Spectroscopic Characterization of Spin-Labeled Magnetically Oriented Phospholipid Bilayers by EPR Spectroscopy

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Abstract: This paper reports the EPR spectroscopic characterization of a recently developed magnetically oriented spin-labeled model membrane system. The oriented membrane system is composed of a mixture of a bilayer forming phospholipid and a short chain phospholipid that breaks up the extended bilayers into bilayered micelles or bicelles that are highly hydrated (approximately 75% aqueous). Paramagnetic lanthanide ions (Tm^{3+}) were added to align the bicelles such that the bilayer normal is collinear with the direction of the static magnetic field. Optimal bicelle alignment was obtained when the temperature was increased slowly (approximately 15 min) from 298 K (gel phase) to 318 K (L_α phase) at 0.64 T. The nitroxide spin probe 3β-doxyl-5α-cholestane (cholestane) was used to demonstrate the effects of macroscopic bilayer alignment through the measurement of orientational dependent hyperfine splittings that were close to A_{yy}. The EPR signals of cholestane inserted into oriented and randomly dispersed DMPC-rich bilayers have been investigated over the temperature range 298−348 K. Also, the time dependence of the loss of orientation upon cessation of the magnetic field has been characterized. Power saturation EPR experiments indicate that for the sample compositions described here, the lanthanide ions do not induce spectral line broadening of the cholestane EPR signal in DMPC-rich lipid bilayers. Recently, there has been a great deal of excitement over the use of magnetically oriented systems for both solution and solid-state NMR spectroscopy. This study demonstrates the feasibility of conducting bicelle experiments in the relatively low magnetic field of a conventional EPR spectrometer. The system offers the opportunity to carry out EPR studies using a well-oriented highly hydrated model membrane system whose preparation is amenable to the reconstitution of labile membrane components such as integral membrane proteins.

Introduction

The utilization of oriented phospholipid bilayers in both NMR[1−11] and EPR[12−14] spectroscopy has provided a wealth of structural and dynamic information about membrane-associated molecules. The orientational dependent behavior of various nitroxide spin labels incorporated into aligned membrane systems has been investigated by several researchers with EPR spectroscopy.[12,13,15−23] Generally, two methods are used for membrane alignment: (1) mechanical orientation on glass plates or Mylar films and (2) the isopotential spin-dry ultracentrifugation (ISDU) technique.[15,22] In oriented phospholipid bilayer samples, the resulting EPR spectra reveal orientational-dependent changes in the hyperfine splitting based upon the alignment of the spin label with respect to the magnetic field as well as a reduction in the spectral line widths. Reduced line widths improve spectral resolution and enable the ^{14}N hyperfine splitting and g tensors to be measured with greater precision. The anisotropic hyperfine coupling of aligned spin-labeled phospholipid bilayers can provide a more detailed structural and orientational picture of the probe with respect to the membrane

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when compared to randomly dispersed bilayer samples. Also, molecular motions can be probed over a broad range of frequencies by examining aligned spin-labeled membrane systems at variable resonant microwave frequencies.[12,19] Thus, structural and dynamic information can be abstracted from the EPR spectrum of oriented spin-labeled phospholipid bilayers to provide a clearer understanding of complex biological membranes at the molecular level.

Membrane systems that spontaneously orient in magnetic fields have been demonstrated to be successful for a wide range of NMR investigations.[2,6,10,11,24−26] In particular, several researchers have investigated membrane proteins and peptides incorporated into magnetically oriented phospholipid bilayers with solid-state NMR spectroscopy.[2,6,27,28] These oriented membrane systems are composed of a mixture of a bilayer forming phospholipid and a short chain phospholipid that breaks up the extended bilayers into bilayered micelles or bicelles that are highly hydrated (approximately 75% aqueous). Generally, the lipid mixture consists of long chain bilayer forming 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) phospholipids and short chain 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) phospholipids. The q ratio (DMPC/DHPC) between the two phospholipids is used to define the structural geometry of the bicelle.[10,29] The morphology of the magnetically aligned phospholipid micelles (bicelles) has been described as disklike with approximate dimensions of 200×40 Å^2 depending upon the long chain/short chain lipid ratio.[29]

The magnetic alignment of bicelles is due to the anisotropy of the overall magnetic susceptibility of the system. The negative sign of the diamagnetic susceptibility tensor ($\Delta\chi < 0$) for phospholipid bilayers dictates that the bicelles align with their bilayer normal oriented perpendicular to the direction of the static magnetic field. The addition of paramagnetic lanthanide ions with large positive magnetic susceptibilities (Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}) can cause the bicelles to flip 90° such that the average bilayer normal is collinear with the direction of the static magnetic field.[30] The ions are thought to associate with the phospholipid head groups of the bicelles, changing the overall magnetic susceptibility.

Although bicelle model membrane systems were initially developed for NMR applications, it has been noted that bicelles hold promise for being well-suited for a wide variety of other biophysical applications such as neutron diffraction, X-ray diffraction, EPR spectroscopy, and several optical spectroscopic techniques.[11,31] In this paper, we extend upon our initial communication and describe how we have optimized and characterized magnetically aligned phospholipid bilayers for spin label X-band EPR spectroscopic studies.[32] We feel that the development of this new spin label method will open up a whole new area of investigation for phospholipid bilayer systems and membrane protein EPR studies.

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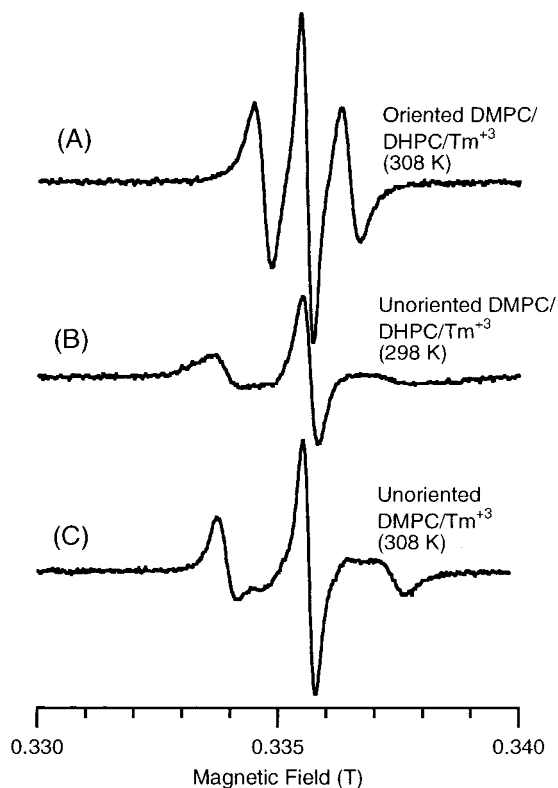

Figure 1. EPR spectra of a cholestane spin label incorporated into oriented and randomly dispersed DMPC-rich phospholipid bilayers. (A) Magnetically aligned phospholipid bilayers (25% w/w lipid) at 308 K consisting of DMPC/DHPC/cholesterol/Tm^{3+}/PEG2000-PE/cholestane in molar ratios of 3.5/1.0/0.35/0.70/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0. The sample temperature was raised from room temperature to 318 K in the EPR cavity with the magnetic field set to 0.64 T prior to taking spectra at the specified temperatures. (B) Same sample as in A except the spectrum was taken at 298 K. (C) Sample at 308 K with the same composition and methods as in A except DHPC was not included.

Results

Oriented and Randomly Dispersed Phospholipid Bilayers. The EPR spectra of 3β-doxyl-5α-cholestane (cholestane) spin labels incorporated into oriented and randomly dispersed DMPC-rich phospholipid bilayers are shown in Figure 1. Figure 1A is the EPR spectrum of magnetically oriented phospholipid bilayers at 308 K. Figure 1B displays an EPR spectrum of the same sample as in Figure 1A taken at a temperature (298 K) where the system loses its orientational features. Figure 1C displays an EPR spectrum of the same sample as in Figure 1A except the short chain lipid (DHPC) necessary to solubilize the extended DMPC bilayers into orientable disks is not included. The line widths are much broader for the two randomly dispersed spectra (Figure 1B,C) when compared to the spectrum consisting of magnetically aligned phospholipid bilayers shown in Figure 1A. The reduced line widths and the reduction of the hyperfine splitting in Figure 1A with respect to the unoriented sample in Figure 1C at the same temperature are clearly indicative of macroscopic orientation of the membrane bilayers.

Magnetically aligned phospholipid bilayer samples doped with Tm^{3+} are oriented such that the normal of the membrane bilayer is parallel with the direction of the static magnetic field.[30] We have chosen Tm^{3+} as an alignment agent over Eu^{3+}, Er^{3+}, and Yb^{3+} for these low-field EPR experiments because this ion has the largest positive $\Delta\chi$ and should yield optimal alignment. Previous experiments have indicated that cholestane aligns with

its long axis parallel to the long axis of the phospholipids and undergoes rapid rotation ($R_{||}$) about this axis.[18] For the nitroxide spin label cholestane, the nitroxide *y*-axis is approximately parallel to the long axis of the steroid derived spin probe. The reduction of the hyperfine splitting in Figure 1A with respect to the unoriented sample in Figure 1C at the same temperature is consistent with macroscopic orientation of the membrane bilayers such that their normals (and hence *y*-axis of associated cholestane spin labels) are nearly parallel with B_0. The experimentally measured hyperfine splitting (measured between the $m_I = +1$ and 0 spectral lines) in the oriented spectrum in Figure 1A is 9.2 G, which is in close agreement with values measured in previously published spectra with cholestane incorporated into phospholipid bilayers containing approximately 10% cholesterol on mechanically oriented glass plates.[18,20]

The composition of bicelles used for the EPR spectra shown is similar to that used for most of the previously published NMR studies of bicelles.[10] However, in addition to the inclusion of cholestane, there are two other components included in our EPR samples that have not been routinely used in NMR bicelle studies. First of all, the bicelles were enriched with 10% molar cholesterol to increase the local order in the membrane. By restricting the movement of the spin probe, the effects of macroscopic bilayer orientation are highlighted in the observed orientational-dependent EPR spectra. Furthermore, a small amount (1% molar to DMPC) of a phospholipid that had a soluble poly(ethylene glycol) polymer tail attached to its head group, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (PEG2000-PE) was added to improve stability of the lanthanide-doped bicelle samples.[32−34]

Optimization of Bicelle Alignment at Low Magnetic Fields (Importance of Temperature). At lower magnetic fields, magnetically oriented phospholipid bilayers are more difficult to align (degree of alignment is proportional to the square of B_0).[10] We have spent a considerable amount of time optimizing the experimental methods necessary to orient nitroxide spin-labeled bicelle systems for X-band EPR studies. The overall degree of bicelle alignment was found to be very sensitive to the temperature and magnetic field strength at which the samples were placed into the EPR spectrometer. Figure 2A displays the EPR spectrum of a bicelle system aligned using an optimized protocol. Bicelle alignment was initiated by placing the sample into the spectrometer with the magnetic field set to 0.64 T at room temperature. The temperature of the sample was then slowly raised (10 to 15 min) from room temperature up to 318 K allowing the phospholipid bilayers to undergo a phase transition from the gel phase to the L_α phase in the presence of the magnetic field. After the sample attained thermal equilibrium at 318 K (approximately 10 min), orientational characteristics were observed by taking a quick EPR spectrum at a center field of 0.3350 T. Under these exact conditions, we were able to consistently reproduce this result with different bicelle samples made up of the same composition.

The sample used to collect the spectrum in Figure 2A was removed from the magnetic field of the EPR spectrometer and mixed at room temperature to completely unorient the sample. The sample was then placed back inside the EPR spectrometer at 0 T, and the temperature was raised from room temperature to 318 K. The sample was allowed to equilibrate at 318 K (about 10 min), and then the magnetic field was turned on and set to 0.64 T. Under these conditions, the sample went through the

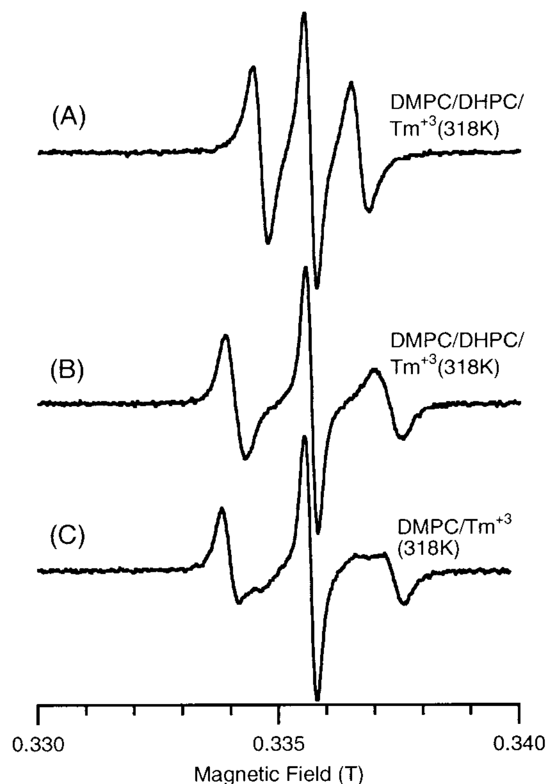

Figure 2. EPR spectra demonstrating the effects of macroscopic phospholipid bilayer alignment utilizing a gel to L_α phase transition in the presence of a magnetic field. (A) Sample consisted of 25% w/w lipid of DMPC/DHPC/cholesterol/Tm^{3+}/PEG2000-PE/cholestane in molar ratios of 3.5/1.0/0.35/0.70/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0, at 318 K. The sample temperature was raised from room temperature to 318 K in the EPR cavity over a 10- to 15-min period with the magnetic field set to 0.64 T. (B) Same sample as in A, except the sample temperature was raised in the EPR cavity from room temperature to 318 K in the absence of a magnetic field. (C) Sample prepared and treated the same as in A except DHPC was excluded.

gel to L_α phase transition in the absence of a magnetic field. After 10 min at 0.64 T, an EPR spectrum was taken, and the result is shown in Figure 2B. Inspection of Figure 2B indicates that under these conditions the phospholipid bilayer disks are not fully aligned. Furthermore, we were able to consistently and reversibly reproduce both the oriented and randomly dispersed spectra shown in Figure 2A and 2B several times with the same sample (data not shown). The minimum magnetic field at which we could initiate complete alignment of the phospholipid bilayer disks was 0.45 T.

Figure 2C represents an EPR spectrum taken from a sample prepared the same way as in Figure 2A except DHPC was not included. The sample was placed into the spectrometer, and the spectrum was gathered utilizing the same method (gel to L_α phase transition at 0.64 T) as described for Figure 2A. The spectrum does not show any orientational characteristics and is similar to the randomly dispersed spectrum displayed in Figure 2B.

Time Scale of the Loss of Alignment Upon Cessation of the Magnetic Field. Next, the time scale at which the phospholipid disks lose orientation in the absence of a magnetic field at 318 K was investigated (Figure 3). The extent of orientation of the phospholipid bilayer disks was monitored as a function of the amount of time that the magnetic field was set equal to 0 T. The data were collected by recording a quick (<1 min) EPR spectrum centered around 0.33 T. Initially, the

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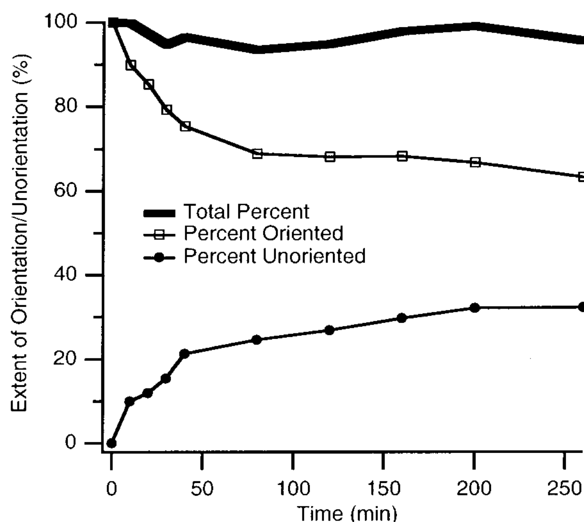

Figure 3. Diagram showing the percentage of oriented and unoriented cholestane spin-labeled bicelles at 318 K in the absence of B_0 as a function of time. The presence of both the oriented component and unoriented component was monitored using the corresponding $m_I = +1$ resonances. The unoriented component is observed at 0.3340 T and is displayed as a series of circles, and the aligned component (A_{yy}) is displayed as a series of squares (0.3346 T). The sample was prepared under the same conditions as Figure 2A. The thick line at the top represents the summation of the total percentage of bicelles (oriented + unoriented).

sample was oriented with the same method described in Figure 2A. Immediately after each spectrum was gathered, the magnetic field was set back to 0 T. Over the time course of 4 h, two distinct spectral components were observed for the $m_I = +1$ transition representing two different bicelle distributions (oriented and unoriented). An inner component was observed which represents the portion of bilayer disks that are still aligned (A_{yy}) with the magnetic field at 0.3346 T (shown as squares). The decay of bicelle alignment is monitored as a function of time by the amplitude of this peak. Initially (0 min), the bicelles are all fully aligned (100% oriented). At 260 min, in the absence of a magnetic field approximately 63% of the disks are still aligned. Alternatively, we can monitor an outer component observed at 0.3340 T (shown as circles) which represents the portion of disks that are unoriented with respect to the direction of the magnetic field. At the beginning (0 min), all of the bilayer disks are completely oriented (0% unoriented). After 260 min, approximately 33% of the bicelles are randomly dispersed throughout the sample. In one sample tube, the total number of bilayer disks remains constant (oriented and unoriented); thus, by adding the percentages of oriented (squares) and randomly dispersed (circles) bicelles together, the total should equal 100%. The thick line at the top of Figure 3 represents this summation. Over the time period studied, the total percentage of bicelles is near 100%. For these experiments, we are assuming that the rapidly scanned spectra gathered around 0.33 T does not cause the phospholipid disks to realign, since the minimum magnetic field needed to initiate alignment for the sample composition used here was found to be 0.45 T.

Cholestane Bicelle Temperature Dependence. Figure 4 shows a series of EPR spectra of magnetically oriented phospholipid bilayers investigated as a function of temperature. The oriented bicelle sample was prepared and aligned utilizing the same orientational technique described in Figure 2A. Inspection of Figure 4 indicates that as the temperature increases from 308 to 348 K the hyperfine splitting increases. For

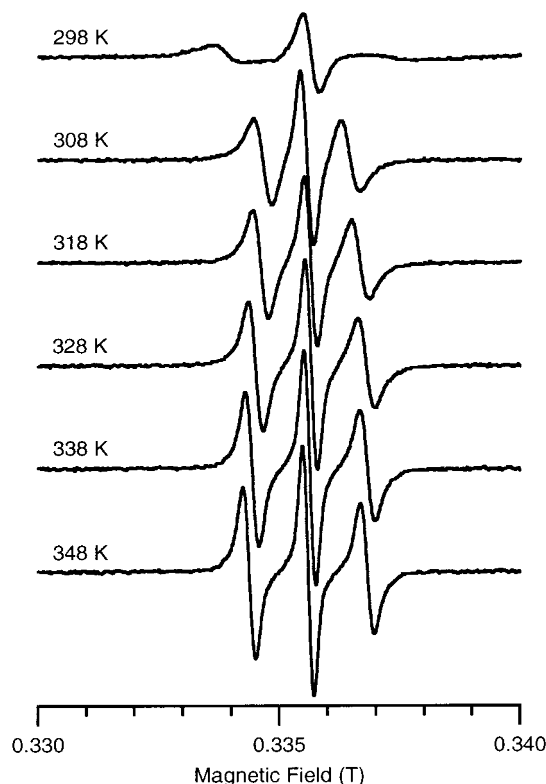

Figure 4. The temperature dependence of the EPR signal of cholestane incorporated into magnetically oriented phospholipid bilayers. Same sample composition as Figure 2A. The first spectrum (unoriented) was collected at 298 K. The sample was then warmed from room temperature to 318 K in the EPR cavity with the magnetic field set to 0.64 T. The temperatures were then adjusted as indicated and allowed to equilibrate for 10 min before the corresponding spectra were taken.

comparison, Figure 5 illustrates a series of EPR spectra gathered as a function of temperature for randomly dispersed DMPC-rich bilayers prepared the same as in Figure 4 except DHPC was not included. Conversely, for the unoriented sample as the temperature increases, the effective hyperfine splitting decreases. Figure 6 displays the hyperfine splittings measured between the $m_I = +1$ and 0 spectral lines of two unoriented samples (Figure 5 and another sample prepared the same as for Figure 4 except Tm^{3+} was excluded) and the oriented bicelle sample (Figure 4) as a function of temperature.

Power Saturation Experiments. CW-EPR power saturation experiments were carried out on cholestane spin-labeled phospholipid bilayer samples (two oriented with Tm^{3+} and two randomly dispersed without Tm^{3+}) in the presence and in the absence of oxygen at 318 K. The results are displayed in Figure 7. For all of the DMPC-rich bilayer samples, the normalized peak-to-peak amplitude of the $m_I = 0$ transition was measured.[35] Any indication of line broadening for this transition would result in a decrease in the amplitude of the spectral line. The power saturation curves for the oriented bicelle samples with Tm^{3+} are displayed as circles and the unoriented samples prepared without Tm^{3+} are shown as squares. The power saturation data arising from samples prepared in the presence of O_2 are shown as open circles and squares, whereas the curves arising from samples prepared in the absence of O_2 are displayed as solid circles and squares. As the microwave power increases, saturation broadening is clearly visible in the power saturation curves

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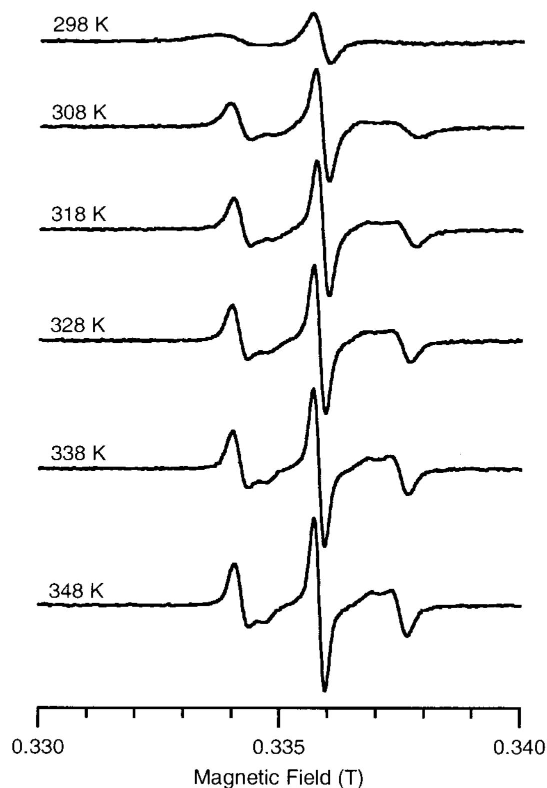

Figure 5. The temperature dependence of the EPR signal of cholestane incorporated into unoriented DMPC-rich bilayers. The sample composition is the same as in Figure 2C. The first spectrum was collected at 298 K. The sample was then warmed from room temperature to 318 K in the EPR cavity with the magnetic field set to 0.64 T. The temperatures were adjusted as indicated and allowed to equilibrate for approximately 10 min before the corresponding spectra were taken.

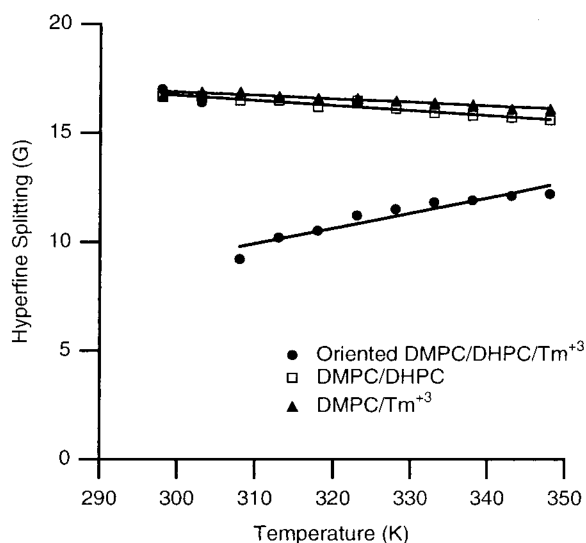

Figure 6. The temperature dependence of the hyperfine splitting of cholestane incorporated into oriented and randomly dispersed phospholipid bilayers. The oriented sample (circles) has the same sample composition and method of preparation as described in Figure 2A. Unoriented samples were prepared exactly the same except Tm^{3+} (squares) or DHPC (triangles) was excluded. The lines represent a linear least-squares fit of the experimental data.

of the samples prepared in the absence of oxygen. Conversely, the data from the samples prepared with oxygen do not show any signs of saturation, indicating that O_2 permeates through the membrane of the bicelle and interacts with the cholestane

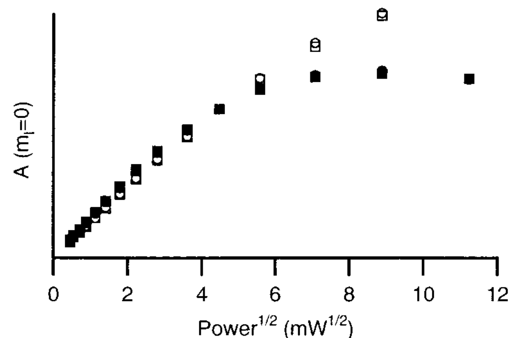

Figure 7. EPR power saturation study of oriented and unoriented phospholipid bilayer samples containing cholestane at 318 K prepared in the presence or absence of oxygen. Oriented samples are shown as circles and unoriented samples are displayed as squares. Samples prepared in the absence of oxygen are shown with solid (circles and squares) symbols. The samples with oxygen were prepared in the same manner as Figure 2A and are shown as open (circles and squares) symbols. The signal amplitude was measured from the peak-to-peak intensity of the $m_I = 0$ center line. The unoriented samples were prepared exactly the same as the oriented samples except Tm^{3+} (squares) was excluded.

spin label. In the presence of paramagnetic O_2, the power required to saturate the cholestane spin label increases due to an enhancement of the spin–lattice relaxation rate ($1/T_1$) of the nitroxide electron.[36,37] The power saturation data indicate that the presence of Tm^{3+} does not significantly alter the relaxation properties of the cholestane spin label in the presence or absence of molecular oxygen.

Discussion

This paper describes the sample composition and experimental conditions necessary to macroscopically align phospholipid bilayers in the magnetic field of an X-band EPR spectrometer. The alignment results presented in Figure 2 indicate that it is essential for the phospholipid bilayer arrays to undergo the gel to L_α phase transition in the presence of a magnetic field greater than 0.45 T to fully align the disks. This behavior suggests that the macroscopic sample alignment observed at temperatures above 308 K requires the preexistent formation of a room temperature magnetically induced phase. Firestone and coworkers have observed an analogous magnetically induced alignment mechanism with a polymer-grafted lipid-based complex fluid.[38,39] Their samples also require preequilibration at a lower temperature in the magnetic field before increasing the temperature to achieve a high degree of alignment as assessed by polarized optical microscopy and small-angle X-ray scattering.[38,39]

For the samples described in this paper, it is unclear what the morphology of our magnetically induced room temperature phase might be, although it is clear that the EPR spectra of cholestane-labeled bicelle samples that have been equilibrated at fields greater than 0.45 T at 298 K are consistent with an unoriented sample. It is conceivable that the magnetically induced room temperature phase facilitates the formation of the higher temperature macroscopically oriented phase, whereas samples that are placed in the high field directly at temperatures

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above 308 K are kinetically trapped in a phase that disfavors the formation of structures required for macroscopic alignment.

With EPR spectroscopy, we were able to monitor the time scale at which magnetically oriented phospholipid bilayers unorient upon cessation of the magnetic field (Figure 3). After 10 min at 0 T and 318 K, approximately 90% of the bicelles are still oriented. After 4 h, roughly 63% of the bicelles are still oriented such that their membrane normals are parallel with the direction of the static magnetic field. The maintenance of orientation outside the magnetic field holds potential for the use of this model membrane system for other biophysical studies.

The tensor parameters associated with a cholestane spin label incorporated into DMPC-rich phospholipid bilayers have been studied by Barnes and Freed at 250 GHz with EPR spectroscopy. In their studies, they utilized macroscopically aligned DMPC samples utilizing the ISDU technique to obtain the following magnetic parameters: $g_{xx} = 2.00871$, $g_{yy} = 2.00573$; $g_{zz} = 2.00210$, $A_{xx} = 4.9$ G, $A_{yy} = 5.5$ G, and $A_{zz} = 33.1$ G.[12] Magnetically oriented phospholipid bilayers containing cholestane that undergo rapid $R_{||}$ motions and that are oriented such that the membrane normal is parallel with the magnetic field should yield tensor values close to A_{yy}. This is the case for the oriented bicelle spectra displayed in Figure 1A which yields a hyperfine splitting of 9.2 G. A randomly dispersed motionally averaged isotropic sample should yield a hyperfine splitting of $(A_{xx} + A_{yy} + A_{zz})/3$, which is approximately equal to 14.5 G. Although the 9.2 G hyperfine splitting is larger than A_{yy} (5.5 G), it is still much smaller than the isotropic value. We can attribute this difference to slight variations in uniform alignment between the various magnetically oriented phospholipid bilayers and a restricted rapid random walk motion of the cholestane spin label that occurs perpendicular to the motion about $R_{||}$.[16,18,19]

Solid-state NMR experiments indicate that magnetically oriented lanthanide-doped phospholipid bilayers are not perfectly aligned with respect to the magnetic field but wobble about their average orientation. For a sample composition similar to that used in our EPR samples, oriented bicelles have been characterized by an order parameter of 0.7 ± 0.05 with respect to a static bilayer for solid-state NMR spectral studies carried out at 8.5 T.[2] The degree of orientation depends on several factors including the q ratio (DMPC/DHPC), sample temperature, level of hydration, and concentration of lanthanide. Also, mechanically oriented cholestane spin-labeled egg lecithin multibilayer studies have indicated that experimentally measured hyperfine values of oriented systems deviate from A_{yy} because the long axis of the cholestane probe is not perfectly aligned with B_0 and undergoes a restricted random walk motion within a cone of angle γ whose axis is perpendicular to the bilayer normal.[18,19] Adding cholesterol to the membrane decreases fluidity and reduces the amplitude of the random walk motion.[18,19] Thus, to minimize this effect and stabilize the cholestane spin label within the bicelle we added 10% molar cholesterol (with respect to DMPC) to all of our samples. In future experiments, we plan on investigating how cholesterol affects the alignment and molecular motion of the cholestane spin label in oriented bicelles by examining the hyperfine splitting as a function of cholesterol concentration.

The temperature dependence of the hyperfine splitting for magnetically oriented phospholipid bilayers and randomly dispersed DMPC-rich bilayers are summarized in the graph shown in Figure 6 over the temperature range from 298K to 348K. As the temperature increases for the oriented EPR spectra (displayed as circles), the hyperfine splitting increases. We attribute the increase in the hyperfine splitting to an enlargement in the amplitude of the random walk motion and a decrease in the overall alignment of the oriented bilayer disks caused by the increase in temperature. Conversely, as the temperature increases for the randomly dispersed bilayer samples, the observed hyperfine splitting decreases. At higher temperatures, the cholestane spin label incorporated into the unoriented phospholipid bilayers undergoes more rapid isotropic molecular motion than at lower temperatures; thus, the hyperfine splitting is approaching the isotropic value of $(A_{xx} + A_{yy} + A_{zz})/3$, which is approximately equal to 14.5 G.

For accurate analysis of spin label spectra collected for the magnetically aligned EPR bicelle samples described here, the possible interfering effects of the paramagnetic lanthanide must be understood. A potential drawback of this system could be that the presence of the paramagnetic lanthanide that is required for sample alignment could cause paramagnetic line broadening and complicate detailed analysis of spin-label EPR spectra.[24] We explored this possibility by performing power saturation studies on cholestane spin-labeled phospholipid bilayer samples both oriented and randomly dispersed and both with and without oxygen at 318 K (Figure 7). The EPR spectra indicate that the presence of Tm^{3+} does not affect the power saturation behavior. Additionally, the spectral line widths of unoriented samples prepared with and without Tm^{3+} were found to be equal. Our line width data are in agreement with similar spectra obtained from n-doxyl stearic acids interacting with lanthanide ions which showed no significant changes in spectral line width.[40] Thus, adding Tm^{3+} at these concentrations does not significantly alter the relaxation properties of the cholestane spin label.

The interaction of a nitroxide spin label with a paramagnetic species such as molecular oxygen which diffuses through the membrane and collides with the spin label is dominated by Heisenberg spin exchange and yields changes in the electron spin−lattice relaxation rate of the spin label.[41] However, our results suggest that the paramagnetic lanthanide ions used in the bicelle samples interact via a different relaxation mechanism. In phospholipid membranes, the positively charged lanthanide ions bind at the surface of the membrane and do not readily diffuse through the bilayer. The interaction between the positively charged lanthanide ions and the negative charges associated with the phospholipid head groups is believed to be electrostatic in nature and involves the formation of coordination complexes with one or more of the phosphate groups.[40,42,43]

For two different paramagnetic species that do not undergo rapid collisions such as a paramagnetic ion and a nitroxide spin label separated by a distance r that is large enough to preclude orbital overlap, the relaxation interaction is governed by a dipolar mechanism.[44,45] The dipolar relaxation mechanism is proportional to $\mu_R^2 T_{1R}/r$,[6] where μ_R and T_{1R} represent the magnetic moment and electron spin−lattice relaxation time of the transition ion probe. Thus, a paramagnetic species such as Gd^{3+} which possesses a large magnetic moment and a relatively long spin−lattice relaxation time can alter the relaxation properties of the spin label. However, depending upon the

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magnitude of *r*, ions with shorter T_{1R} values such as Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, and Tm^{3+} will minimize or eliminate the effects of the dipolar relaxation mechanism when compared to Gd^{3+}.[46] Gd^{3+} has a T_{1R} value of 5.3×10^{-10} s, whereas Tm^{3+} has a much shorter T_{1R} value of 2.8×10^{-13} s.[46,47] Thus, the fact that Tm^{3+} does not significantly alter the relaxation properties of the spin label can be explained by the short T_{1R} of Tm^{3+} and the concomitant minimized dipolar relaxation mechanism.

The development of magnetically oriented phospholipid bilayers at low magnetic fields provides enormous potential for investigating membrane protein systems with spin label EPR spectroscopy. A wide variety of spin labels including site-directed spin labels attached to integral membrane proteins or peptides, steroid derivatives, and fatty acid labels can be easily integrated into aligned phospholipid bicelle systems and studied via EPR spectroscopy. Thus, this new technique will enable the development of a more accurate and detailed understanding of complex biological mixtures found in membrane protein environments.

Experimental Section

Sample Preparation. DMPC, DHPC, and PEG2000-PE were purchased from Avanti Polar Lipids (Alabaster, AL). Thulium(III) chloride hexahydrate, cholestane, and HEPES were obtained from Sigma/Aldrich. The cholesterol was obtained from Avocado Research Chemicals, Ltd. All lipids were dissolved in chloroform and stored at −20 °C prior to use. An aqueous solution of thulium chloride hexahydrate was prepared fresh each day. All aqueous solutions were prepared with Nanopure filtered water.

The standard bicelle sample, consisting of 25% (w/w) phospholipid to solution with a $q = 3.5$, was made in two separate 15 or 25 mL pear-shaped flasks. In one flask DMPC, PEG2000-PE, and cholesterol were mixed together at ratios of 3.5/0.035/0.35, while in the second flask DHPC and cholestane were combined at ratios of 1/0.0056, respectively. The chloroform in both flasks was blown off by a constant low pressure stream of nitrogen gas (approximately 20 min), and both flasks were placed under high vacuum overnight.

The following day, an appropriate amount of 100 mM HEPES buffer at pH 7.0 for a single sample was halved and added to each flask. The two flasks were then vortexed briefly, sonicated for about 30 min, and vortexed again. The samples were sonicated with a FS30 (Fisher Scientific) bath sonicator with the heater turned off. Occasionally, brief (10−20 s) heating in a 60 °C water bath was needed to remove all of the material from the sides and bottom of the flask. Next, the DHPC and cholestane solution was added to the flask containing the DMPC, PEG2000-PE, and cholesterol and vortexed until homogeneous. The combined sample was subjected to two freeze (77 K)/thaw cycles (room temperature) to homogenize the sample and remove any air bubbles. Finally, at 0 °C (ice bucket), an appropriate aliquot of a concentrated aqueous solution of thulium(III) chloride hexahydrate was added and mixed into the sample. Typically, the total mass of the prepared samples was 200 mg.

The bicelle samples were drawn into 1 mm ID capillary tubes (Kimax) via a syringe. Both ends of the capillary tube were sealed with Critoseal (Fisher Scientific) and placed inside standard quartz EPR tubes (Wilmad, 707-SQ-250M) filled with light mineral oil.

EPR Spectroscopy. All EPR experiments were carried out on a Bruker EMX X-band CW-EPR spectrometer consisting of an ER 041XG microwave bridge and a TE_{102} cavity coupled with a BVT 3000 nitrogen gas temperature controller (temperature stability of ± 0.2 °C). All EPR spectra were gathered with a center field of 0.3350 T, sweep width of 100 G, a microwave frequency of 9.39 GHz, modulation frequency of 100 kHz, modulation amplitude of 1.0 Gpp, and a power of 6.3 mW (except for the power saturation study). All oriented samples were aligned at a maximum magnetic field strength of 0.64 T. All of the EPR spectra and resulting graphs were processed on a 300 MHz G3 Macintosh computer utilizing the Igor software package (Wavemetrics, Lake Oswego, OR).

The data in Figure 3 illustrating the percentage of oriented and unoriented cholestane spin-labeled bicelles at 318 K in the absence of a magnetic field as a function of time were obtained by subtracting out the individual unoriented and oriented spectral components. Specifically, the data representing the percentage of oriented bicelles (squares) were calculated by subtracting out the unoriented component taken from a randomly dispersed sample (318 K) at the same magnetic field position (0.3346 T). Conversely, the data representing the percentage of randomly dispersed bicelles (circles) were calculated by subtracting out the oriented component taken from a perfectly aligned bicelle sample (318 K and 0 min) at the same magnetic field position (0.3340 T). The initial data points at the two field positions were set to 100% for the oriented component at 0.3346 T and 0% for the unoriented component at 0.3340 T and the remaining points were scaled accordingly. The method is validated by summing the two percentages together (thick line) at each data point.

The DMPC-rich bilayer samples for the CW-EPR power saturation experiments were prepared with or without Tm^{3+}, and in the presence or absence of O_2. Samples with O_2 were prepared by exposing the samples to air for at least 20 min before gathering the spectra. Degassed samples (absence of O_2) were prepared by bubbling N_2 gas through the HEPES buffer solution for approximately 20 min. Next, the degassed solution was transferred to the pear-shaped flask containing the phospholipids and sealed with a rubber septum. Under a N_2 atmosphere, the sample was vortexed, sonicated, and subjected to freeze/thaw cycles as described above. Finally, N_2 gas was blown over the sample for at least 20 min and the sample was drawn into the capillary tube as described previously.

Power saturation experiments were carried out by gradually increasing the microwave power from 0.2 to 80 mW (for bicelle samples made in the presence of O_2) and from 0.2 to 126 mW (for bicelle samples made in the absence of O_2). The peak-to-peak amplitude of the $m_1 = 0$ transition was measured.[35] At a given microwave power, the spectra were normalized to the same spin concentration.

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