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Investigating Magnetically Aligned Phospholipid Bilayers with EPR Spectroscopy at 94 GHz

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In this paper, we report our initial results on studying magnetically aligned phospholipid bilayers (bicelles) at high magnetic fields (∼3.4 T) with electron paramagnetic resonance (EPR) spectroscopy at 95 GHz (W-band). In order to characterize this system for W-band EPR studies, we have utilized the nitroxide spin probe 3β-doxyl-5α-cholestane to demonstrate the effects of macroscopic bilayer alignment. At W-band due to the increase in magnetic field strength (when compared to X-band studies at 9.5 GHz) (S. M. Garber *et al., J. Am. Chem. Soc.* 121, 3240–3241 (1999)), we were able to examine magnetically aligned phospholipid bilayers at two orientations with the bilayer normal oriented either perpendicular or parallel (upon addition of $YbCl_3$) with respect to the direction of the static magnetic field. Additionally, at a magnetic field of 3.4 T ($g = 2$ resonance at W-band), we were able to study the parallel alignment with a lower concentration of Yb^{3+}, thereby eliminating the possible unwanted effects associated with lanthanide–protein interactions and paramagnetic shifts and/or line broadening induced by the lanthanide ions. The development of this new spin label alignment technique will open up a whole new area of investigation for phospholipid bilayer systems and membrane protein EPR studies at high magnetic fields. © 2001 Academic Press

Key Words: **model membrane; nitroxide spin label; EPR spectroscopy; high-field EPR; orientation.**

INTRODUCTION

A promising new technique for studying uniaxially aligned phospholipid bilayers is based on a magnetic alignment of the membrane bilayers of a certain composition in static magnetic fields of high strengths (*1–6*). These bilayered micelles or bicelles serve as an excellent model for membrane studies, because they represent an intermediate morphology between lipid vesicles and mixed micelles (*4*). Bicelles are believed to be aqueous lipid-detergent assemblies in which lipid bilayer sections are stabilized near their edges by various detergents (*4, 6, 7*). For solid-state NMR studies, the bicelles are still mobile enough (approximately 70% aqueous) to provide a high degree of resolution, yet still retain a high degree of ordering. Magnetically aligned phospholipid bilayers are disc-like with an approximate diameter of 200 Å and a thickness of 40 Å, depending upon the long-chain/short-chain lipid ratio (*7*). Generally, the lipid mixture consists of short-chain 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) and long-chain 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) phospholipids (*6, 7*). Fortunately, the bicelle technique is well suited for a wide variety of spectroscopic techniques that can be applied to biophysical research: NMR spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, neutron diffraction, X-ray diffraction, and several optical spectroscopic techniques (*4, 8*). One recent review article pointed out the enormous potential for examining oriented protein/phospholipid bicelle systems with spin label EPR spectroscopy (*4*).

The magnetic alignment of the bicelle discs results from the anisotropy of the magnetic susceptibility tensor ($\Delta\chi$) (*6*). The negative sign of this tensor for phospholipid bilayers dictates that the bicelles align with their bilayer normal, or director, oriented perpendicular to the direction of the static magnetic field. The degree of ordering depends upon several factors including the strength of the magnetic field (B_o), the sign and magnitude of the magnetic susceptibility anisotropy tensor, and the amount of material (*6*). The strength of the magnetic field has the dominant effect because the degree of ordering depends upon the square of B_o. Recently, it was discovered that the addition of certain types of paramagnetic lanthanide ions shifts $\Delta\chi$ to a positive value, thus causing the bicelles to flip 90° such that the membrane normal is parallel with the direction of the magnetic field (*9*). Thus, spectroscopic studies of magnetically aligned phospholipid bilayers can be carried out at two different orientations with respect to the magnetic field. In uniaxially aligned systems, the NMR data can yield the orientation of the molecular fragments with respect to the magnetic field and the lipid bilayer (*2, 10–12*). Similarly, the orientation of spin-labeled molecules and protein fragments site-specifically labeled with nitroxides could also be deduced from EPR spectra from uniaxially aligned systems. The overall goal of our research is to develop new

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techniques that will extend our knowledge of membrane protein systems by investigating magnetically aligned phospholipid bilayers with EPR spectroscopy.

Almost all spin-labeling EPR studies of membranes are currently carried out at X-band (0.3 T resonance field) and only a handful have been conducted at higher magnetic fields particularly with bilayers under physiologically relevant temperatures (*13–17*). However, no EPR studies to date have been carried out on phospholipid bilayers aligned by high magnetic fields. Investigating magnetically oriented phospholipid bilayers at higher magnetic fields with EPR spectroscopy has several advantages over parallel studies performed at X-band. First of all, the bicelle discs are easier to align because the degree of bicelle ordering increases as square of B_0 (*4, 6*). Obtaining well-oriented bicelle systems has never been a problem in NMR spectroscopy because the magnetic field strengths are much greater than those typically used in EPR spectroscopy. Recently, we first demonstrated the feasibility of conducting EPR studies on magnetically aligned phospholipid bilayers at X-band (*18*). However, the magnetic field was ramped up to 0.72 T to initiate the alignment process and then lowered down to 0.34 T in order to observe the $g = 2$ nitroxide spin label EPR signal at X-band. The jump in magnetic field could cause slight misalignment of the bicelle discs, thus, resulting in additional inhomogeneous broadening. By performing these experiments at higher magnetic fields of approximately 3.4 T we will be able to analyze the oriented spectra at the same magnetic field at which the phospholipid bilayer discs align. Also, at higher magnetic fields less lanthanide is needed to successfully flip the bicelles so that the membrane normal is parallel with the magnetic field. This is advantageous because at lower lanthanide concentrations complications caused by lanthanide–protein interactions, paramagnetic shifts, and line broadening are avoided (*5*). Furthermore, at higher magnetic fields ($\geq$3 T) the *g*-anisotropy of nitroxide spin labels dominates over that of the nitrogen hyperfine interaction, yielding new information on the motion and environment of the spin label (*17, 19*). In addition, the absolute point sensitivity increases at W-band (*14*). Thus, membrane protein samples can be prepared on a much smaller scale, when compared to samples needed for X-band experiments. Finally, higher magnetic field experiments are more sensitive to faster rotational motions than X-band studies (*20*). Rapid molecular motions observed at X-band through motionally averaged nitroxide spectra may not be rapid enough to cause motional averaging at high magnetic fields. Thus, by comparing spectra at different field strengths we can map out a wider range of rotational frequencies and build up a better model of motion (*14, 21*).

MATERIALS AND METHODS

Sample preparation. DMPC, DHPC, and 1,2-dimyristoyl-*sn*-glycero-3-phosphoetholamine-*N*-[Poly(ethylene glycol) 2000] (PEG2000-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Ytterbium (III) chloride hexahydrate, cholestane spin-label (CSL), and *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (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 ytterbium chloride hexahydrate was prepared fresh before each experiment.

The standard bicelle sample, consisting of 25% (w/w) phospholipid to solution with a DMPC/DHPC ratio of 3.5, was made in two separate 15- and 25-mL pear-shaped flasks. In one flask DMPC, PEG2000-PE, and cholesterol were mixed together at molar ratios of 3.5/0.035/0.35, while in the second flask DHPC and CSL were combined at molar 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 stored under vacuum overnight in a desiccator connected to a mechanical vacuum pump. The following day, the total amount of 100 mM HEPES pH 7.0 buffer needed for the entire sample was halved, and an aliquot was added to each flask. The two flasks were then vortexed briefly, sonicated for about 30 min, and vortexed again. Next, the DHPC and cholestane solution was added to the flask containing the DMPC, PEG2000-PE, and cholesterol, and vortexed until homogeneous. Finally, the combined sample (approx. 200 mg) was subjected to two freeze/thaw cycles (77 K/room temperature).

Clear fused quartz capillary tubes (0.20 mm ID) (CV2033Q, VitroCom, Mountain Lakes, NJ) were used for the W-band EPR experiments. The bicelle samples were loaded by placing the end of the capillary tube into the membrane-containing flask, which was kept at 0°C in an ice bucket. Critoseal (Fisher) was used to seal both ends of the capillary tubes. Typical sample volumes inside the W-band EPR cavity was 150–300 nL.

EPR spectroscopy. Preliminary experiments (data not shown) were carried out at Miami University on a Bruker EMX X-band CW EPR spectrometer consisting of an ER 041XG microwave bridge, a TE_{102} cavity, and a BVT 3000 nitrogen gas temperature controller (temperature stability of $\pm$0.2°C).

For W-band measurements, spin-labeled bicelle samples were prepared at Miami University and shipped overnight on dry ice to the Illinois EPR Research Center. Before shipment, some samples were checked for magnetic field alignment with solid-state NMR spectroscopy (^{31}P and ^{2}H) and signal intensity with X-band EPR spectroscopy.

W-band (94 GHz) experiments were carried out with a homebuilt EPR spectrometer at the University of Illinois EPR Research Center (*22, 23*). The sample temperature was maintained with an Oxford variable temperature system outfitted with a modified constant flow CF1200 cryostat and ITC-4 digital temperature controller (all supplied by Oxford Instruments, Ltd.) A constant flow of nitrogen gas was supplied directly to the cryostat and heated to the desired temperature. The sample temperature ($\pm$0.1°C) was monitored with two thermocouples directly mounted in the brass block surrounding

the resonance cavity and thus providing excellent temperature stability.

RESULTS

Figures 1 and 2 show a series of W-band EPR spectra of a 3β-doxyl-5α-cholestane spin label (CSL) incorporated into magnetically aligned and randomly dispersed DMPC-rich bilayers at 40 and 60°C, respectively. Figure 1A is the EPR spectrum of a magnetically oriented phospholipid bilayer sample consisting of DMPC/DHPC/cholesterol/$YbCl_3$/PEG2000-PE/cholestane in molar ratios of 3.5/1.0/0.35/0.35/0.035/0.0056 in 100 mM HEPES buffer at pH 7.0. Solid-state NMR studies have indicated that magnetically aligned phospholipid bilayers doped with Yb^{3+} are oriented such that the normal of the membrane bilayer is parallel to the static magnetic field direction. For CSL, the nitroxide *y* axis is approximately parallel to the long axis of the steroid-derived spin probe. Previous studies have shown that CSL aligns with its long axis parallel to the long axis of the phospholipids and undergoes a rapid rotation ($R_{||}$) about this axis (*24*).

First, we observed a reduction of the hyperfine splitting and the linewidth in Fig. 1A with respect to those of the unoriented sample in Fig. 1C. This is clearly indicative of a macroscopic alignment of the phospholipid bilayers such that their normals (and hence *y*-axis of associated cholestane spin labels) are parallel with B_o. Although the spectrum in Fig. 1A appears to be close to the fast motional limit, least-squares simulations with Lorentzian–Gaussian convolution lines (i.e., Voigt lineshape) revealed that three main spectral components are not equally spaced (Fig. 3A). This inequality in spacing is solely due to slow-motional effects (second-order hyperfine shifts for

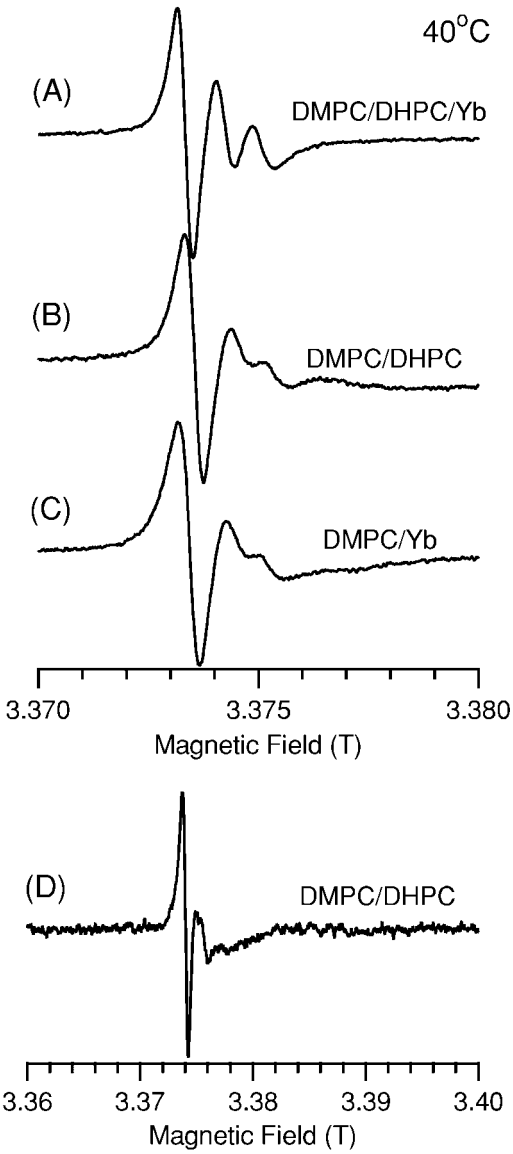

FIG. 1. W-band (95 GHz) EPR spectra (40°C) of a cholestane spin label incorporated into phospholipid bilayers. (A) Magnetically aligned phospholipid bilayers consisting of DMPC/DHPC/cholesterol/$YbCl_3$/PEG2000-PE/cholestane in the molar ratios 3.5/1.0/0.35/0.35/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0 at 25% w/w. (B) Phospholipid bilayers at 25% w/w with the same composition as (A) except $YbCl_3$ was not included in the mixture. (C) Unoriented phospholipid bilayers at 25% w/w with the same composition as (A) except DHPC was not included. (D) Phospholipid bilayers of the same composition as (B) but at 20% w/w. This spectrum was acquired over a broader spectral window. All spectra were amplitude normalized.

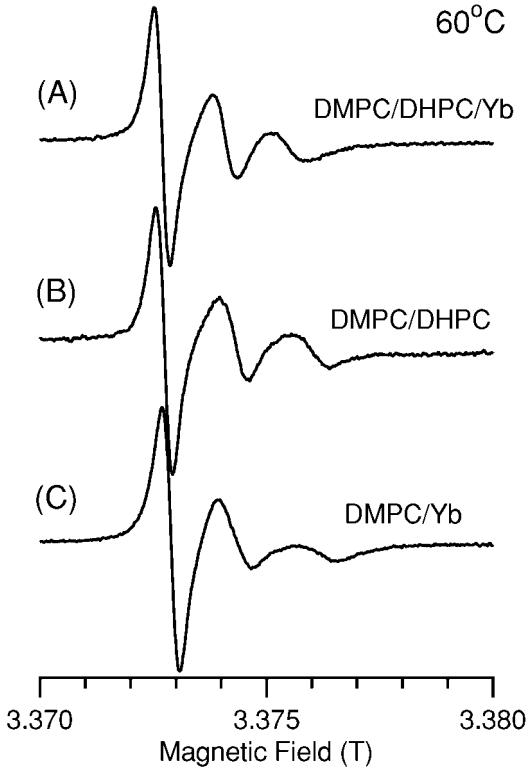

FIG. 2. Same as described in the legend to Fig. 1 except the sample temperature was 60°C and (D) is omitted.

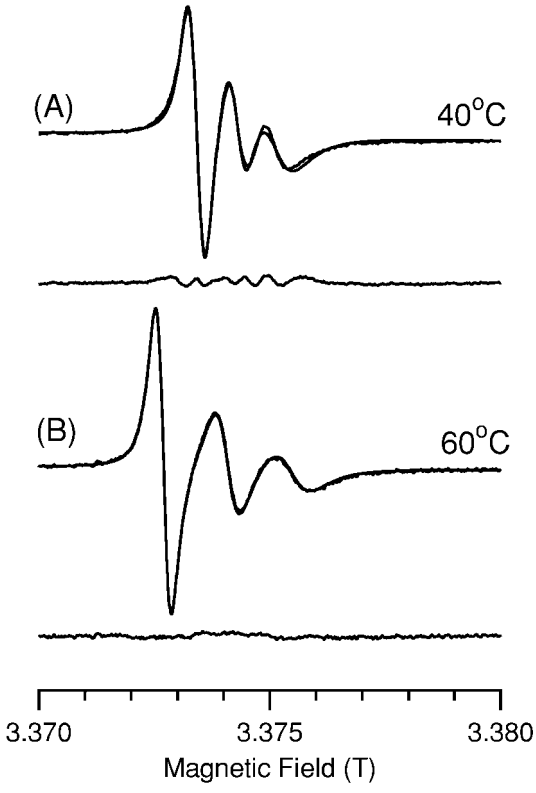

FIG. 3. Comparison between simulated and experimental 95-GHz EPR spectra of lanthanide-doped magnetically aligned bicelle samples (membrane normal is parallel with B_0) from Figs. 1A and 2A. The experimental spectra and the simulated are superimposed on top of each other. The residuals (experimental-simulated) are shown below the corresponding spectra. Temperature: (A) 40°C and (B) 60°C.

nitroxides at W-band are on the order of 3 mG and can be neglected for practical purposes). The best fit is shown in Fig. 3A where the experimental spectrum is superimposed on the simulations and the residual (a difference between experiment and simulations) is shown below. From simulations the splitting between the two low field components were 8.85 ± 0.06 and 8.05 ± 0.06 G for the high-field pair. The simulations were carried out with a least-squares convolution-based fitting program (*25*). The results closely agree with the values previously reported for cholestane incorporated into phospholipid bilayers with 10% cholesterol, which were mechanically aligned on glass plates (*24, 26*). Furthermore, this agrees well with the value of 8.7 G obtained from our previous oriented bicelle study performed at X-band (*18*). This indicates very similar effective order parameters for CSL obtained for this and earlier studies utilizing magnetic field alignment.

Figure 2A displays an EPR spectrum of the same sample as in Fig. 1A, except that the temperature has been raised to 60°C. Visual examination indicates that the splittings between the nitrogen hyperfine components are greater at 60°C than at 40°C. We have found that spectral simulations of the 60°C cholestane EPR spectrum using a fast-motional model with equal spacing between the components fits the data exceptionally well. The best fit is shown in Fig. 3B where the experimental spectrum is superimposed on the simulations and the residual is shown at the bottom. The splitting between spectral components determined from the simulations was 13.84 ± 0.02 G.

Figures 1B and 2B display the EPR spectra of magnetically aligned phospholipid bilayers of the same composition as in Figs. 1A and 2A except $YbCl_3$ was not added at 40 and 60°C. A broad scan from a sample of similar DMPC/DHPC composition is shown in Fig. 1D at 40°C. At this bicelle composition, ^2H and ^{31}P solid-state NMR studies have indicated that the membrane normals are perpendicular with respect to the direction of B_0 (*1, 2*). The slow-motional effects are clearly seen in the spectra at 40°C (Figs. 1B and 1D). It is worthwhile to note here that the spectra at 40°C can be considered as a superposition of relatively narrow three-line features (as clearly seen in the narrow-scan spectrum of Fig. 1B) and much broader three-line features as seen in Fig. 1D. The corresponding splittings are 10.5 ± 0.2 and 17.5 ± 1 G respectively (measured between the corresponding two low-field components). At 60°C this broad spectrum collapses into one set of three lines with almost equal spacing of 15.6 G (60°C). The randomly dispersed phospholipid bilayer samples (Figs. 1C and 2C) were prepared from DMPC/ cholesterol/$YbCl_3$/PEG2000-PE/cholestane in molar ratios of 3.5/0.35/0.35/0.035/0.0056. For the randomly dispersed samples, the experimentally measured hyperfine splittings were 10.5 G (40°C) and 13.9 G (60°C), respectively (measured between the two low-field components).

DISCUSSION

The dynamics and ordering of CSL in phospholipid membranes has been well studied by conventional EPR techniques (*13, 26–29*). Recently, Barnes and Freed utilized DMPC-rich phospholipid bilayers mechanically aligned by the isopotential spin-dry ultracentrifugation (ISDU) technique to study the dynamics and ordering of CSL with EPR spectroscopy at 250 GHz (*13*). They obtained the following magnetic parameters for cholestane: $g_{xx} = 2.00871$, $g_{yy} = 2.00573$, $g_{zz} = 2.00210$, $A_{xx}/\gamma_e = 4.9$ G, $A_{yy}/\gamma_e = 5.5$ G, and $A_{zz}/\gamma_e = 33.1$ G (*13*). We will use these literature values to compare with our 95-GHz EPR results obtained by a novel alignment method based upon the magnetic ordering of mixed DMPC/DHPC bicelles in a magnetic field.

For our magnetically oriented phospholipid bilayers containing CSL and oriented such that the membrane normal is parallel with the magnetic field, the EPR spectrum should consist of three lines separated by A_{yy}, if the cholestane is aligned along the bilayer normal and all effects of motion except $R_{||}$ are negligible. These conditions are close to what we observed for the Yb^{3+}-doped bicelle sample (Fig. 1A), which displays a hyperfine splitting of 8.85 G. A randomly dispersed motionally averaged isotropic sample should yield an isotropic hyperfine

value $(A_{xx} + A_{yy} + A_{zz})/3\gamma_e$ approximately equal to 14.5 G. Although the 8.85-G hyperfine splitting we observed is larger than A_{yy} (5.5 G), it is still much smaller than the isotropic value. We can attribute this difference to imperfect alignment of our bicelle discs (tilt between the bilayer normal and the magnetic axis of CSL) and/or a restricted rapid random walk motion of the cholestane spin label that occurs perpendicular ($R_\perp$) to the surface of the bilayer (*21, 24, 30*). Previous NMR studies have indicated that DMPC/DHPC bicelles are not perfectly aligned with respect to the direction of the static magnetic field (*1, 6, 31*). The degree of alignment as defined through the S_{bilayer} order parameter depends upon several factors including the sample phospholipid ratio, temperature, and water content (*1, 4, 6*). For a sample composition similar to that used in our high-field EPR samples, S_{bilayer} has been found equal to 0.7 ± 0.05 with respect to a static bilayer for solid-state NMR spectral studies carried out at 8.5 T (*2*). Additionally, studies of mechanically aligned cholestane egg lecithin multibilayers also show a significant deviation of experimentally measured splittings between the spectral components from A_{yy} because of nonnegligible $R_\perp$ cholestane motion (*21, 24*). Depending upon the morphology of the membrane, the addition of cholesterol to the membrane can decrease fluidity and reduce the amplitude of the $R_\perp$ motion (*21, 24, 27–29, 32*). Thus, in an effort to minimize this effect and reduce the $R_\perp$ motion we have added 10% molar cholesterol (with respect to DMPC) to all of our samples.

In general, an oriented membrane sample is characterized by at least three parameters: (1) a dynamic order parameter S_{dyn} describing restricted molecular motion of the spin-labeled probe, (2) an order parameter S_{bic} attributed to the alignment of the bicelles in the magnetic field, and (3) a tilt angle of the probe. The overall order parameter S is a function of dynamic ordering effects and the bicelle alignment and can be approximated as

$$S \approx S_{\text{dyn}} \cdot S_{\text{bic}}. \quad [1]$$

The overall order parameter S can be estimated from the experimental spectral at two perpendicular orientations as (*33*)

$$S = \Delta A/\Delta A_{\text{max}}, \quad [2]$$

where ΔA is the difference between the splittings observed when magnetic field is perpendicular and parallel to the plane of the sample and ΔA_{max} is the maximum observable anisotropy, which for CSL is $\Delta A_{\text{max}} = (A_{xx} + A_{zz})/2 - A_{yy} = 13.5$ G. Recent X-band EPR experiments with DMPC/DHPC bicelles doped with Tm^{3+} and Dy^{3+} aligned by magnetic field ramping indicated that $\Delta A = 9.4$ G, which gives $S = 0.7$ assuming that the overall order parameter is the same for the two bilayer orientations achieved by doping with different lanthanide ions (*34*).

In our W-band experiment the splitting corresponding to the bicelle discs orientation with the director parallel to the magnetic field is 8.85 G, which is only marginally greater than 8.7 G reported for X-band alignment with ramping the magnetic field (*34, 35*). This alone demonstrates a very similar overall order parameter. After combining with measurements at perpendicular orientation, we estimate the overall order parameter $S = 0.64 \pm 0.04$ for the sample at 40°C.

Previous X-band EPR studies have investigated the effect of cholesterol on the phospholipid bilayers prepared from egg lecitin and dipalmitoyl lecitin using mechanically aligned bilayers and indicated that at approximately 10% molar cholesterol concentration and room temperature the order parameter of CSL is about $S = 0.6$. The overall order parameter we measured here ($S = 0.64$) and from previous X-band experiments with magnetic field ramping ($S = 0.7$) are somewhat greater. This could be due to a difference in the bilayer composition and/or due to some increase of the lipid order in the membrane polar head region upon lanthanide doping. The latter could increase S_{dyn} for CSL and, thus, increase the overall order parameter S. If indeed the lanthanide ions increase the CSL order parameter, this would also explain why an S was observed in our W-band experiment somewhat lower than that in previous X-band studies that utilized higher lanthanide concentrations.

Overall, the high-order parameters observed for CSL in magnetically aligned bicelles indicate that overall alignment is similar to that previously achieved with the membranes deposited on the quartz surfaces (*26*).

When the sample temperature was raised from 40 to 60°C, the measured hyperfine splitting increased from 8.85 to 13.7 G for the Yb^{3+}-doped bicelle sample in which the bilayer normal is colinear with the direction of the static magnetic field (Figs. 1A and 2A). We attribute the increase to an enlargement in the amplitude or rate of the random walk motion (particularly $R_\perp$) and a decrease in the overall alignment of the oriented bilayer discs caused by the increase in temperature. Previous solid-state NMR studies of lanthanide-doped bicelles indicate that phase instability can occur at temperatures greater than 50°C, which would lead to a decrease in bicelle alignment (*36–38*). This is also justified by the least-squares simulations shown in Fig. 3B, which demonstrates an almost perfect fit to the fast-motion model in which the nitrogen hyperfine components are equally spaced. The fact that the CSL EPR spectrum at 60°C falls into a fast-motion limit indicates a faster rate of rotational diffusion. The splitting determined from this fit (13.84 ± 0.02 G) approaches the isotropic hyperfine value (14.23 G) derived for cholestane in the DMPC-rich bilayer environment from the 250-GHz study (*13*). The increase in the apparent hyperfine splitting is indicative of a more disordered sample.

Figures 1B and 2B represent the W-band EPR spectra of CSL in oriented phospholipid bilayers whose membrane normals are perpendicular to the magnetic field direction at 40 and 60°C. At this orientation, it is much more difficult to distinguish between magnetically aligned and randomly dispersed bicelle samples (Figs. 1C and 2C) at the same temperatures. The similarity between the perpendicular alignment and the randomly dispersed samples was also previously observed for mechanically aligned

membranes containing CSL (*26, 39*). At this orientation, CSL is undergoing a rapid rotational motion coincident with the bilayer normal (y axis of CSL).

In order for the EPR spectrum to appear close to the fast-motion regime the rate of $R_{||}$ motion (i.e., about the long cholestane y axis) should be sufficient to effectively average out the corresponding anisotropies $(g_{xx} - g_{zz})\beta_e B_o/h$ and $(A_{xx} - A_{zz})$. For the nitroxides, both g and A anisotropies are the largest for x and z pairs; thus, this condition is hard to satisfy especially at high magnetic fields. At W-band, $(g_{xx} - g_{zz})\beta_e B_o/h$ exceeds $(A_{xx} - A_{zz})$ by a factor of 4. Thus, this motion is occurring at an intermediate rather than a rapid frequency. This results in a line broadening and a decrease in the observed splitting between the components due to nitrogen hyperfine (*39*). Our observation of 15.6 G for the splitting at 60°C is in accord with these considerations. At the lower temperature (40°C), the rate of cholestane motion slows down even more. The x and z components are not effectively averaged, resulting in a broader spectrum that spreads between g_{xx} and g_{zz} regions with a distribution of components with splittings of 10.5 ± 0.2 and 17.5 ± 1 G. Under these conditions, both the perpendicular alignment (Fig. 1B) and a randomly dispersed sample (Fig. 1C) reveal comparable hyperfine splittings of 10.5 G. For cholestane inserted into unoriented DMPC-rich lipid bilayers, the tumbling rate is faster about y than about the x and z directions (*24*). Thus, for disordered samples the spectrum will be an axial motionally averaged "powder" spectrum with $A_{||} = A_{yy}$, $A_{\perp} = (A_{xx} + A_{zz})/2$. At X-band, the anisotropy of the Zeeman term is smaller than A, and the spectrum is primarily determined by $A_{||}$ and $A_{\perp}$. However, at W-band the spectral lines are more sensitive to faster rotational rates because of the enhanced contribution from the motionally modulated Zeeman term. Thus, the tumbling rate could be insufficient to effectively preaverage the x and z features. If so, that would result in broader lines, so for this axial spectrum the $A_{\perp} = (A_{xx} + A_{yy})/2$ component should generally be broader than $A_{||}$. At 40°C, the observed splitting is smaller and closer to A_{yy} for the unoriented W-band spectrum (Fig. 1C) (the motionally averaged $A_{\perp}$ component contributes as a broader line). When the temperature is increased to 60°C, the spectrum is getting closer to the fast-motion limit, resulting in a spectrum with splittings that approach the isotropic value of 14.5 G. The overall order parameter for CSL is clearly decreasing from $S = 0.64 \pm 0.04$ at 40°C to $S = 0.14$ at 60°C. We speculate that this 4.6-fold decrease is due to a decrease in S_{bic} accompanied by a decrease in the CSL dynamic order parameter S_{dyn}.

It is beneficial to perform these magnetically aligned experiments with the lowest possible concentration of lanthanide ions in order to prevent lanthanide–protein interactions and to significantly reduce paramagnetic shifts and/or line broadening induced by the lanthanide ions. At W-band, we were able to magnetically orient phospholipid bilayers such that the membrane normal is parallel with the direction of the magnetic field with only 1% molar Yb^{3+} to DMPC as compared to >10% molar Yb^{3+} at X-band (data not shown). Also, one of the disadvantages of performing these experiments at X-band is that the field must be ramped up to at least 7200 G in order to magnetically align the phospholipid discs and then allow the sample to anneal in the L_{α} phase for approximately 2 h. Furthermore, in order to observe the nitroxide EPR signal the field must be lowered to approximately 3300 G. Alternatively, the advantage of W-band EPR is that the bicelle discs align spontaneously in the 3.4-T magnetic field and the aligned spectra are gathered at the same field strength at with they were aligned (no field ramping is needed).

To our knowledge, this manuscript represents the first time that magnetically aligned phospholipid bilayers have been investigated with EPR spectroscopy at 94 GHz. This method represents an attractive alternative to the ISDU alignment technique because the sample preparation and conditions for alignment are much easier.

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