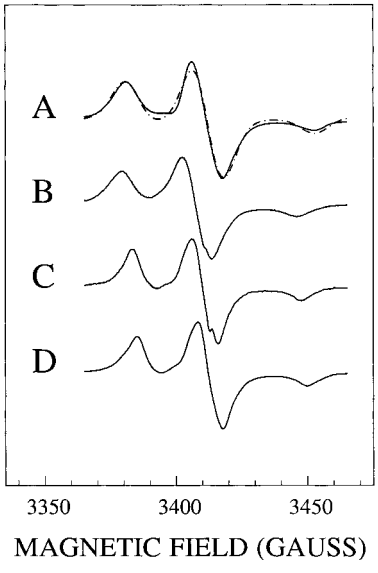

FIGURE 4 (*A*) Effective powder ESR spectra of DPPTC in ISDU-aligned DPPC membranes in the presence of excess water. ——, Experimental; – · –, simulated. The best fit parameters of the *g* and *A* tensor components obtained from this simulation were used for fitting the ESR spectra in Fig. 5. (*B*) Experimental rigid limit ESR spectrum of 16PC in DPPC dispersions. (*C* and *D*) Effective powder ESR spectra of 16PC in pressure-annealed and ISDU-aligned DPPC bilayers, respectively.

TABLE 1 A_{zz} of 16PC in dispersions and macroscopically oriented DPPC and DMPC membranes

Membrane	Dispersions	ISDU-aligned	Pressure-annealed
DPPC	33.7	32.0	32.4
DMPC	33.8	33.1	32.3

The unit of A_{zz} is Gauss. The estimated error in A_{zz} is ±0.2 G.

It can be seen that for the dispersions, the A_{zz} values of both spin labels in DMPC are the same as in DPPC, but for the ISDU-aligned membranes, the situation is different. The differences in A_{zz} of both spin labels between the ISDU-aligned membranes and the dispersions are smaller for DMPC (0.7–0.8G) than for DPPC (1.4–1.5G). These differences are not likely to arise from the difference in the acyl chain length between DMPC and DPPC. We noticed that after hydration the ISDU-aligned DMPC membranes become very soft and deformed to some extent under the weight of the covering glass plate, which can be seen from the smearing of sample edges and extension of the area of the sample. Therefore, we ascribe the differences between A_{zz} values of ISDU-aligned DMPC and DPPC membranes to a reduced macroscopic ordering of the ISDU-aligned DMPC membranes compared to ISDU-aligned DPPC membranes.

Taken together, the above results show that the polarity profile from the headgroup to the center of the bilayer is different for oriented bilayers and dispersions. That is, the headgroup (chain) region is more (less) polar in the oriented than in the dispersed bilayers. We also measured the A_{zz} of 16PC in ISDU-aligned and "dispersed" DPPC membranes containing 15 mol% cholesterol, which are 32.3 and 32.4 G, respectively, or equal within experimental error. Thus, addition of cholesterol reduces the A_{zz} for the chain region of

TABLE 2 A_{zz} of DPPTC in dispersions and ISDU-aligned DPPC and DMPC membranes

Membrane	Dispersions	ISDU-aligned
DPPC	34.8	36.0
DMPC	34.8	35.6

Units are the same as in Table 1.

the "dispersed" membrane, such that its reduced polarity is comparable to that of a macroscopically aligned membrane.

Note that once we have simulated the effective powder spectrum, it is then convenient to return to the original set of oriented spectra as a function of tilt angle and to fit them simultaneously to obtain the ordering in the frozen membrane. The fits for 16PC in pressure-annealed DPPC bilayers are shown in Fig. 2. We obtained an order parameter, $S = 0.60$, corresponding to the fact that even at the center of the frozen pressure-annealed DPPC bilayers, the membrane is highly ordered. In these simulations it was also necessary to vary the angle between the z' or principal axis of ordering, and the magnetic z''' axis. The best fit value of the ordering tilt angle was found to be 43°, indicating that the end of the chain is tilted by this amount relative to the normal to the bilayer, consistent with previous studies (e.g., Meirovitch and Freed, 1980). This result is consistent with the fact that the outer-peak separation of the experimental spectra has a maximum at ~45°, as shown in Fig. 1. (Note also that the g and A tensor components obtained from the effective powder spectra proved useful in a study of chain dynamics using oriented membranes, (Cassol et al., 1997).) The variation in outer-peak separation of the ISDU-aligned DPPC membrane sample with angle is much less than the pressure-annealed sample. This corresponds to a significant loss of macroscopic alignment in the frozen state. We tentatively attribute this to the fact that in a pressure-annealed sample, both upper and lower plates serve as anchors that maintain the alignment. In the ISDU sample, the upper plate must not touch the membrane, or it would spoil the alignment. Thus it cannot constrain the membrane to remain aligned when it is frozen. However, there is no reason to assume that there is a real reduction in microscopic alignment. Furthermore, because the ESR spectrum from a frozen MOMD sample is unable to provide information on microscopic ordering, it is not possible to compare the value of $S = 0.60$ for the pressure-annealed sample with the (unknown) value for the MOMD sample.

The angle-dependent spectra of DPPTC in ISDU-aligned DPPC bilayers at 22°C and their simulations using nonlinear least-squares fitting are shown in Fig. 5. To the best of our knowledge, these are the first published ESR spectra from a headgroup spin label in a macroscopically oriented membrane. The g tensor and A tensor components used in the simulation, $g_{xx} = 2.0082$, $g_{yy} = 2.0053$, $g_{zz} = 2.0015$, $A_{xx} = 7.0$ G, $A_{yy} = 7.0$ G, $A_{zz} = 36.0$ G, are the best fit values of the simulation of the effective powder spectrum (Fig. 4 *A*). The spectra of DPPTC in DPPC dispersions in the presence of excess water at 25°C and 50°C, and their simulations, are plotted in Fig. 6. The best fit parameters of rotational diffusion rates, $R_\perp$, $R_\parallel$, potential coefficients ϵ_0^2, ϵ_2^2, and the calculated order parameters S and S_2 are listed in Table 3. A striking feature revealed from these results is that the spectrum of DPPTC in DPPC dispersions at 25°C (gel phase) has a large order parameter $S = 0.50$, whereas at 50°C (LC phase), it has a negative order parameter $S = -0.32$.

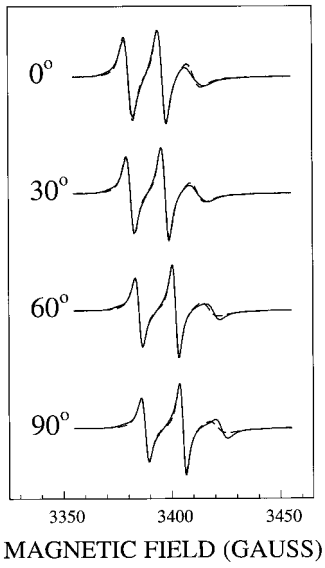

FIGURE 5 Experimental (——) and simulated (– – –) ESR spectra of headgroup spin-labeled DPPTC in ISDU-aligned DPPC membranes hydrated in 100% RH at 22°C. The values in the figure are tilt angles. The g and A tensor components used were $g_{xx} = 2.0082$, $g_{yy} = 2.0053$, $g_{zz} = 2.0015$, $A_{xx} = A_{yy} = 7.0$ G, $A_{zz} = 36.0$ G.

To explain the significance of a negative order parameter, let us compare the orienting potentials along three specific directions, $(\theta,\phi) = (90°,0°),(90°, 90°),(0°,0°)$, which specifies the orientations of the x',y', and then the z' axis being parallel to the z'' axis, respectively. One can easily verify from the symmetry of Eq. 1 that the minimum of the orienting potential $U(\theta,\phi)$ must always lie along one of the three directions. We obtained the potential coefficients from

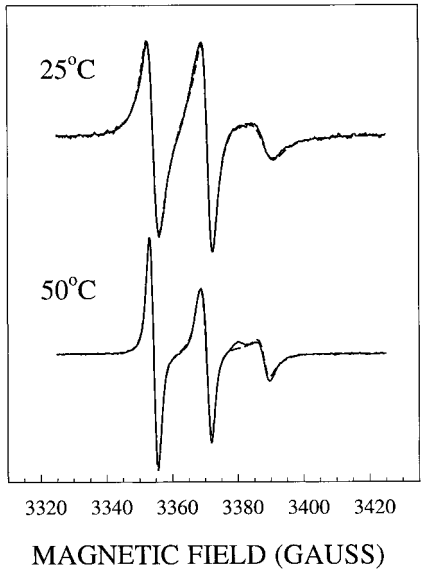

FIGURE 6 Experimental (——) and simulated (– – –) ESR spectra of DPPTC in DPPC dispersions at 25° and 50°C. The g and A tensor components used are $g_{xx} = 2.0082$, $g_{yy} = 2.0053$, $g_{zz} = 2.0015$, $A_{xx} = A_{yy} = 5.6$ G, $A_{zz} = 34.6$ G.

TABLE 3 Comparison of the best fit parameters from nonlinear least-squares fit of ESR spectra of DPPTC in DPPC dispersions and ISDU-aligned bilayers

	T (°C)	$R_\perp$ (s^{-1})	$R_\parallel$ (s^{-1})	ϵ_0^2	ϵ_2^2	S	S_2
Dispersions	25	3.17×10^7	2.57×10^8	3.07	−2.04	0.50	−0.22
	50	3.31×10^7	6.54×10^8	−2.59	0.21	−0.32	0.13
ISDU	22	8.51×10^7	6.46×10^7	0.33	0.17	0.07	−0.04

the simulation of the 50°C spectrum: $\epsilon_0^2 = -2.59$, $\epsilon_2^2 = 0.21$ (cf. Table 3). The corresponding orienting potentials are: $u_x = U(90°,0°)/kT = -1.04$, $u_y = U(90°, 90°)/kT = -1.55$, and $u_z = U(0°,0°)/kT = 2.59$. This shows that there is a strong preference for the y' and the x' axes of the nitroxide radical moiety to align along the z'' axis (the normal to the bilayer surface). The alignment of the y' axis along the bilayer normal is the most preferred, indicating that the plane of the piperidine ring of the tempo moiety is preferentially parallel to the bilayer surface. The orientation of the z' axis along the z'' axis is the most unfavorable. In other words, in the LC phase there is a force field on the DPPC bilayer surface, which tends to align the TMA group parallel to the membrane surface. Because an order parameter of $S = -0.5$ means a perpendicular alignment of a molecular moiety relative to the main ordering axis (de Gennes, 1974; Freed et al., 1994), a value of $S = -0.32$ for the tempo group of DPPTC from our simulation indicates that the TMA group has a strong preference for an alignment parallel to the surface of DPPC bilayers. A value of $S_2 = 0.13$ shows that the y' axis is the one more preferred to lie along the z'' axis than is the x', as we already noted. In the gel phase, on the other hand, $\epsilon_0^2 = 3.07$ and $\epsilon_2^2 = -2.04$, which corresponds to $u_x = -0.86$, $u_y = 4.03$, and $u_z = -3.07$. This shows that the z' axis is preferentially aligned along the z'' axis. That is, the surface-orienting potential tends to align the TMA group parallel to the normal to the bilayer.

The ESR spectra of DPPTC in ISDU-aligned DPPC bilayers hydrated in 100% RH of tilt angle 0°, 30°, 60°, 90° were fit simultaneously. The orienting potentials for the nitroxide radical calculated from the simulation are $u_x = 0.37$, $u_y = -0.04$, $u_z = -0.33$, which shows that the tempo group is only weakly ordered along the normal to the bilayer surface. This is also reflected by a very small order parameter, $S = 0.07$. These results indicate that there is no significant aligning potential in the headgroup region of oriented membranes, which are hydrated at 100% RH. In several experiments under different conditions, we have observed that this orienting potential is quite sensitive to the morphology of the bilayer and temperature. As a result, we are not ready to conclusively state that it is weak in all cases in aligned samples.

DISCUSSION AND CONCLUSIONS

Most relevant to our present study are the measurements of partial molecular volumes of water ($\bar{v}_{wm}$) and lipid ($\bar{v}_{lm}$) by White and co-workers. White and King (1985) found that before the primary hydration shell of the headgroup of oriented egg yolk phosphatidylcholine (EY-PC) bilayers is completed, i.e., when the number of water molecules per lipid is less than 10, $\bar{v}_{wm}$ and $\bar{v}_{lm}$ are 7 $Å^3$ and 1609 $Å^3$, respectively. These values are quite different from the 30 $Å^3$ and 1270 $Å^3$ observed in the EY-PC/water dispersions in a range of hydrations, 5–55 wt% water (excess water appears at water content >35 wt%), over which the volumetric mixing of lipid and water is nearly ideal (White et al., 1987). At hydration levels above 10 waters per lipid, the partial molecular volumes of water and lipid for the oriented EY-PC bilayers are much closer to, but are still significantly different from, the values in the dispersions. The small $\bar{v}_{wm}$ and large $\bar{v}_{lm}$ in oriented EY-PC bilayers reveal that the molecular packing of the headgroups in the macroscopically oriented membranes is much looser, which results in much larger free volumes between the headgroups in the oriented bilayers than in the dispersions (White et al., 1987). Our measurements of A_{zz} of DPPTC in oriented bilayers versus dispersions suggest that the headgroups are more hydrated in oriented bilayers than in dispersions, which is consistent with the model of looser packing and larger volume of hydrated headgroups in oriented membranes than in dispersions (White and King, 1985; White et al., 1987). Because the partial molecular volumes $\bar{v}_{wm}$ and $\bar{v}_{lm}$ were measured in the liquid crystalline phase for EY-PC, whereas the A_{zz} values were measured in the gel phase for DMPC and DPPC in this work, the consistency in the interpretation from the two types of measurements suggests that these characteristics are quite similar for both gel and liquid crystalline phases.

In DPPC and DMPC, an increase by ~1.0 G in the A_{zz} of 16PC from oriented bilayers to dispersions most likely means that significantly more water penetrates to the center of the hydrophobic core in dispersions than in planar multilamellar stacks. The fact that DPPC and DMPC are the most common saturated phosphatidylcholines in biological membranes, and that unsaturation of acyl chain increases the water penetration (Wiener and White, 1992; Ho et al., 1994) may suggest that water penetration into the interior of the hydrophobic core is a fundamental property of biological membranes. We may define ΔA_{zz} as the difference in A_{zz} for a spin label measured from oriented bilayers versus dispersions, i.e., $\Delta A_{zz} \equiv A_{zz}(\text{oriented}) - A_{zz}(\text{dispersion})$. Interestingly, we noticed that the magnitude of ΔA_{zz} of DPPTC in the headgroup region, (A_{zz}(oriented) > A_{zz}(dispersion)), is opposite that of ΔA_{zz} of 16PC in the chain region, (A_{zz}(oriented) < A_{zz}(dispersion)). The data pre-

sented here suggest, but are not yet sufficient for us to conclude, that there is a correlation in the polarity between the headgroup and chain regions, i.e., a relation between the hydration of the headgroup and the water penetration into the hydrophobic core. Furthermore, we are not sure how the water is partitioned between the headgroup and chain regions. The same smaller values of A_{zz} of 16PC in ISDU-aligned and dispersed DPPC membranes containing 15 mol% cholesterol (32.2 G) versus that in pure DPPC dispersions (33.8 G) confirmed that cholesterol reduces the water penetration into bilayers (Simon et al., 1982).

It would, of course, be of some interest to observe the polarity gradient along the acyl chain by labeling different positions. In the 250 GHz ESR study by Earle et al. (1994) on frozen DPPC and on frozen POPC dispersions using the *n*-PC's, where $n = 5$, 7, 10, 12, and 16, they found the expected (Griffith et al., 1974) systematic polarity gradient as a function of *n*, where 16PC is the least polar, as expected for its location near the middle of the bilayer, and 5PC is the most polar. One would want to map out this polarity gradient for the oriented bilayers. (Note that some other experiments, such as those on collision rates of the nitroxide with oxygen around room temperature (Altenbach et al., 1994), indicate a deviation for the 16PC, but the results on polarity gradient (Earle et al., 1994) clearly demonstrate that 16PC is at the most nonpolar position in the frozen dispersions, as noted.)

The distinct polarity profiles in oriented bilayers versus dispersions might originate from two types of effects. One is the effect of morphology. Specifically, the differences in morphology between the two bilayer systems are as follows:

1. The edges of oriented bilayers are open. Thus, in the case of oriented bilayers hydrated in a 100% RH, the edges of lipid monolayers and of water layers are in contact with the surrounding vapors. In contrast, lipid/vapor and water/vapor interfaces do not exist in dispersions, which are a closed structure. The different interface structures inevitably result in different molecular interactions in bilayers, including headgroup/headgroup and headgroup/water interactions.

2. The monolayer curvatures in the two bilayer systems are different: they are uniformly zero throughout the sample for macroscopically oriented bilayers, but they vary with the depth from the surface layer to the innermost layer for dispersions. According to Dill and Flory (1981), the chain disorder gradients in the inner and outer monolayers of a closed bilayer, which have opposite signs of curvature, are quite different, so that chain packing is more inhomogeneous in vesicles than in oriented bilayers. Such disorder would be expected to allow greater water penetration. It has even been postulated that aqueous channels or pores may exist in membranes, in consideration of the exceptionally large permeability of the membrane for water (Hofer, 1981). However, curvature effects should only be significant in vesicles with a small radius of curvature ($<$400 Å) (Brouillette et al., 1982), so that for lipid dispersions, which usually have a radius of 1 μm or greater, curvature effects would not be expected to be very important. However, undulations and/or deformations in the stucture of the dispersion could enhance curvature effects.

3. In the alignment procedures of pressure annealing and ISDU, external forces are brought to bear that could guarantee greater regularity in the multibilayers (e.g., fewer defects), especially in the interbilayer interactions, than for the spontaneously forming dispersions (e.g., the observed differences in behavior of DPPTC shown in Table 3). The need to form a closed vesicle structure could impose additional frustrations and/or deformations not present in the aligned membranes. Instead, the latter has rigid boundary constraints imposed by the surfaces of the holder.

A second type of effect might be the degree of hydration. However, as shown in Tables 1 and 2, for both 16PC and DPPTC, the values of A_{zz} in ISDU-aligned DPPC bilayers do not depend on whether they are hydrated in excess water or in 100% RH. For 16PC, the values of A_{zz} in ISDU-aligned bilayers and pressure-annealed DPPC bilayers are also the same. Thus the so-called vapor pressure paradox, which refers to the phenomenon of lower hydration of lipid dispersions hydrated in a 100% RH than in excess water (Rand and Parsegian, 1989), is not seen for these macroscopically oriented membranes. It implies that a comparison between the A_{zz} values of the spin labels measured from the dispersions in excess water and those measured from the macroscopically oriented membranes hydrated either in excess water or in a 100% RH is appropriate. This indicates that the different hydration characteristics observed for the oriented and dispersed bilayers are principally due to the effect of the morphology. Although we cannot be sure which of the morphological effects noted above is dominant, we do note that results from previous studies on 16PC in DPPC in the liquid crystalline (LC) phase, just above the main phase transition, are consistent with more disorder in the chain packing for dispersions than for aligned samples. Thus Patyal et al. (1997) found an order parameter $S = 0.09$ for the dispersions, and Ge et al. (1994) found $S = 0.22$ for an ISDU-aligned sample (at 100% RH).

Water penetration in the chain region should be facilitated by the reduced order parameter in the dispersions. Moreover, reduced order parameters could increase the fluctuations of the 16PC end-chain into regions of greater polarity in this fluid LC phase. If in the frozen gel phase there is a static (on the ESR time scale) distribution of 16PC end chains with respect to regions of differing polarity, then it should be possible to distinguish it by enhanced broadening of the g_{xx} region of the 250 GHz spectrum, given its great sensitivity to polarity effects (Earle et al., 1994). In fact, Earle et al. (1994) observed that the g_{xx} region for 16PC in dispersions was sharper, implying a smaller distribution in polarity, than for the label on any other chain position. Thus it is not likely that such a distribution is the source of the more polar result for 16PC in dispersions than in aligned membranes that we found in the present studies.

In sum, our results show that differences in morphology of bilayers are likely important in determining the hydration

properties in both the headgroup and acyl chain regions, which are closely associated with different molecular packing, and ultimately with different molecular interactions in both regions. In addition, for spin label studies on membranes, the values of the magnetic parameters that are needed for spectral simulation are themselves dependent on this morphology. Our finding of the presence of a substantial orienting potential in the polar interface of dispersions, which is apparently very weak in oriented membranes, indicates that the interactions between headgroups in dispersions could be quite different from that in the oriented multilayers.

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