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ABSTRACT

X-BAND EPR SPECTROSCOPY OF SPIN-LABELED MEMBRANE BIOMOLECULES INCORPORATED INTO MAGNETICALLY ALIGNED PHOSPHOLIPID BILAYERS

By Thomas B. Cardon

The orientation, structure and dynamics of spin-labeled integral membrane peptides/proteins can be obtained directly from the orientation-dependent hyperfine splittings of the spin-labeled EPR spectra. Various spin-labeled biomolecules were incorporated into magnetically alignable phospholipid bilayers called bicelles. Bicelles are a binary mixture of long-chain and short-chain phospholipids. Dimyristoyl phosphatidylcholine (DMPC) and dihexanoyl phosphatidylcholine (DHPC) were used as the long-chain and short-chain phospholipids, respectively. The mole ratio of DMPC/DHPC is referred to as the q-ratio. The normal to the phospholipid bilayers, \( \mathbf{n} \), can be induced to align either parallel or perpendicular to the direction of the applied magnetic field, \( \mathbf{B}_0 \), when the bicelles are doped with either \( \text{Tm}^{3+} \) or \( \text{Dy}^{3+} \) cations, respectively (Chapters 1 and 2). The affects on the relaxation rates of the spin label and the magnetic alignment behavior of bicelles caused by doping the bicelles with strong paramagnetic lanthanide cations were investigated in Chapters 1 and 3. Chapters 4 and 5 illustrate how varying the experimental conditions and sample composition affect the magnetic alignment behavior of the bicelles and the molecular ordering, \( S_{\text{mol}} \), of the spin label. \( S_{\text{mol}} \) and the temperature range over which the magnetic alignment was stable increased as the q-ratio increased. The magnetic alignment of samples having a q-ratio of 9.5 was stable at temperatures between 298 K (gel phase) to 318 K (liquid crystalline phase), whereas the magnetic alignment of samples having a q-ratio of 2.5 was stable at temperatures between 308 K and 318 K.
For the first time, highly orientation-dependent hyperfine splittings were observed for a spin-labeled peptide (TOAC18 AChR M2), corresponding to the pore-lining transmembrane domain of the nicotinic acetylcholine receptor, reconstituted into magnetically aligned bicelles (Chapter 6). The EPR spectra of TOAC18 AChR M2 was found to be transmembrane with a helical tilt of 10° and the principal z-axis of the TOAC spin label made a 18.9° angle with the helical axis, which agrees very well with results from solid-state NMR spectroscopic and X-ray crystallographic studies. Thus, spin-labeled EPR spectroscopy could be a powerful structural biology tool that is more efficient and less expensive than solid-state NMR spectroscopy.
X-band EPR Spectroscopy of Spin-labeled Membrane Biomolecules Incorporated into Magnetically Aligned Phospholipid Bilayers

A DISSERTATION

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DEDICATION AND ACKNOWLEDGEMENTS

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I have had many people tell me I was crazy to be married and have a family (four boys and another, this time a girl, on the way!) while going to school. Those who say such things truly do not understand the miracle and joy of having children—at least, they do not understand what it means to me. My children are the greatest achievements I will ever make in this life. My research is nothing compared to my work with my family. They have made life as a graduate student worth doing because they gave me a purpose upon which my energies were drawn forward. When I would come home after having a hard day at school, those little eyes would light up, they’d throw their arms around me, kiss me, and nearly bust my eardrums with the chant, “Daddy’s home, Daddy’s home, Daddy’s home….!!!!” Nothing could melt away the stresses of school life and the doubts about one’s abilities like that kind of love and admiration, which never fails.

As the saying goes, “Blood is thicker than water” has proved to be true. Thank heavens that my extended family members were not “turnips” for they have squeezed their life’s blood out to support my efforts and pull me out of hard times. I acknowledge the instrumental hand that colleagues, friends, and wonderful mentors like Dr. Lorigan have had in my successes. They have been my surrogate family and have given me more than I could have ever imagined. I count myself blessed for my association with such fine people. I will miss my daily interaction with them.
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CHAPTER 1

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

1.1 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 ($L_n$ phase) at 0.64 T. The nitrooxide spin probe $3\beta$-doxyl-$5\alpha$-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.

1.2 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.\textsuperscript{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.\textsuperscript{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 $^{14}\text{N}$ 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 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{10,29} The morphology of the magnetically aligned phospholipid micelles (bicelles) has been described as disk-like with approximate dimensions of $200 \times 40 \text{Å}^2$ depending upon the long chain/short chain lipid ratio.\textsuperscript{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 $90^\circ$ such that the average bilayer normal is collinear with the direction of the static magnetic field. 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. 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. 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.

1.3 Experimental

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 $q = 3.5$, 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 TE102 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 1.3 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 Tm³⁺, and in the presence or absence of O₂. Samples with O₂ were prepared by exposing the samples to air for at least 20 min before gathering the spectra. Degassed samples (absence of O₂) were prepared by bubbling N₂ 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 N₂ atmosphere, the sample was vortexed, sonicated, and subjected to freeze/thaw cycles as described above. Finally, N₂ 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 O₂) and from 0.2 to 126 mW (for bicelle samples made in the absence of O₂). The peak-to-peak amplitude of the $m_1 = 0$ transition was measured. At a given microwave power, the spectra were normalized to the same spin concentration.

1.4 Results

Oriented and Randomly Dispersed Phospholipid Bilayers. The EPR spectra of 3β-doxyl-5α-cholestanate (cholestanate) spin labels incorporated into oriented and randomly
dispersed DMPC-rich phospholipid bilayers are shown in Figure 1.1. Figure 1.1A is the EPR spectrum of magnetically oriented phospholipid bilayers at 308 K. Figure 1.1B displays an EPR spectrum of the same sample as in Figure 1.1A taken at a temperature (298 K) where the system loses its orientational features. Figure 1.1C displays an EPR spectrum of the same sample as in Figure 1.1A 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 (Figures 1.1B and 1.1C) when compared to the spectrum consisting of magnetically aligned phospholipid bilayers shown in Figure 1.1A. The reduced line widths and the reduction of the hyperfine splitting in Figure 1.1A with respect to the unoriented sample in Figure 1.1C 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 ($R_\parallel$) 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 1.1A with respect to the unoriented sample in Figure 1.1C 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 1.1A 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
Figure 1.1. 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.
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.\textsuperscript{32-34}

\textit{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 $B_0$).\textsuperscript{10} We have spent a considerable amount of time optimizing the experimental methods necessary to orient nitrooxide 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 1.2A 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 $L_\alpha$ 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 1.2A 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 gel to Lα 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 1.2B. Inspection of Figure 1.2B 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 1.2A and 1.2B 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 1.2C represents an EPR spectrum taken from a sample prepared the same way as in Figure 1.2A except DHPC was not included. The sample was placed into the spectrometer, and the spectrum was gathered utilizing the same method (gel to Lα phase transition at 0.64 T) as described for Figure 1.2A. The spectrum does not show any orientational characteristics and is similar to the randomly dispersed spectrum displayed in Figure 1.2B.

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 1.3). 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 sample was oriented with the same method described in Figure 1.2A. 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 $m_t = +1$ transition representing two different bicelle distributions (oriented and unoriented). The $m_t = +1$ transition of the oriented component was observed at a magnetic field of 0.3346 T (shown as squares), which represents the portion of bilayer disks that are still aligned ($A_y$, parallel with $B_0$). The decay of bicelle alignment is monitored as a function of time by the decrease in 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 the emergence of a component with an observed $m_t = +1$ transition at 0.3340 T (shown as circles), which represents the portion of disks that are
Figure 1.2. EPR spectra demonstrating the effects of macroscopic phospholipid bilayer alignment utilizing a gel to Lα phase transition in the presence of a magnetic field. (A) Sample consisted of 25% w/w lipid of DMPC/DHPC/cholesterol/Tm3+/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.
Figure 1.3. Diagram showing the percentage of oriented and unoriented cholestanol 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 1.2A. The thick line at the top represents the summation of the total percentage of bicelles (oriented + unoriented).
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 1.3 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 1.4 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 1.2A. Inspection of Figure 1.4 indicates that as the temperature increases from 308 to 348 K the hyperfine splitting increases. For comparison, Figure 1.5 illustrates a series of EPR spectra gathered as a function of temperature for randomly dispersed DMPC-rich bilayers prepared the same as in Figure 1.4 except DHPC was not included. Conversely, for the unoriented sample as the temperature increases, the effective hyperfine splitting decreases. Figure 1.6 displays the hyperfine splittings measured between the $m_i = +1$ and 0 spectral lines of two unoriented samples (Figure 1.5 and another sample prepared the same as for Figure 1.4 except $\mathrm{Tm}_{3+}$ was excluded) and the oriented bicelle sample (Figure 1.4) 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 $\mathrm{Tm}_{3+}$ and two randomly dispersed without $\mathrm{Tm}_{3+}$) in the presence and in the absence of oxygen at 318 K. The results are displayed in Figure 1.7. For all of the DMPC-rich bilayer samples, the normalized peak-to-peak amplitude of the $m_i = 0$ transition was measured. 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 $\mathrm{Tm}_{3+}$ are displayed as circles and the unoriented samples prepared without
Figure 1.4. The temperature dependence of the EPR signal of cholestane incorporated into magnetically oriented phospholipid bilayers. Same sample composition as Figure 1.2A. 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.
Figure 1.5. The temperature dependence of the EPR signal of cholestane incorporated into unoriented DMPC-rich bilayers. The sample composition is the same as in Figure 1.2C. 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 1.6. 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 1.2A. 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.
**Figure 1.7.** 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 1.2A 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.
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 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 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 absence or presence of molecular oxygen.

### 1.5 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 1.2 indicate that it is essential for the phospholipid bilayer arrays to undergo the gel to L\(_\alpha\) 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 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 1.3). 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 $R_{ij}$ 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 1.1A 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 $R_{ij}$.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. 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 $\gamma$ whose axis is perpendicular to the bilayer normal.° Adding cholesterol to the membrane decreases fluidity and reduces the amplitude of the random walk motion.°° 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 1.6 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.\textsuperscript{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 1.7). The EPR spectra indicate that the presence of Tm\textsuperscript{3+} does not affect the power saturation behavior. Additionally, the spectral line widths of unoriented samples prepared with and without Tm\textsuperscript{3+} were found to be equal. Our line width data are in agreement with similar spectra obtained from \textit{n}-doxyl stearic acids interacting with lanthanide ions, which showed no significant changes in spectral line width.\textsuperscript{40} Thus, adding Tm\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{44,45} The dipolar relaxation mechanism is proportional to \( \mu_r^2 T_{1R}/r^6 \), where \( \mu_r \) 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\textsuperscript{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 magnitude of \( r \), ions with shorter \( T_{1R} \) values such as Tb\textsuperscript{3+}, Dy\textsuperscript{3+}, Ho\textsuperscript{3+}, Er\textsuperscript{3+}, and Tm\textsuperscript{3+} will minimize or eliminate the effects of the dipolar relaxation mechanism when compared to Gd\textsuperscript{3+}.\textsuperscript{46} Gd\textsuperscript{3+} has a \( T_{1R} \) value of 5.3 \( \times \) 10\textsuperscript{-10} s, whereas Tm\textsuperscript{3+} has a much shorter \( T_{1R} \) value of 2.8 \( \times \) 10\textsuperscript{-13}
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.

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CHAPTER 2

Magnetically Aligned Phospholipid Bilayers at the Parallel and Perpendicular Orientations for X-Band Spin-Label EPR Studies

2.1 Results and Discussion.

Magnetically aligned phospholipid bilayers (bicelles) have been successfully used in a range of solid-state and solution NMR studies to macroscopically order both membrane-bound\textsuperscript{1-5} and water-soluble macromolecules.\textsuperscript{6-9} Sample orientation enables the efficient high-resolution measurement of anisotropic spectral parameters that provide valuable structural and dynamic information for both NMR and EPR studies.\textsuperscript{6,10-13} A standard bicelle consists of a combination of long-chain bilayer forming 1,2-dimyristoyl-\textit{sn}-glycero-3-phosphocholine (DMPC) phospholipids and short-chain 1,2-dihexanoyl-\textit{sn}-glycero-3-phosphocholine (DHPC) phospholipids that when mixed together under the correct conditions magnetically align. The negative sign of the magnetic susceptibility anisotropy tensor for the phospholipid bilayers dictates that the bicelles align with their bilayer normal oriented perpendicular to the direction of the static magnetic field.\textsuperscript{4} Unfortunately, the standard DMPC/DHPC bicelle disks used in NMR studies do not completely align at the perpendicular orientation at low magnetic fields used for X-band EPR studies presumably due to the lack of sufficient negative magnetic susceptibility anisotropy. The inability to study magnetically aligned phospholipid bilayers at this orientation at low magnetic fields dramatically limits the usefulness of this technique for spin-label EPR studies. In this communication, we successfully demonstrate with spin-label EPR spectroscopy that magnetically aligned phospholipid bilayers can be investigated with their bilayer normals perpendicular with the magnetic field in a conventional X-band EPR spectrometer. The addition of paramagnetic lanthanide alignment reagents with large positive magnetic susceptibilities (Eu\textsuperscript{3+}, Er\textsuperscript{3+}, Tm\textsuperscript{3+}, or Yb\textsuperscript{3+}) causes the phospholipid bilayer disks to align with their bilayer normals parallel with \( B_0 \).\textsuperscript{14,15} Previously, we demonstrated that phospholipid bilayers doped with either Tm\textsuperscript{3+} or Yb\textsuperscript{3+} magnetically align at this orientation using spin-label EPR spectroscopy at X-band and discussed conditions for optimal alignment.\textsuperscript{16,17} Here, with the aim of increasing the magnitude of the overall negative \( \Delta \chi \) of the disks to induce a perpendicular bicelle alignment, we have added Dy\textsuperscript{3+} to the DMPC/DHPC bicelle matrix, which alternatively possess a large negative \( \Delta \chi \).\textsuperscript{14,18} The ability to align lipid bilayer systems in the perpendicular as well as parallel orientation is valuable because the combination of
both types of anisotropic spectra provides a more detailed and complete description of the structural and motional properties of the membrane-associated spin label than either type alone.\textsuperscript{19,20} The orientational characteristics of the nitrooxide label can be described by the molecular axes (x, y, and z) and are defined relative to the doxyl ring.\textsuperscript{20} The x-axis is along the N-O bond, the z-axis is along the 2p \(\pi\) orbital of the nitrogen, and the y-axis is perpendicular to the x, z plane. Typical principal values for a nitrooxide hyperfine tensor are \(A_{xx} = 6\) G, \(A_{yy} = 6\) G, and \(A_{zz} = 32\) G.\textsuperscript{20} In an aligned spectrum, the orientation that the spin label makes with respect to the magnetic field and the motion about its molecular axis will determine the observed hyperfine splitting. Figure 2.1 illustrates the effects of bicelle alignment (parallel and perpendicular) on the observed hyperfine splittings of two different spin labels (3\(\beta\)-doxyl-5\(\alpha\)-cholestanate (CLS) and 5-doxylstearic acid). EPR spectra A and D of Figure 2.1 display randomly dispersed bicelle disks and serve as an excellent basis for comparison between the subsequent perpendicular and parallel aligned bicelle EPR spectra.\textsuperscript{17,21} The line shape and hyperfine splitting of the spectrum shown in Figure 2.1A is similar to previous data gathered at 318 K.\textsuperscript{17} As illustrated in Figure 2.1, CLS aligns with its long axis (dotted line) parallel to the long axis of the phospholipids and undergoes rapid rotation \(R_y\) about this axis.\textsuperscript{22} The nitrooxide y-axis is approximately parallel to the long axis of the steroid derived spin probe.\textsuperscript{22} Figure 2.1B shows the EPR spectrum of a DMPC/DHPC/Dy\textsuperscript{3+} bicelle sample doped with CLS. The observed increase in hyperfine splitting when compared to the randomly dispersed spectrum indicates that the bilayer normal of the Dy\textsuperscript{3+}-bicelle is aligned perpendicular to the magnetic field. At this orientation, the y-axis and axis of rotation are approximately perpendicular to the magnetic field; thus, the x- and z-tensoral components are averaged. The measured hyperfine splitting of 18.1 G is close to the theoretical value of \((A_{zz} + A_{yy})/2 = 19\) G. Conversely, Figure 2.1C shows an EPR spectrum of a Tm\textsuperscript{3+}-bicelle sample containing CLS. The observed 9.2 G hyperfine splitting is significantly reduced with respect to the unoriented and perpendicular aligned samples in spectra A and B of Figure 2.1 and is consistent with macroscopic orientation of the membrane bilayers such that their normals (and hence y-axis of associated CLS spin labels) are nearly parallel with \(B_0\). The experimentally measured hyperfine splittings agree with values ranging from 17.5 to 19.0 G (perpendicular) and 8.0-10.0 G (parallel) in previously published mechanically
oriented spectra with varying degrees of cholesterol. The alignment of the bicelle disks was further confirmed with a 5-doxylstearic acid spin label in which the long axis of the fatty acid is collinear with the long chains of the DMPC phospholipids. For the 5-doxylstearic acid in the parallel Tm3+-bicelle alignment, the z-component and the axis of rotation are approximately parallel with the bilayer normal and the magnetic field (Figure 2.1F). The measured hyperfine splitting is equal to 22.0 G and matches a similar value of 22.5 G obtained at this orientation in the literature. Conversely, for the perpendicular Dy3+-bicelle alignment (Figure 2.1E) the x- and y-tensoral components are motionally averaged to reveal a measured hyperfine splitting of 11.4 G. This splitting agrees well with a literature value of 11.9 G. The dynamics of the 5-doxylstearic acid are more complicated than CLS because rapid segmental motion occurs due to a large number of gauche-trans interconversions of the fatty acid chain resulting in a partial averaging of the hyperfine tensors. Clearly, the orientational-dependent hyperfine splittings observed in spectra E and F in Figure 2.1 demonstrate the effects of macroscopic bilayer alignment when compared to the randomly dispersed spectrum in Figure 2.1D. All of the hyperfine splittings and the shape of the observed spectra displayed in Figure 2.1 closely resemble the EPR spectra in the literature obtained from mechanically aligned phospholipid bilayers on glass plates. As a control, we prepared a DMPC/DMPC54/DHPC/Dy3+ bicelle sample and checked for alignment via NMR spectroscopy. The corresponding spectrum yielded a well-resolved 2H NMR spectrum that matches the shape, breadth, and resolution of magnetically aligned bicelle spectra shown in the literature. Conformational changes within membranes have been shown to be small upon Ln3+ binding; however, minimizing these changes is important. A phospholipid that chelates Ln3+ ions will be employed in future studies. The methodology described in this report enables high-resolution oriented EPR studies to be carried out at two different orientations with respect to the direction of the membrane normal and B0. The anisotropic EPR data gleaned from these two distinct orientations will greatly enhance our ability to study membrane proteins with spin-label EPR spectroscopy and provide a more detailed structural and orientational picture of the probe...
Figure 2.1. Comparison of magnetically aligned and randomly dispersed phospholipid bilayers with spin-label EPR spectroscopy. (A) EPR spectrum of randomly dispersed bicelle disks containing CLS. (B) EPR spectrum of magnetically aligned phospholipid bilayers (membrane normal is perpendicular with $B_0$) containing CLS and Dy$^{3+}$. (C) EPR spectrum of magnetically aligned bicelles (membrane normal is parallel with $B_0$) containing CLS and Tm$^{3+}$. (D) Same as spectrum A except 5-doxysteric acid was used. (E) Same as spectrum B except 5-doxysteric acid was used. (F) Same as spectrum C except 5-doxysteric acid was used. The orientation and axis of rotation of the two spin labels with respect to the magnetic field and membrane normal of the bicelle are displayed on the edges of the figure. In the center, cartoons depicting the orientation of the disks are shown (bilayer normals shown as arrows). All samples consisted of DMPC/DHPC/cholesterol/Ln$^{3+}$/PEG2000-PE/spin label in molar ratios of 3.5/1.0/0.35/0.70/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0. 10% molar cholesterol was added to all of our samples to decrease fluidity and stabilize the spin label within the bicelle. For the randomly dispersed spectra, the sample temperature was raised from 298 to 318K in the absence of $B_0$, prior to taking spectra at 308 K. All spectra were collected with a center field of 3350 G, sweep width of 100 G, $\nu = 9.39$ GHz, modulation amplitude of 1.0 G, and a microwave power of 2.5 mW. Conditions for magnetically aligning the phospholipid bilayer disks has been described previously.\textsuperscript{17}
with respect to the membrane when compared to randomly dispersed bilayers.\textsuperscript{16,17} The bilayers in magnetically aligned bicelles are in the $L_\alpha$ phase and have the capacity to incorporate integral membrane proteins.\textsuperscript{4,5} Thus, this alignment method will be useful for investigating site-specific spin-labeled membrane proteins. Finally, this magnetically aligned system can be used for both NMR and EPR studies and several published reports have already explicitly called for studies on phospholipid-protein interactions that combine well-resolved $^2$H NMR results with EPR spin label studies.\textsuperscript{25,26}

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2.2 References


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CHAPTER 3

Investigating Magnetically Aligned Phospholipid Bilayers with Various Lanthanide Ions for X-band Spin-label EPR Studies

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3.1 Abstract

This paper reports the EPR spectroscopic characterization of a model membrane system that magnetically aligns with a variety of different lanthanide ions in the applied magnetic field (< 1 T) of an X-band EPR spectrometer. The ability to align phospholipid bilayer systems is valuable because the anisotropic spectra provide a more detailed and complete description of the structural and motional properties of the membrane-associated spin label when compared to randomly dispersed EPR spectra. The nitrooxide spin probe 3β-doxyl-5α-cholestanе (cholestanе or CLS) was inserted into the bilayer discs to demonstrate the effects of macroscopic bilayer alignment through the measurement of orientational dependent hyperfine splittings. The effects of different lanthanide ions with varying degrees of magnetic susceptibility anisotropy and relaxation properties were examined. For X-band EPR studies, the minimal amounts of the Tm³⁺, Yb³⁺, and Dy³⁺ lanthanide ions needed to align the phospholipid bilayers were determined. Power saturation EPR experiments indicate that for the sample compositions described here, the spin-lattice relaxation rate of the CLS spin label was increased by varying amounts in the presence of different lanthanide (Gd³⁺, Dy³⁺, Er³⁺, Yb³⁺, and Tm³⁺) ions, and in the presence of molecular oxygen. The addition of Gd³⁺ caused a significant increase in the spin-lattice relaxation rate of CLS when compared to the other lanthanide ions tested.

3.2 Introduction

The utilization of aligned model membranes in both NMR¹⁻¹¹ spectroscopy and EPR¹²⁻¹⁴ spectroscopy has provided a wealth of structural and dynamic information about membrane associated molecules. The orientational dependent behavior of various nitrooxide spin labels incorporated into aligned membrane systems has been investigated by several researchers with EPR spectroscopy.¹²⁻²¹ Membrane alignment is usually carried out using two methods: (1) mechanical orientation on glass plates or mylar films and (2) the isopotential spin-dry ultracentrifugation (ISDU) technique.¹³,²⁰ In aligned
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 splitting of aligned spin-labeled phospholipid bilayers can provide a more detailed structural picture of the probe with respect to the membrane when compared to randomly dispersed phospholipid 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. Thus, structural and dynamic information can be gleaned from the EPR spectrum of oriented spin-labeled phospholipid bilayers to provide a more detailed understanding of complex biological membranes at the molecular level.

Membrane systems that spontaneously align in magnetic fields have been demonstrated to be successful for a wide range of NMR studies for membrane and integral membrane proteins and peptides. 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. The morphology of the magnetically aligned phospholipid micelles (bicelles) has been described as disc-like with approximate dimensions of $200 \times 40 \ \text{Å}$ depending upon the long-chain/short-chain lipid ratio and the temperature.

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 anisotropy 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 ($\text{Eu}^{3+}$, $\text{Er}^{3+}$, $\text{Tm}^{3+}$, and $\text{Yb}^{3+}$) can cause the bicelles to flip $90^\circ$ such that the average bilayer
normal is collinear with the direction of the static magnetic field. The ions are thought to associate with the phospholipid headgroups of the bicelles, changing the overall $\Delta \chi$.

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. In this paper, we extend upon our previous work and describe the effect that different lanthanide ions have on magnetically aligned and randomly dispersed phospholipid bilayers for spin-labeled X-band EPR spectroscopic studies. 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.

3.3 Experimental

Sample preparation. DMPC, DHPC, and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (PEG2000-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Thulium (III) chloride hexahydrate, dysprosium (III) chloride hexahydrate, erbium (III) chloride hexahydrate, ytterbium (III) chloride hexahydrate, gadolinium (III) chloride hexahydrate, cholesterol, 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. Aqueous solutions of lanthanide ions were 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 15- or 25-ml pear-shaped flasks. In the flask DMPC, PEG2000-PE, DHPC, cholesterol, and cholestane were mixed in ratios of 3.5:0.035:1:0.35:0.0196, respectively. The chloroform in the flask was rotovapped off using a Buchi R-3000 rotovap (approximately 20 min), and the flask was placed under high vacuum overnight.

The following day, an appropriate amount of 100 mM HEPES buffer at pH 7.0 was
added to the flask. The flask was then vortexed briefly, sonicated for about 30 min, and vortexed again. The samples were sonicated with a Fisher Scientific FS30 bath sonicator (Florence, KY) with the heater turned off. Occasionally, brief (10–20 s) cooling in an ice water bath was needed to remove all of the material from the sides and bottom of the flask. The combined sample was subjected to two freeze (77 K)/thaw (room temperature) cycles to homogenize the sample and remove any air bubbles. Finally, at 0 °C (ice bucket), an appropriate aliquot of a concentrated aqueous solution of lanthanide ion 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-250 M) 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 0.1 mT (pp), and a power of 6.3 mW (except for the power saturation studies). All oriented samples were aligned by warming the sample from 298 to 318 K at a maximum magnetic field strength of 0.64 T.$^{32}$ 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 DMPC-rich bilayer samples for the CW-EPR power saturation experiments were prepared with various lanthanide ions (Gd$^{3+}$, Dy$^{3+}$, Er$^{3+}$, Yb$^{3+}$, and Tm$^{3+}$) at a concentration of 20% (mol% lanthanide to DMPC), 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 200 mW for all samples. The peak-to-peak amplitude of the $m_1 = 0$ transition was measured according to procedures established in the literature. Spectra recorded from the same sample over increasing microwave power were normalized to the same spin concentration at $P_0 = 1.30$ mW. The resulting saturation curves for different samples were then scaled to the same amplitude at $P_0$.

3.4 Results

The spin-labeled EPR spectra of 3β-doxyl-5α-cholestanol (CLS or cholestane) incorporated into DMPC/DHPC/cholesterol/phospholipid bilayer discs are displayed in Figure 3.1 as a function of the concentration of Tm$^{3+}$ at 318 K. At 0% and 5% molar Tm$^{3+}$ with respect to DMPC, the EPR spectra (Figures 3.1(A) and (B)) indicate that the bicelle discs are not aligned with respect to the direction of the static magnetic field. At 10% molar Tm$^{3+}$ with respect to DMPC, the hyperfine splitting observed in the EPR spectrum of Figure 3.1(C) is significantly reduced when compared to the EPR spectra in Figures 3.1(A) and (B). CLS has been shown to align with its long axis parallel to the long axis of the phospholipids and undergoes rapid rotational motion ($R_{||}$) about this axis. The nitroxide y-axis is nearly parallel to the long axis of the steroid-derived spin probe. Thus, the line shape and reduced hyperfine splitting observed in Figure 3.1(C) indicate macroscopic orientation of the membrane bilayers such that their normals (and hence y-axis of associated cholestane spin labels) are nearly parallel with the static magnetic field. Additional DMPC/DHPC/cholesterol/Tm$^{3+}$ bicelle EPR spectra were collected up to 25% molar Tm$^{3+}$ with respect to DMPC (data not shown).
**Figure 3.1.** EPR spectra of a cholestane spin label incorporated into oriented and randomly dispersed DMPC/DHPC/cholesterol phospholipid bilayers at concentrations of 0%, 5%, and 10% molar Tm\(^{3+}\) with respect to DMPC. Spectra were taken at 318 K and a static magnetic field strength of 0.64 T was used to align the samples. (A) 0% Tm\(^{3+}\) spectrum was typical of unoriented DMPC/DHPC phospholipid bilayers. (B) 5% Tm\(^{3+}\) spectrum showed some signs of parallel phospholipid bilayer alignment but remained mostly unaligned. (C) 10% Tm\(^{3+}\) spectrum revealed a fully aligned phospholipid bilayer system that was aligned such that the bilayer normal was collinear with the static magnetic field.
Similarly, Figure 3.2 displays the EPR spectra of CLS incorporated into DMPC/DHPC/cholesterol phospholipid bilayers as a function of the concentration of Dy$^{3+}$. The EPR spectrum displayed in Figure 3.2(A) with 0% Dy$^{3+}$ indicates that phospholipid bilayer arrays are randomly dispersed with respect to the direction of the static magnetic field. At the higher magnetic fields used in NMR studies, the phospholipid bilayered discs of this composition were aligned such that the membrane normal was perpendicular to the direction of the static magnetic field. At 0.3 T (X-band, $g = 2$ resonance), additional lanthanide alignment reagents were needed to align the phospholipid bilayers.$^{38}$

Figure 3.3 summarizes the results from Figures 3.1 and 3.2 by showing the EPR spectroscopic data from a series of Tm$^{3+}$ and Dy$^{3+}$ bicelle titration experiments. The hyperfine splitting from various DMPC/DHPC bicelle samples was plotted versus the concentration of Tm$^{3+}$ and Dy$^{3+}$ with respect to DMPC.

In order to probe the effect that different lanthanide ions have on the electron spin-lattice relaxation rate of the cholestane spin label, a series of CW-EPR power saturation experiments were carried out on cholestane spin-labeled phospholipid bilayer samples in the presence and absence of oxygen at 318 K. Figure 3.4 shows a series of power saturation curves for DMPC/DHPC/cholestan e bicelle samples with Gd$^{3+}$, Dy$^{3+}$, and Tm$^{3+}$ with and without O$_2$ compared to the control (no lanthanide, O$_2$ depleted). For all of the DMPC/DHPC samples, the normalized peak-to-peak amplitude of the $m_I = 0$ transition was measured.$^{33}$ The power saturation curves for the various DMPC/DHPC samples are displayed as down triangles for the control sample, up triangles for the Gd$^{3+}$ samples, squares for the Dy$^{3+}$ samples, and circles for the Tm$^{3+}$ samples. Saturation curves from samples prepared in the absence of O$_2$ are displayed as closed-face symbols, while samples prepared in the presence of O$_2$ are displayed as open-faced symbols.
Figure 3.2. EPR spectra of a cholestane spin label incorporated into oriented and randomly dispersed DMPC/DHPC/cholesterol phospholipid bilayers at concentrations of 0%, 5%, and 10% molar Dy$^{3+}$ with respect to DMPC. Spectra were taken at 318 K and a static magnetic field strength of 0.64 T was used to align the samples. (A) 0% Dy$^{3+}$ spectrum was typical of unoriented DMPC/DHPC phospholipid bilayers. (B) 5% Dy$^{3+}$ spectrum indicated that the phospholipid bilayers were aligned such that the bilayer normal was perpendicular to the static magnetic field. (C) 10% Dy$^{3+}$ spectrum indicated that the phospholipid bilayers were aligned such that the bilayer normal was perpendicular to the static magnetic field.
Figure 3.3. Diagram showing the dependence of the hyperfine splitting on the concentration of Tm$^{3+}$ and Dy$^{3+}$ lanthanide ions added to DMPC/DHPC phospholipid bilayers. The squares represent the hyperfine splitting values measured from EPR spectra with Dy$^{3+}$ and the circles are the resultant hyperfine splittings measured from EPR spectra with Tm$^{3+}$. 
Figure 3.4. EPR power saturation study of cholestane incorporated into DMPC/DHPC phospholipid bilayer samples with various lanthanide ions at 20% (mol% lanthanide to DMPC). Samples prepared with oxygen are shown with open-faced symbols, oxygen-depleted samples are shown with closed-faced symbols. The signal amplitude was measured from the peak-to-peak amplitude of the $m_i = 0$ center line.
For comparison with a DMPC/DHPC bicelle model membrane system, similar CW-EPR power saturation studies were conducted on DHPC micelle samples. Figure 3.5 shows a series of power saturation curves for DHPC micelle samples with and without oxygen and various different lanthanide ions (Gd$^{3+}$, Dy$^{3+}$, and Tm$^{3+}$). DHPC/cholestanate samples are displayed as down triangles for the control sample, up triangles for the Gd$^{3+}$ samples, squares for the Dy$^{3+}$ samples, and circles for Tm$^{3+}$ samples. The power saturation data arising from samples prepared in the presence of O$_2$ are shown as open-faced symbols; whereas, the curves arising from samples prepared in the absence of O$_2$ (O$_2$ depleted) are displayed as closed-faced symbols. As with the DMPC/DHPC samples, varying degrees of spin-lattice relaxation enhancement were observed for all of the lanthanide samples tested. The power saturation results for the DHPC micelle samples were comparable to the results from the DMPC/DHPC bicelle samples.

The saturation curves were then fitted to the function

$A(P) = IP^{1/2}[1+(2^{1/ε}-1)P/P_{1/2}]^{ε}$

where $A$ is the peak-to-peak amplitude, $P$ is microwave power, $I$ is a scaling factor, $ε$ is a measure of the inhomogeneity of the sample, and $P_{1/2}$ is the power at half saturation. $ΔP_{1/2}$ values for the bicelle samples were found by subtracting the $P_{1/2}$ of the control (DMPC/DHPC sample with no lanthanide and no O$_2$) from the $P_{1/2}$ of the sample. Table 1 shows the $ΔP_{1/2}$ values ($P_{1/2}(\text{sample}) - P_{1/2}(\text{control})$) for various lanthanide ions with (shown as O$_2$) and without O$_2$ (shown as N$_2$). The $ΔP_{1/2}$ values for bicelle samples containing Gd$^{3+}$ could not be determined due to the lack of saturation observed. The lanthanide samples showed varying degrees of $1/T_1$ enhancement as indicated by the increasing $ΔP_{1/2}$ values, which was further enhanced by the addition of O$_2$.

### 3.5 Discussion

As illustrated in Figure 3.1(C), magnetically aligned phospholipid bilayer samples doped with Tm$^{3+}$ align such that the normal of the membrane bilayer is collinear with the direction of the static magnetic field. The preferred orientation of the bicelle disc director $n$ in the presence of a magnetic field is dependent upon the sign of $Δ\chi$ of the bicelle (the
Figure 3.5. EPR power saturation study of cholestane incorporated into DHPC micelles with various lanthanide ions at 20% (mol% lanthanide to DHPC). Samples prepared with oxygen are shown with open-faced symbols, oxygen-depleted samples are shown with closed-faced symbols. The signal amplitude was measured from the peak-to-peak amplitude of the $m_1 = 0$ center line.
TABLE 3.1

Power saturation parameters for various lanthanides ($\Delta P_{1/2}$)

<table>
<thead>
<tr>
<th>Lanthanide</th>
<th>DHPC</th>
<th>DMPC/DHPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N₂)</td>
<td>(O₂)</td>
</tr>
<tr>
<td>Dy³⁺</td>
<td>101</td>
<td>284</td>
</tr>
<tr>
<td>Er³⁺</td>
<td>55.6</td>
<td>235</td>
</tr>
<tr>
<td>Yb³⁺</td>
<td>20.4</td>
<td>192</td>
</tr>
<tr>
<td>Tm³⁺</td>
<td>19.5</td>
<td>224</td>
</tr>
</tbody>
</table>

The $\Delta P_{1/2}$ values of both DMCP/DHPC bicelle and DHPC micelle samples with various lanthanides at 20% (mol% lanthanide to DMPC for bicelles, and DHPC sample with no lanthanide and no O₂) from the $P_{1/2}$ of the sample. $\Delta P_{1/2}$ values for the micelle samples were found similarly, except a DHPC sample with no lanthanide and no O₂ was used as a control.
magnetic susceptibility anisotropy tensor of the bicelle disc). The sign of $\Delta \chi$ of the bicelle can be changed from negative to positive by adding paramagnetic lanthanide ions (i.e., Tm$^{3+}$, Yb$^{3+}$, Er$^{3+}$, Eu$^{3+}$), which bind to the phosphatidylcholine headgroups.$^{23,34,35}$ The magnitude and sign of $\Delta \chi$ for the bicelle are dependent upon the orientation of the principal magnetic axes of the lanthanide cation with respect to the long molecular axis of the lanthanide–phospholipid complex.$^{36}$ Inspection of the line shape and resultant hyperfine splitting in Figure 3.1 indicates that the bicelle discs are not aligned at this orientation until 10% molar Tm$^{3+}$ with respect to DMPC is added to the bicelle sample. Under identical alignment and magnetic field conditions, the phospholipid bilayer arrays do not fully align at this orientation until 15–20% molar Yb$^{3+}$ with respect to DMPC is added to the bicelle matrix (data not shown). This is not surprising because the Tm$^{3+}$ ion has the largest positive $\Delta \chi$ and should yield optimal alignment at lower concentrations when compared to Eu$^{3+}$, Er$^{3+}$, and Yb$^{3+}$ ions which possess smaller positive $\Delta \chi$ values.$^{23,24}$

At the high magnetic fields typically used in NMR studies (greater than 7.0 T), the sign of $\Delta \chi$ for the bicelle discs is negative (in the absence of lanthanide ions). Thus, in the presence of a static magnetic field the preferred alignment of the bicelle disc director is oriented perpendicular to the applied magnetic field. For low field X-band EPR experiments (approximately 0.64 T), the phospholipid bilayer discs do not fully align at this orientation without additional alignment reagents.$^{37}$ Dy$^{3+}$ ions must be added to the bicelle samples to align them at this orientation (Figures 3.2(B) and (C)).$^{38}$ The magnitude of the overall negative $\Delta \chi$ of the discs is increased by adding Dy$^{3+}$ to the DMPC/DHPC bicelle matrix, which alternatively possesses a large negative $\Delta \chi$.$^{23,24}$ The resultant line shape and hyperfine splitting of the EPR spectra displayed in Figure 3.2 indicate that the phospholipid bilayer arrays are aligned when only 5% molar Dy$^{3+}$ with respect to DMPC is added to the sample.

Figure 3.3 compares the resultant hyperfine splitting of CLS incorporated into DMPC/DHPC phospholipid bilayer arrays with varying Dy$^{3+}$ and Tm$^{3+}$ concentrations. The bicelle samples are almost fully aligned at 7.5% molar Tm$^{3+}$ with respect to DMPC or at 2.5% molar Dy$^{3+}$ with respect to DMPC as observed by the resultant line shape and hyperfine splitting. Complete bicelle disc alignment is observed at 10% molar Tm$^{3+}$ or 5% molar Dy$^{3+}$ with respect to DMPC. The data indicate that it takes approximately 50%
less Dy\(^{3+}\) ions to align the bicelle discs at the perpendicular orientation when compared to Tm\(^{3+}\) ions in the parallel alignment. Less Dy\(^{3+}\) ions (which have a large negative $\Delta \chi$) are needed for alignment because the phospholipid bilayer arrays already possess a negative $\Delta \chi$; whereas a greater amount of Tm\(^{3+}\) ions (which have a smaller $|\Delta \chi|$ than Dy\(^{3+}\)) are needed to flip the bicelle discs by changing the magnitude and sign of $\Delta \chi$ for the bicelle system to a positive value. These results place a threshold on the minimal amount of lanthanide ions that are needed to align the DMPC/DHPC bilayers in the L\(_a\) phase for X-band EPR studies. The phospholipid bilayers in magnetically aligned bicelles are in the L\(_a\) phase at 318 K and have the capacity to incorporate integral membrane proteins.\(^{22-24}\)

Thus, this study will be useful for investigating the structural and dynamical properties of membrane proteins with site-specific spin-labeled EPR studies with the smallest amount of paramagnetic lanthanide alignment reagents as possible to minimize potential protein–lanthanide ion interactions.

For complete and accurate analysis of spin-label EPR spectra collected for the magnetically aligned 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 significant paramagnetic line broadening and complicate detailed analysis of spin-label EPR spectra. We explored this possibility by performing power saturation studies on cholestane spin-labeled phospholipid bilayer samples (DMPC/DHPC) and also micelle samples (DHPC) with a variety of different lanthanide ions with and without oxygen at 318 K (Figures 3.4 and 3.5, and Table 3.1). The addition of lanthanide ions increased the electron spin-lattice relaxation rate of the spin label as shown by the increase in $P_{1/2}$ values. The relaxation enhancement was the greatest for Gd\(^{3+}\), which had a $\Delta P_{1/2}$ at least one order of magnitude larger than the other lanthanides because it was still in the linear region of the curve at all the power levels studied (Figures 3.4 and 3.5). The $1/T_1$ of the spin label was increased by the other lanthanide ions with Dy\(^{3+}\) giving the greatest enhancement followed by Er\(^{3+}\), Yb\(^{3+}\), and Tm\(^{3+}\). The addition of Tm\(^{3+}\) and Yb\(^{3+}\) ions to the bicelle samples caused a minimal change in $1/T_1$ of CLS. In the presence of paramagnetic O\(_2\), the power required to saturate the cholestane spin label increased due to an enhancement of the spin-lattice relaxation rate ($1/T_1$) of the nitrooxide electron.\(^{40-42}\) The
interaction of a nitroxide spin label with a paramagnetic species like 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.\textsuperscript{43} The data presented here suggest that the paramagnetic lanthanide ions used in the bicelle samples interact via an alternative 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.\textsuperscript{35,39}

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.\textsuperscript{42,44–46} The dipolar relaxation mechanism is proportional to $\mu_r^2 T_{1R} / r^6$, where $\mu_r$ and $T_{1R}$ represent the magnetic moment and electron spin-lattice relaxation time of the transition ion probe. Thus, a paramagnetic species like Gd$^{3+}$, which possesses a large magnetic moment and a relatively long spin-lattice relaxation time, significantly alters the relaxation properties of the spin label. However, depending upon the magnitude of $r$, ions with shorter $T_{1R}$ values such as Yb$^{3+}$, Dy$^{3+}$, Ho$^{3+}$, Er$^{3+}$, and Tm$^{3+}$ will have less significant effects on the dipolar relaxation mechanism when compared to Gd$^{3+}$.\textsuperscript{42,46} Gd$^{3+}$ has a $T_{1R}$ value of $1 \times 10^{-8}$ s, whereas Tm$^{3+}$ and Yb$^{3+}$ have much shorter $T_{1R}$ values of approximately $2.8 \times 10^{-13}$ s.\textsuperscript{47,48} Thus, the increased relaxation rates (observed through $\Delta P_{1/2}$ values) of the spin label caused by a particular lanthanide ion are correlated to the $T_{1R}$ times, supporting the dipolar relaxation interaction with the lanthanides being bound to the surface of the bilayer and not coming in direct contact with the spin label. The development of magnetically aligned phospholipid bilayers utilizing lanthanide alignment reagents 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.

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(30) Katsaras, J.; Donaberger, R. L.; Swainson, I. P.; Tennant, D. C.; Tun, Z.; Vold,


CHAPTER 4

Magnetically Aligned Phospholipid Bilayers in Weak Magnetic Fields: Optimization, Mechanism, and Advantages for X-band EPR Studies

4.1 Abstract

Our lab is developing a spin-labeled EPR spectroscopic technique complementary to solid-state NMR studies to study the structure, orientation, and dynamics of uniaxially aligned integral membrane proteins inserted into magnetically aligned discotic phospholipid bilayers, or bicelles. The focus of this study is to optimize and understand the mechanisms involved in the magnetic alignment process of bicelle disks in weak magnetic fields. Developing experimental conditions for optimized magnetic alignment of bicelles in low magnetic fields may prove useful to study the dynamics of membrane proteins and its interactions with lipids, drugs, steroids, signaling events, other proteins, etc. In weak magnetic fields, the magnetic alignment of Tm³⁺-doped bicelle disks was thermodynamically and kinetically very sensitive to experimental conditions. Tm³⁺-doped bicelles were magnetically aligned using the following optimized procedure: the temperature was slowly raised at a rate of 1.9 K/min from an initial temperature being between 298 and 307 K to a final temperature of 318 K in the presence of a static magnetic field of 6300 G. The spin probe 3β-doxyl-5α-cholestan (cholestan) was inserted into the bicelle disks and utilized to monitor bicelle alignment by analyzing the anisotropic hyperfine splitting for the corresponding EPR spectra. The phases of the bicelles were determined using solid-state ²H NMR spectroscopy and compared with the corresponding EPR spectra. Macroscopic alignment commenced in the liquid crystalline nematic phase (307 K), continued to increase upon slowly raising the temperature, and was well-aligned in the liquid crystalline lamellar smectic phase (318 K).

4.2 Introduction

Membrane proteins consist of approximately 1/3 of all the known proteins, but only approximately 0.2% of these proteins (< 30 proteins) have had their structure determined.¹ New biophysical techniques are urgently needed to support, enhance, and add additional knowledge about the structure, orientation in the membrane, and dynamics of integral membrane proteins.
A recently developed model membrane system called bilayered micelles, or bicelles,\textsuperscript{2,3,5} which aligns in the presence of a magnetic field, has great promise in NMR spectroscopic studies of membrane and globular proteins.\textsuperscript{4,6-13} Bicelles consist of a binary mixture of diacyl long-chain and diacyl short-chain phospholipids and water. The diacyl long-chain phospholipids form disk-shaped bilayers and the diacyl short-chain phospholipids pack around the hydrophobic edge of the bilayered disks.\textsuperscript{2} The normal to the bicelle disk is perpendicular to the plane of the bilayered region and is parallel with the director, \textbf{n}. The dimensions of the bicelle disks are dependent on the molar ratio (\textit{q}-ratio) of the long-chain to short-chain phospholipids.\textsuperscript{14} Bicelle disks are stable over a large range of \textit{q}-ratios (0.2–6) and hydration (40–95\%, wt\%).\textsuperscript{15-18} This study used 1,2-dimyrstioyl-\textit{sn}-glycerol phosphatidylcholine (DMPC) and 1,2-dihexanoyl-\textit{sn}-glycerol phosphatidylcholine (DHPC) as the long-chain and short-chain phospholipids, respectively, with a mole ratio of DMPC:DHPC of 3.5 (\textit{q}-ratio) and 75\% hydrated (wt\%).

Magnetically aligned phospholipid bilayers with nitroxide spin-labeled probes incorporated into the bicelle disks can provide a powerful means of obtaining orientational information of the membrane due to the anisotropy of the hyperfine splitting term of the spin probe.\textsuperscript{19-21} The spin probe 3\textbeta- doxyl-5\alpha-cholestan (cholestan or CLS) inserts into the bilayered region of the bicelle disk with its long molecular axis nearly parallel with the normal to the bicelle disks and rapidly rotates about this long molecular axis.\textsuperscript{22-24} Previously, we showed three cholestan spin-labeled EPR spectra when the bicelle disks are randomly dispersed, or uniaxially aligned with the average normal to the bicelle disks being parallel or perpendicular to the magnetic field.\textsuperscript{24} Also, a detailed discussion on the anisotropic hyperfine splitting tensorial values for the cholestan spin label was previously given.\textsuperscript{24} When the bicelle disks are randomly dispersed and motionally averaged, the observed hyperfine splitting, \textit{A}_{\text{obs}}\textsuperscript{, for the cholestan spin-labeled EPR spectra is the average of the hyperfine splitting tensors (i.e., A}_{\text{obs}} = (A_{xx} + A_{yy} + A_{zz})/3) with a theoretical value of 14.5 G. When the bicelle disks magnetically align in the perpendicular alignment, the normal to the bicelle disks and the long molecular axis of cholestan are nearly perpendicular to the static magnetic field. Because cholestan rapidly rotates around the long molecular axis, the observed hyperfine splitting is \textit{A}_{\text{obs}} = (A_{xx} + A_{zz})/2 with a theoretical value of 19 G. Alternatively, when the bicelle disks
magnetically align in the parallel alignment, the normal to the bicelle disks and the long molecular axis of cholestane are parallel to the magnetic field; thus, the y-axis of cholestane is nearly parallel with the magnetic field and $A_{\text{obs}} \approx A_{yy}$. At a temperature of 308 K, the experimental values of $A_{\text{obs}}$ for randomly dispersed, perpendicular-aligned, and parallel-aligned bicelle disks were, respectively, 16.0, 18.1, and 9.8 G.\textsuperscript{24} Deviations from the theoretical hyperfine splitting values are due to the long molecular axis of cholestane not being perfectly parallel to the bicelle normal, and the spin label undergoes a random walk motion perpendicular to the long molecular axis within the limit of a cone.\textsuperscript{22,25} Ten percentage of cholesterol (mol% of cholesterol to DMPC) is added to the bicelle mixture to inhibit this motion. Also, the director of the bicelle disks does not align perfectly in the parallel or perpendicular alignment.\textsuperscript{24}

Bicelle disks do not spontaneously align at either the perpendicular or the parallel alignment when placed into an X-band EPR spectrometer at a temperature corresponding to either the gel or liquid crystalline ($L_a$) phase.\textsuperscript{23,24} Previously, we reported that to magnetically align bicelle disks for X-band EPR spectroscopic studies a lanthanide-doped bicelle sample must be placed into the EPR cavity at a temperature of 298 K, the magnetic field was set to at least 4500 G, and the temperature was slowly raised (~10–15 min) to 318K.\textsuperscript{23} Paramagnetic lanthanide cations (i.e., Tm$^{3+}$ or Dy$^{3+}$) were used as alignment reagents to magnetically align the bicelle disks either in the parallel (Tm$^{3+}$) or perpendicular alignment (Dy$^{3+}$).\textsuperscript{24}

The preferred orientation of the bicelle disk director $\mathbf{n}$ in the presence of a magnetic field is dependent upon the sign of $\Delta\chi_{\text{bicelle}}$ (the magnetic susceptibility anisotropy tensor of the bicelle disk). The magnetic anisotropy tensor is given by the following:

$$\Delta\chi_{\text{bicelle}} = \chi_{\parallel\text{bicelle}} - \chi_{\perp\text{bicelle}}$$

(1)

where $\chi_{\parallel\text{bicelle}}$ and $\chi_{\perp\text{bicelle}}$ are the magnetic susceptibility anisotropy tensors directed, respectively, parallel and perpendicular to the bicelle disk director $\mathbf{n}$. Without added lanthanide cations, the sign of $\Delta\chi_{\text{bicelle}}$ is negative; therefore, in the presence of a magnetic field the preferred alignment of the bicelle disk director is oriented perpendicular to the applied magnetic field. The sign of $\Delta\chi_{\text{bicelle}}$ can be changed from negative to positive by
adding certain types of paramagnetic lanthanide cations (i.e., Tm$^{3+}$, Yb$^{3+}$, Er$^{3+}$, Eu$^{3+}$), which bind to the phosphatidylcholine headgroups. The magnitude and sign of $\Delta \chi_{\text{bicelle}}$ is dependent upon the orientation of the principal magnetic axes of the lanthanide cation with respect to the long molecular axis of the lanthanide–phospholipid complex. Mironov and co-workers and Prosser and co-workers give excellent discussions on the magnetic susceptibility anisotropy of lanthanide-containing liquid crystals and the factors that govern the magnitude and sign of the microscopic and macroscopic magnetic susceptibility anisotropy. The degree of magnetic alignment or whether magnetic alignment of Tm$^{3+}$-doped bicelle disks is thermodynamically or kinetically obtainable under a given set of experimental conditions is dependent upon several factors. The magnitude of the molecular $\Delta \chi$ value of Tm$^{3+}$, the orientation of the long molecular axis of the lanthanide–phospholipid complex with the principal magnetic axes, microscopic and macroscopic disorder effects, the strength of the applied magnetic field, the viscosity of the bicelle system, and hysteresis effects.

Liquid crystalline systems are known to have many phases characterized by different viscosities, hydration, and ordering and motion on the microscopic, mesoscopic, and macroscopic levels which can affect the cooperativity in alignment and reorientation of the system. Therefore, phase and viscosity changes have a bearing on the magnetic alignment process of bicelle disks in an X-band EPR spectrometer. It has been shown that other liquid crystals can magnetically align upon cooling the sample from the isotropic phase to the smectic phase or upon heating from the hexagonal phase to the smectic phase. The phases of phospholipid membranes and the accompanying viscosities, ordering, and phase transition temperatures can be modified upon incorporating integral membrane proteins and other biomolecules into the membrane system and/or changing other membrane components (i.e., salt concentrations, mol% cholesterol, etc.). If these membrane component modifications are incorporated into bicelle disks, it is assumed that the experimental conditions for optimal magnetic alignment of the bicelle system in low magnetic fields will also change. If the magnetic alignment of bicelle disks in low magnetic fields is dependent upon the membrane composition, then developing experimental conditions for optimized magnetic alignment of bicelles in low magnetic fields may prove useful to study the dynamics of membrane...
proteins and its interactions with lipids, drugs, steroids, signaling events, other proteins, etc.

Magnetically aligned bicelle disks could expand current spin-labeling techniques\textsuperscript{42–49} to determine the orientation of a variety of membrane-associated spin-labeled peptides or proteins. For example, a cysteine specific nitrooxide spin-label reagent (MTSSL) can be attached to site-directed cysteine mutations of recombinant and synthesized proteins and peptides via formation of a disulfide bridge between the spin label and the cysteine residue(s)\textsuperscript{42,45,48} or by incorporating TOAC or POAC spin-labeled amino acids into the sequence of synthesized peptides.\textsuperscript{50,51} The orientation of a spin-labeled protein that has been incorporated into bicelles with respect to the membrane could be determined by measuring the observed hyperfine splitting from the spin-labeled EPR spectra when the bicelle disks are in a known orientation with respect to the magnetic field.\textsuperscript{42,43,46,49,51} Our lab is developing new spin-labeled EPR spectroscopic techniques to complement solid-state NMR spectroscopic techniques using bicelles as model membrane systems to study the structure, dynamics and orientation of membrane protein systems.\textsuperscript{23,24,52–55} Spin-label EPR spectroscopy has a greater sensitivity when compared to solid-state NMR spectroscopy because the magnetic moment of an electron is orders of magnitude greater than the magnetic moment of NMR active nuclei. An increase in sensitivity allows for shorter data acquisition times and smaller amounts of spin-labeled probes and peptides are needed to obtain spin-labeled EPR spectra with high signal-to-noise ratios.

The focus of this study is three fold. First, investigate the optimized conditions for magnetically aligning lanthanide-doped bicelle disks in weak magnetic fields using spin-labeled X-band EPR spectroscopy. More specifically, investigate what is the maximum initial temperature, the minimum final temperature, and the minimum time over which the sample temperature is raised from the initial temperature to the final temperature in the presence of a static magnetic field that would result in well-aligned lanthanide-doped bicelle disks. Second, investigate the temperatures at which different phases are detected using solid-state NMR spectroscopy. Third, compare and correlate the optimized conditions for magnetic alignment with the observed phases and propose a mechanism for the magnetic alignment process based on the knowledge of various factors that possibly govern the kinetic and thermodynamic properties of the system over the range of
temperatures used during the procedure for magnetic alignment. The results of this study will give a better understanding of the mechanisms involved in the magnetic alignment process of lanthanide-doped bicelle systems at low magnetic fields. Also, understanding the mechanism of this magnetic alignment process and the factors that affect the degree and ease of alignment may aid in addressing possible future problems when developing an optimized procedure for magnetically aligning bicelle disks with incorporated integral membrane proteins.

4.3 Experimental

Materials. 1,2-Dihexanoyl-sn-glycerol phosphatidylcholine (DHPC), 1,2-dimyristoyl-sn-glycerol phosphatidylcholine (DMPC), deuterated 1,2-dimyristoyl-sn-glycerol phosphatidylcholine (DMPCd54), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000] (PEG 2000 PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Thulium (III) chloride hexahydrate, 3β-doxyl-5α-cholestan (cholestan or CLS), and Hepes buffer were obtained from Sigma–Aldrich (St. Louis, MO). Cholesterol was obtained from Avocado Research Chemicals (Ward Hill, MA).

Sample preparation for EPR studies. Stock solutions of DMPC, DHPC, PEG 2000 PE, CLS, and cholesterol were prepared in separate amber colored bottles, dissolving each compound in chloroform, and storing the stock solutions at −20 °C. Aqueous solutions of Hepes buffer and Tm³⁺ were prepared fresh on the day of sample preparation and were adjusted to pH 7. All aqueous solutions were prepared with NANOpure filtered water.

Bicelle samples with a q-ratio of 3.5 and 75% (wt%) hydrated were prepared by dispensing the appropriate volumes of the DMPC, DHPC, PEG 2000 PE, cholesterol, and CLS stock solutions into a 25- or 35-ml pear-shaped flask having molar ratios of 3.5/1.0/0.035/0.35/0.0056, respectively. The lipids were rotovapped at room temperature and vacuum desiccated overnight. One hundred mM Hepes buffer was added to the pear-shaped flask so the amount of lipid in the sample was 25% (wt%). The samples were chilled in an ice bath and vortexed until all of the lipids were solublized. The samples
were intermittently chilled in an ice bath during vortexing to maintain fluidity in the samples. The samples were sonicated in a Fisher Scientific FS30 bath sonicator (Florence, KY) for 30 min with the heater turned off and ice added to the bath. The samples were subjected to at least three freeze–thaw cycles (frozen at 77 K and thawed at 318 K). The samples were cooled in an ice bath and Tm$^{3+}$ (aq) was added to some samples so that the amount of Tm$^{3+}$ was 20% (mol% of Tm$^{3+}$ to DMPC). The Tm$^{3+}$-doped samples were gently mixed by tilting and slowly rotating the flask for a few minutes until the sample looked homogenous.

The bicelle samples were drawn into 1 mm ID capillary tubes by attaching a syringe to one end of the tube. Both ends of the tube were sealed off with Critoseal purchased from Fisher Scientific (Florence, KY). The capillary tube was placed into a standard quartz EPR tube (707-SQ-250M) purchased from Wilmad Glass (Buena, NJ) 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 K). Each cholestane spin-labeled EPR spectrum was acquired by taking a 42-s field-swept scan with the center field set to 3350 G, a sweep width of 100 G, a microwave frequency of 9.39 GHz, the modulation frequency was set to 100 kHz, a modulation amplitude of 1.0 G, and the microwave power set to 2.5mW.

**Solid-state $^2$H NMR studies.** The sample composition was the same as that for the EPR studies except cholestane was omitted from the sample and DMPC$_{d54}$ (8% molar ratio of DMPC$_{d54}$ to DMPC) was added as an isotopic label. Also, deuterium-depleted water purchased from Isotec (Miamisburg, OH) was used to make the aqueous solutions. Samples were loaded into a NMR flat-bottom tube with an o.d. of 5 mm purchased from Wilmad Glass (Buena, NJ) using a pipetman.

Samples were inserted into a static double-tuned 5-mm round-coil solid-state NMR probe purchased from Doty Scientific (Columbia, SC). Solid-state $^2$H NMR spectra were recorded using a quadecho pulse sequence (4-$\mu$s 90° pulses, 45-$\mu$s delay, 5.12-ms acquisition time, 1024 scans) at a $^2$H resonance frequency of 46.07 MHz. Solid-state $^2$H NMR experiments were carried out on a modified Bruker AVANCE 7.05 T narrow bore
300/54 magnet configured to conduct high-power solid-state NMR studies. The sample was allowed to sit in the spectrometer for approximately 10 min to ensure equilibration before obtaining a spectrum.

**EPR and NMR data processing.** Processing of all cholestane EPR spectra and generated graphs were performed using the Igor software package from Wavemetrics (Lake Oswego, OR) installed on a 300 MHz G3 Macintosh computer. NMR data were processed on the same computer described above using the Igor software package described above and MacNuts software from Acorn NMR (Livermore, CA).

**4.4 Results**

In this study, the general outline of the procedure used to magnetically align Tm$^{3+}$-doped bicelle disks has been discussed in our previous study$^{23}$ and will be called the magnetic alignment procedure. A magnetic alignment procedure consists of slowly raising the sample temperature from an initial temperature to a final temperature in the presence of an applied magnetic field resulting in well-aligned bicelle disks. The time to raise the sample temperature will be called the temperature-ramping time. The magnetic alignment process refers to the thermodynamic and kinetic mechanisms affecting the magnetic alignment of bicelle disks in the presence of a static magnetic field.

**Time dependence of the magnetic alignment procedure.** In our previous work, we showed that Tm$^{3+}$-doped bicelle disks magnetically aligned in the presence of a static magnetic field of 6300 G when the magnetic alignment procedure was performed on the sample with a temperature-ramping time of approximately 15 min with the initial and final temperatures being 298 and 318 K, respectively.$^{23}$ However, we did not investigate the minimum required temperature-ramping time over which Tm$^{3+}$-doped bicelle disks magnetically align. In Figure 4.1, the minimum temperature-ramping time required to obtain
**Figure 4.1.** A stacked plot of normalized cholestane spin-labeled EPR spectra of Tm$^{3+}$-doped bicelle disks showing the time dependence of the macroscopic magnetic alignment procedure. The bicelle sample composition consisted of DMPC/DHPC/cholesterol/PEG 2000 PE/cholestane/Tm$^{3+}$ in molar ratios of 3.5/1.0/0.35/0.035/0.0056/0.70 in 100mM Hepes buffer, pH 7. The magnetic alignment procedure was performed several times on the bicelle sample and consisted of the following steps. The sample temperature was slowly raised from 298 to 318K in the EPR cavity in the presence of a static magnetic field set to 6300 G. The time it took to raise the sample temperature from the initial to the final temperature (temperature-ramping time) was varied and was either < 5, 7, 11, or 14 min. The corresponding EPR spectra are denoted according to this temperature-ramping time. All EPR spectra were gathered at a final temperature of 318 K.
well-aligned Tm$^{3+}$-doped bicelle disks was investigated by performing the magnetic alignment procedure on a Tm$^{3+}$-doped bicelle sample several times with the initial and final temperatures being 298 and 318 K, respectively, and the temperature-ramping time being varied in length (i.e., < 5, 7, 11, and 14 min).

Cholestane spin-labeled EPR bicelle spectra were obtained at 318 K and are denoted according to the temperature-ramping time used and the spectra are displayed in Figure 4.1. The hyperfine splitting was measured for each EPR spectrum shown in Figure 4.1 (measured between the down-field and center peaks). The hyperfine splittings range from 15.5 to 10.6 G and generally decreased as the temperature-ramping time increased from < 5 to 11 min. The cholestane spin-labeled < 5 min EPR spectrum shows more than one spectral component arising from partial bicelle alignment and randomly dispersed bicelle disks. The presence of partial bicelle alignment and randomly dispersed bicelle disks in the < 5 min EPR spectrum is also supported by its broader linewidth as compared to the narrower linewidth of the 11 min EPR spectrum. The hyperfine splitting of all spectral components in the < 5 min EPR spectrum were approximately 12.6 – 15.5 G which corresponds to a mixture of partially oriented and randomly dispersed bicelle disks. The 7 min EPR spectrum has a lineshape and linewidth that suggests a more homogenous bicelle disk alignment as compared to the lineshape and linewidth of the < 5 min EPR spectrum. The 7 min EPR spectrum resulted in partially aligned bicelle disks in the parallel alignment with an average hyperfine splitting of 13.1 G. The argument for partial alignment is that a hyperfine splitting of 13.1 G is significantly less than the experimentally obtained hyperfine splitting from randomly dispersed bicelle disks at 318 K (~16.3 G, see Figure 4.3), but significantly larger than that obtained from bicelle disks oriented in the parallel alignment at 318K (10.6 G for the 11 min EPR spectrum). For the 11 min EPR spectrum, the lineshape of the spectrum and the hyperfine splitting of 10.6 G is typical for cholestane spin probes incorporated into bicelle disks oriented with the director parallel to the static magnetic field. Temperature-ramping times greater than 11 min resulted in EPR spectra with hyperfine splittings and lineshapes similar to the 11 min EPR spectrum. It was determined that the temperature-ramping time of 11 min was optimal for fully aligning the bicelle disks in the parallel alignment in the shortest time. The rate the temperature was increased (temperature-ramping rate) for the 11 min EPR
spectrum was approximately 1.9 K/min.

Optimization of the initial and final temperatures of the magnetic alignment procedure. Although we have previously shown that it was necessary to raise the temperature to induce magnetic alignment of the bicelle disks in weak magnetic fields, we did not have a reasonable explanation for the mechanism involved in this magnetic alignment process.\textsuperscript{23} It was hypothesized that a magnetically induced phase or a pretransition phase occurred between the gel phase (298 K) and liquid crystalline phase (318 K) that allowed the Tm\textsuperscript{3+}-doped DMPC/DHPC bicelle disks to magnetically align. If there is a magnetically induced phase that is critical to the magnetic alignment process, then the optimized initial and final temperatures of the magnetic alignment procedure must at least span this magnetically induced phase and possibly one or more other phases of our bicelle system. Also, temperatures close to the optimized initial and final temperatures may coincide with a phase transition(s) that occur(s) for our bicelle system. The purpose of the following experiments was to optimize the initial and final temperatures of the magnetic alignment procedure used to obtain the 11 min EPR spectrum in Figure 4.1.

To determine the maximum initial temperature that results in well-aligned bicelle disks, the magnetic alignment procedure was performed several times on a Tm\textsuperscript{3+}-doped bicelle sample with an initial temperature between 298 and 318 K, the final temperature was 318 K, and the temperature-ramping time was 11 min. After performing the magnetic alignment procedure a cholestane spin-labeled EPR spectrum was obtained at 318 K and denoted according to the corresponding initial temperature employed.

Figure 4.2 shows a select few of the spin-labeled EPR spectra from the experiment described above. All experiments performed using the magnetic alignment procedure with an initial temperature between 298 and 307 K were identical in their hyperfine splitting (~10.6 G) and lineshape as the 11 min EPR spectrum in Figure 4.1 and is typical of cholestane spin-labeled EPR spectra for parallel-aligned bicelle disks at 318 K.\textsuperscript{23} Experiments with initial temperatures > 307 K have spin-labeled EPR spectra with a hyperfine splitting that increased as the initial temperature was set at increasingly higher temperatures between 308 and 318 K and approached a hyperfine splitting of approximately 13.7 G. Subsequently, peak intensities for the down-field peak became
Figure 4.2. A stacked plot of normalized cholestane spin-labeled EPR spectra of Tm$^{3+}$-doped bicelle disks showing that the magnetic alignment process of the Tm$^{3+}$-doped bicelle sample is dependent upon the initial temperature of the magnetic alignment procedure. The magnetic alignment procedure was performed several times on a Tm$^{3+}$-doped bicelle sample (same sample composition as in Figure 4.1). The initial temperature of the magnetic alignment procedure was varied between 298 and 318 K and the final temperature was 318 K. All experiments were performed with a temperature-ramping time of 11 min to allow sufficient time for the reorientation of the bicelle disks during the magnetic alignment procedure. All EPR spectra were taken at the final temperature of 318 K and are denoted according to the initial temperature of the magnetic alignment procedure used.
weaker relative to the center peak and linewidths became broader. This suggests that experiments with a magnetic alignment procedure performed at initial temperatures between 308 and 318 K were not fully capable of magnetically inducing complete parallel alignment of the bicelle disks. Therefore, the maximum initial temperature resulting in aligned bicelle disks was 307 K and may signify a magnetically inducible phase or phase transition near 307 K.

Figure 4.3 shows a more convenient way of looking at the results of the EPR spectra obtained in Figure 4.2 and compares it to parallel studies on randomly dispersed bicelle disks. For each cholestane spin-labeled EPR spectrum of Tm$^{3+}$-doped bicelle disks (Figure 4.2) the hyperfine splitting was measured and plotted as a function of the corresponding initial temperature employed (solid diamonds). Cholestane spin-labeled EPR spectra were acquired for two samples of randomly dispersed bicelle disks obtained by the following two methods. In the first method (open-faced diamonds), a Tm$^{3+}$-doped bicelle sample (same composition as described in Figure 4.1) was subjected several times to the same magnetic alignment procedure described in Figure 4.2, but in the absence of a static magnetic field. In the second method (solid circles), the same magnetic alignment procedure described in Figure 4.2 was performed several times on a bicelle sample with the same sample composition as described in Figure 4.1 except that the alignment reagent Tm$^{3+}$ was omitted. All EPR spectra of randomly dispersed bicelle disks were obtained at the final temperature of 318 K, the hyperfine splitting for each EPR spectrum was measured and plotted in Figure 4.3 as a function of the corresponding initial temperature used in the magnetic alignment procedure (open-faced diamonds and solid circles).

Figure 4.3 shows there was little variation in the hyperfine splitting (~10.6 G) and lineshape for the magnetic alignment procedure performed on Tm$^{3+}$-doped bicelle disks (solid diamonds) with initial temperatures between 298 and 307 K, which was to be expected. However, Figure 4.3 shows more clearly than Figure 4.2 the abrupt increase in the hyperfine splitting for the magnetic alignment procedure performed on Tm$^{3+}$-doped bicelle disks (solid diamonds) with initial temperatures between 307 and 308 K and approached a maximum hyperfine splitting of 13.7 G at an initial temperature of 318 K. Although this increase in hyperfine splittings approaches the theoretical isotropic
Figure 4.3. A graph showing the initial temperature dependence on the magnetic alignment process of Tm$^{3+}$-doped bicelle disks using the magnetic alignment procedure described in Figure 4.2. The hyperfine splitting of the cholestane spin-labeled EPR spectra from Figure 4.2 (measured between the down-field and center peaks) was plotted as a function of the corresponding initial temperature of the magnetic alignment procedure (solid diamonds). Similar plots were obtained for randomly dispersed bicelle disks obtained by the following two methods. In the first method (open-faced diamonds), a Tm$^{3+}$-doped bicelle sample (same composition as described in Figure 4.1) was subjected several times to the same magnetic alignment procedure described in Figure 4.2, but in the absence of a magnetic field. In the second method (solid circles), the same magnetic alignment procedure described in Figure 4.2 was performed several times on a bicelle sample with the same sample composition as described in Figure 4.1 except the alignment reagent Tm$^{3+}$ was omitted. All EPR spectra of randomly dispersed bicelle disks were obtained at the final temperature of 318 K and the hyperfine splitting of each EPR spectrum was measured and plotted as a function of the initial temperature used (open-faced diamonds and solid circles).
hyperfine splitting of 14.5 G, the increase in hyperfine splittings arises from partial alignment of the bicelle disks and not from increased isotropic motion when compared to a sample of randomly dispersed bicelle disks at 318 K. For the randomly dispersed bicelle disks (solid circles and open-faced diamonds), the hyperfine splitting was approximately 16.3 G and was fairly constant for all experiments, which, from our previous studies, is typical for EPR spectra of randomly dispersed bicelle disks collected at a temperature of 318 K. The deviation of the experimentally obtained hyperfine splitting for randomly dispersed bicelle disks (~16.3 G) from the theoretically obtained isotropic value of 14.5 G arises because the isotropic motion of the cholestane spin label and the motion of the bicelle disks at 318 K is not rapid enough on the X-band EPR time scale to completely average out the anisotropic components of the hyperfine splitting. In fact, it was previously shown that the experimental hyperfine splitting for randomly dispersed and magnetically aligned bicelle disks approached the theoretical isotropic value in a linear relationship upon increasing the sample temperature.

To determine the minimum final temperature that results in well-aligned bicelle disks, the magnetic alignment procedure was performed several times on a Tm$^{3+}$-doped bicelle sample with the temperature being raised slowly (1.9 K/min) from an initial temperature of 298 K to a variable final temperature between 298 and 318 K. Cholestane spin-labeled EPR spectra were obtained at the corresponding final temperature and the hyperfine splitting for each EPR spectrum was plotted in Figure 4.4 as a function of that final temperature (open-faced circles). A few of these EPR spectra (i.e., 306, 310, 314, and 318 K) are shown in Figure 4.4 (inset). As a control, cholestane spin-labeled EPR spectra were acquired for a sample of randomly dispersed bicelle disks. The sample composition of the randomly dispersed bicelle disks was the same as the Tm$^{3+}$-doped bicelle sample (open-faced circles) except Tm$^{3+}$ was omitted and obtained by the following method. The sample was inserted into the EPR cavity at a static temperature in the absence of a magnetic field (no magnetic alignment procedure was performed). Cholestane spin-labeled EPR spectra were obtained at the respective static final temperatures. The hyperfine splitting for each EPR spectrum is plotted in Figure 4.4 as a function of the temperature at which the spectra were acquired (solid circles). For the cholestane
Figure 4.4. A graph showing the final temperature dependence on the magnetic alignment process of Tm$^{3+}$-doped bicelle disks. The magnetic alignment procedure was performed several times on a Tm$^{3+}$-doped bicelle sample (same sample composition as described in Figure 4.1). The initial temperature of the magnetic alignment procedure was 298 K and the final temperature was varied between a temperature of 298 and 318 K (open-faced circles). The temperature-ramping rate was 1.9 K/ min for all experiments. Cholestane spin-labeled EPR spectra were collected at the corresponding final temperature of the magnetic alignment procedure used and denoted according to this final temperature. The hyperfine splitting was measured for each EPR spectrum in the same manner described in Figure 4.3 and plotted as a function of the corresponding final temperature of the magnetic alignment procedure used (open-faced circles). The graph also shows results for randomly dispersed bicelle disks (solid circles). The sample composition of the randomly dispersed bicelle disks was the same as in Figure 4.1 except the alignment reagent Tm$^{3+}$ was omitted.
spin-labeled EPR spectra of the randomly dispersed bicelle disks (solid circles) the hyperfine splitting decreased in a linear relationship as the static temperature was set at increasing temperatures, which was expected from our previous studies on the temperature-dependent hyperfine splitting for randomly dispersed bicelle disks. For the magnetic alignment procedure performed on Tm\(^{3+}\)-doped bicelle disks with a final temperature between 298 and 306 K (open-faced circles) the average hyperfine splitting of these EPR spectra was approximately 17.0 G. This hyperfine splitting is similar to randomly dispersed bicelle disks at a temperature between 298 and 306 K (solid circles) and agrees with the extrapolation of our previous work for the temperature-dependent hyperfine splitting curve for randomly dispersed bicelle disks at these same temperatures. Combining these results with the results in Figures 4.2 and 4.3 may suggest that the magnetic alignment process was independent of the phase(s) or phase transition(s) present at temperatures between 298 and 306 K. At the final temperatures of 310 and 314 K the corresponding cholestane spin-labeled EPR spectra (Figure 4.4, inset) show the presence of coexisting randomly dispersed and partially aligned bicelle disks. This suggest that the bicelle disks were beginning to align between a temperature of 306 and 310 K and the intensity of the spectral component associated with the partially aligned bicelle disks increased as the final temperature increased (Figure 4.4, inset). At a final temperature of 318 K the cholestane spin-labeled EPR spectrum (Figure 4.4, inset) had a hyperfine splitting of 10.7 G (open-faced circles) and had no detectable coexisting spectral components in the corresponding EPR spectra (Figure 4, inset), which is in agreement of well-aligned Tm\(^{3+}\)-doped bicelle disks in the parallel alignment at a temperature of 318 K (Figures 1–3). Therefore, it was determined that the minimum final temperature of the optimized magnetic alignment procedure was 318 K.

**Determination of the phase changes for Tm\(^{3+}\)-doped bicelle disks using solid-state \(^2\)H NMR spectroscopy.** Previously, Mironov and co-workers\(^{29}\) stated that for some liquid crystalline systems the nematic phase was much easier to magnetically align than the smectic phase. This argument was used to explain the results of the magnetic alignment behavior for their lanthanide-containing metallomesogens.\(^{29}\) Mironov and co-workers\(^{29,32}\) further stated that many liquid crystalline substances magnetically align when the samples are cooled while undergoing phase transitions from the isotropic phase →
nematic phase → smectic phase. This magnetic alignment process in many cases does not occur if the sample is heated to the smectic phase due to hysteresis effects.\textsuperscript{29,32} Firestone and co-workers\textsuperscript{34} showed that their liquid crystalline system magnetically aligned at a low temperature corresponding to a hexagonal phase and that it was necessary to raise the temperature slowly from the hexagonal phase to a highly viscous lamellar phase which “locked in” the macroscopic alignment. A $^2$H NMR study\textsuperscript{31} and a neutron diffraction study\textsuperscript{35} observed a lanthanide-induced nematic-to-smectic phase transition for parallel-aligned Tm$^{3+}$-doped bicelle disks.

Figure 4.5 shows several solid-state $^2$H NMR spectra of a Tm$^{3+}$-doped bicelle sample containing a small amount of DMPC$_{d54}$ taken at various static temperatures from 298 to 323 K. The sample was allowed to thermally equilibrate at these various static temperatures for 10 min before a $^2$H NMR spectrum was acquired. The solid-state $^2$H NMR spectra taken between 298 and 302 K exhibit an isotropic phase (only the spectra 298 K is shown in Figure 4.5), which has been described as either rapidly tumbling bicelle disks accompanied by higher fluidity of the sample or a mixed micelle phase.\textsuperscript{18} Recent studies of the morphology of bicelle phases using pulsed field gradient NMR spectroscopy (PFG NMR) and small angular neutron scattering (SANS) techniques support the rapidly tumbling-bicelle-disks model.\textsuperscript{15,17} Solid-state $^2$H NMR spectra taken at temperatures between 303 and 305 K showed broad unresolved peaks indicative of randomly dispersed bicelle disks. This powder-like spectrum at a temperature of 303 K marks the gel-to-liquid crystalline phase transition for our bicelle system but was not stable in the L$_a$ phase at these temperatures. This main phase transition temperature is greater than that observed for pure DMPC without added Tm$^{3+}$,\textsuperscript{28} which may be explained by the Tm$^{3+}$ cations invoking greater order and packing of the phospholipid acyl chains.\textsuperscript{28,31} $^2$H NMR spectra taken at temperatures between 307 and 311 K exhibited well-resolved peaks and are in good agreement with parallel-aligned bicelle disks in the nematic L$_a$ phase. $^2$H NMR spectra taken at a temperature of 313 K or above showed increased resolution and order and was ascribed by Prosser and co-workers\textsuperscript{31} and Katsaras and co-workers\textsuperscript{35} as the formation of a lanthanide-induced lamellar L$_a$ smectic phase.
Figure 4.5. Normalized solid-state \(^2\)H NMR spectra of a Tm\(^{3+}\)-doped bicelle sample showing the various temperature-dependent phases of the Tm\(^{3+}\)-doped bicelle system. The sample composition was the same as in Figure 4.1 except cholestane is omitted and the isotopic label DMPC\(_{d54}\) was added (8% DMPC\(_{d54}\)/DMPC molar ratio). \(^2\)H NMR spectra were taken at various static temperatures in a resonant frequency of 46.07 MHz. The temperature at which each spectrum was taken is noted on the left of the spectrum.
Magnetic alignment of the smectic phase near the isotropic phase. Prosser and co-workers\textsuperscript{31} showed that a lanthanide-induced lamellar L\textsubscript{α} smectic phase could be cooled slowly to a temperature close to the isotropic phase without the recovery of the L\textsubscript{α} nematic phase and retain its high degree of order and alignment. If a smectic phase had formed for the experiments described in Figures 4.2–4.4, then it may be possible to cool an aligned bicelle sample in the X-band EPR spectrometer near the isotropic phase (i.e., ~303 K, Figure 4.5) and retain its macroscopic alignment. Also, it is important to note that a temperature of 303 K was below the temperature at which bicelle alignment commenced (i.e., ~307 K, Figures 4.4 and 4.5). Therefore, the magnetic alignment procedure was performed on a Tm\textsuperscript{3+}-doped bicelle sample in the X-band EPR spectrometer with the initial and final temperatures being, respectively, 298 and 318 K. The temperature-ramping rate was 1.9 K/min. After the final temperature was reached and the sample equilibrated for 10 min, a cholestane spin-labeled EPR spectrum was taken at 318 K (spectrum not shown) to check that the sample was well aligned. Then the temperature was lowered slowly to a temperature of 304 K in the presence of a magnetic field of 6300 G and making sure the temperature did not go below 304 K. After the sample temperature reached 304 K, a cholestane spin-labeled EPR spectrum was taken every 5 min and the lineshape and hyperfine splitting of each EPR spectrum was qualitatively monitored to assess the stability of the macroscopic alignment of the bicelle sample at temperatures near the isotropic-nematic phase transition. Throughout the magnetic alignment, cooling, and monitoring steps of the experiment described above the magnetic field was kept at a static magnetic field strength of 6300 G except periodically during the acquisition of an EPR spectrum. Figure 4.6 shows the cholestane spin-labeled EPR spectrum taken at 304 K. Because the hyperfine splitting and lineshape did not change over the course of an hour, only the first EPR spectrum taken at 304 K is shown in Figure 4.6. This EPR spectrum at 304 K showed a significant portion of the sample had retained a parallel alignment and had a hyperfine splitting of approximately 8.5 G. This hyperfine splitting agrees with the extrapolation of our previous work for the temperature-dependent hyperfine splitting curve for well-aligned bicelle disks at 304 K.\textsuperscript{23} This EPR spectrum exhibited a broad down-field peak as compared to the cholestane spin-labeled EPR
Figure 4.6. A stacked plot of cholestane spin-labeled EPR spectra showing the retention of well-aligned phospholipid bilayers upon lowering the sample temperature close to the main liquid crystalline phase–gel phase transition temperature (303 K). The sample composition and magnetic alignment procedure were the same as described for the 11 min EPR spectrum in Figure 1. After a well-aligned Tm$^{3+}$-doped bicelle sample had been obtained, the sample temperature was slowly decreased from 318 to 304 K. An EPR spectrum was obtained at 304 K after the sample temperature was allowed to equilibrate for 10 min at 304 K. After 1 h, the sample temperature was lowered down to 303 K and an EPR spectrum was collected at 303 K after the sample had equilibrated for 10 min.
spectrum of a well-aligned sample at 318 K (Figures 4.1–4.4). One possible explanation for the broadness of the down-field peak was that some of the macroscopic alignment had been partially lost resulting in varying degrees of macroscopic alignment. An alternative or additional explanation might be that the motions are slower at this lower temperature. Next, the temperature was lowered to 303 K and the macroscopic alignment was monitored over time. EPR spectra taken at 303 K resulted in a hyperfine splitting of 16.8 G with no change in the EPR spectra over the course of 30 min; therefore, only the first EPR spectrum taken at 303 K is shown in Figure 4.6. Upon raising the temperature again to 304 K and monitoring the alignment over time, the EPR spectra were identical in hyperfine splitting and lineshape to the EPR spectra taken at 303 K; therefore, these EPR spectra were not included in Figure 4.6. This indicated that the macroscopic alignment of the model membrane system had completely collapsed to randomly dispersed bicelle disks. This collapse may have been caused by a decrease in the viscosity upon formation of the gel phase. This agrees very well with the formation of the isotropic phase at 303 K in Figure 4.5.

4.5 Discussion

Previously, it was observed that bicelle disks do not spontaneously align when placed into the X-band EPR spectrometer at 298 K in the presence of a magnetic field and no or partial orientation was detected over the course of an hour or more. From the solid-state $^2$H NMR spectra in Figure 5, the bicelle disks are in an isotropic phase at 298 K. The nature of this isotropic phase may either be a phase separated mixture of DHPC–DMPC mixed micelles and/or DMPC bilayers in the gel phase, or small, rapidly tumbling bicelle disks in the gel phase. In the former case the spherical geometry of mixed micelles lack magnetic anisotropy and therefore will not align in the presence of a magnetic field. Lipid dispersions of pure DMPC (i.e., multi lamellar DMPC bilayers) also will not align in the gel phase. In the latter case, small, rapidly tumbling bicelle disks accompanied by low viscosity similar to that of pure water have a high degree of thermal motion and cannot maintain any degree of alignment. The low viscosity also lowers the degree of
cooperativity between bicelle disks possibly due to significant amounts of bulk water filling the interbicelle space.

In the gel phase, the phospholipid acyl chains and long molecular axis are tilted 30° with respect to the normal of the phospholipid bilayers.\textsuperscript{38,56,57} Tilting may also be present in bicelles and the angle may be even greater than 30° because of the greater hydration levels used for our bicelle samples as compared to that in the literature.\textsuperscript{57} Tilting of the phospholipid acyl chains would significantly decrease the magnitude of the magnetic susceptibility anisotropy of the bicelle disk relative to its maximum limit (i.e., when the long molecular axis is parallel with the bicelle normal).\textsuperscript{29} Also, the partially buried phospholipid headgoups may reduce the lanthanide binding affinity to the headgroups. This would effectively decrease the magnitude of the magnetic susceptibility anisotropy of the bicelle disks and make alignment more difficult at low magnetic fields for X-band EPR studies.

Also, it was shown previously that Tm\textsuperscript{3+}-doped bicelle disks did not spontaneously align when placed into the X-band EPR spectrometer at a static temperature of 318 K and thermally equilibrated before applying a static magnetic field; instead, a partial alignment is observed (Figures 4.2 and 4.3).\textsuperscript{23} In the absence of a magnetic field, our bicelle system is presumably in a poorly aligned smectic liquid crystalline phase at 318 K, which has been described as perforated lamellar sheets with DHPC lining the edges of the sheets and pores.\textsuperscript{15,17,31,35} Struppe and Vold\textsuperscript{6} showed that a maximum viscosity is observed upon forming the nematic liquid crystalline phase (i.e., between 304 and 307 K for our system) the viscosity decreased monotonically with increasing temperature. It is assumed that the viscosity of our bicelle system at 318 K is still significantly greater than the viscosity of the bicelle sample in the isotropic phase. High viscosity causes the reorientation of the system to be kinetically slow and the degree of bicelle alignment is severely reduced.\textsuperscript{29} If the bicelle disks were randomly dispersed before the formation of a smectic phase (i.e., smectic phase forms in the absence of the magnetic field),\textsuperscript{17} then small polydomains of stacked lamellar sheets may have formed in the smectic phase.\textsuperscript{29} This would be the case if randomly dispersed bicelle disks were introduced into the X-band EPR spectrometer at a static temperature of 318 K in the absence of a magnetic field or if the temperature is increased from 298 to 318 K too quickly in the presence or absence of the magnetic field.
At 318 K, these stacked lamellae have been described as having undulations (ripples) and/or may have toroidal defects in the surface of the phospholipid bilayers. Formation of polydomains, undulations, and defects in the phospholipid bilayered lamellae would increase the macroscopic disorder of the system, which would decrease the magnitude of the macroscopic magnetic susceptibility anisotropy. This is supported by a study, which observed a low effective magnetic moment $\mu_{\text{eff}}$ for the system upon heating an analogous lanthanide-containing liquid crystalline sample from the solid phase to the isotropic phase. Upon heating the liquid crystalline samples studied by Mironov and co-workers and Binnemans and co-workers the order of phases encountered were the solid phase, smectic phase, nematic phase, and the isotropic phase. In contrast, the order of phases encountered upon heating our bicelle sample were the isotropic phase, the nematic L$_{\alpha}$ phase, and the smectic L$_{\alpha}$ phase.

Also, the molecular order parameter of the bound lanthanide cation decreases with increasing temperature due to the increased flexibility and motion in the bonds between the phospholipid headgroups and lanthanide cations. A lower molecular order parameter would contribute to an increase in microscopic disorder accompanied with a decrease in the magnitude of the overall magnetic susceptibility anisotropy of the bicelle system.

The combination of the various microscopic and macroscopic disorder effects described above coupled with the difficulty in reorienting smectic phases in a highly viscous medium disallow Tm$^{3+}$-doped bicelles in the smectic phase to fully align in the weak magnetic field strengths used in X-band EPR studies.

Mironov and co-workers stated that it is much easier to magnetically align liquid crystals when they are in the nematic phase than when they are in the smectic phase. They further stated that magnetic alignment was observed when their liquid crystalline sample was cooled from the isotropic phase to the nematic phase then to the smectic phase, but that no magnetic alignment occurred upon heating from the solid phase to the smectic phase.

Binnemans and co-workers showed that for their liquid crystalline sample there was a large increase in effective magnetic moment $\mu_{\text{eff}}$ near the isotropic-to-nematic phase transition and that this occurred over a very narrow temperature range. This may explain why the bicelle sample must be inserted into the EPR spectrometer at a maximum initial
temperature of 307 K to achieve fully aligned bicelle disks; whereas, the magnetic
alignment procedure with an initial temperature of 308 K showed a significant decrease
in the degree of magnetic alignment of the bicelle disks (Figures 4.2 and 4.3).

At the gel-to-L_α phase transition temperature of 304 K, the phospholipid acyl chains
begin to melt causing the long molecular axis to go from tilted to being statistically
parallel to the normal of the bicelle disk, which would increase the magnitude of the
magnetic susceptibility anisotropy. Also, the formation of a stable liquid crystalline phase
at 307 K may allow the phospholipid headgroups to become fully accessible to lanthanide
binding, which would increase the magnitude of the magnetic susceptibility anisotropy
tensor. The stabilization of the liquid crystalline nematic phase at 307 K was assumed to
be accompanied by an increase in the viscosity of the system.\textsuperscript{2,6,18,29} The increased
viscosity of the system may have increased the degree of cooperativity to reorient the
bicelle disks by increasing the hydration attraction forces between the bicelle disks.\textsuperscript{18,39}
Also, the presence of lanthanide cations may also aid in increasing the hydration
attraction forces between bicelle disks,\textsuperscript{39} and may act to stabilize the formation of the
bicelle disks.\textsuperscript{18} Gaemers and Bax\textsuperscript{15} described this nematic phase morphology as bicelle
disks transiently coming into contact with each other in an end-to-end manner and
approach the equivalent of strongly perforated lamellae.

An increase in viscosity would also cause this reorientation to be kinetically slow.\textsuperscript{29}
This was supported by the results in Figure 4.1 where increasing the temperature from the
isotropic phase to the smectic phase too quickly disrupted the complete macroscopic
alignment of the bicelle disks. As the temperature is increased, there would be an increase
in the fluidity of the sample allowing for the bicelle disks to reorient more easily. It is
apparent that viscosity may play a crucial role in the ability to magnetically align bicelle
disks in the presence of weak magnetic fields. On one hand it appears that magnetic
alignment at too low a viscosity suffers from too much thermal motion and too little
cooperativity, but on the other hand a viscosity that is too high is kinetically unfavorable.

As the temperature was increased from 307 to 318 K it is assumed that the bicelle
disks gradually got larger (i.e., an increase in the effective q-ratio of the bicelle disks) due
to the increased solubility of DHPC into the aqueous solution\textsuperscript{6,10} and/or possibly results in
increased transient end-to-end contacts between bicelle disks that would result in the
system becoming more smectic-like in character.\textsuperscript{15} An increase in the diameter of the bicelle disks would increase the magnitude of the magnetic susceptibility of the individual bicelle disks. An increase in temperature from 307 to 318 K would also lower the viscosity of the system allowing for the reorientation process to progress more easily. This was supported by the results in Figure 4.4 where a gradual increase in the macroscopic alignment was seen between the temperatures of 306 and 318 K.

The formation of a smectic phase between 311 and 313 K (Figure 5) after partial or complete alignment of the Tm\textsuperscript{3+}-doped bicelle disks would serve to stabilize macroscopic alignment. Partial alignment of Tm\textsuperscript{3+}-doped bicelle disks is observed in the EPR spectra in Figure 4.4 (310 and 314 K, \textit{inset}) and the degree of partially aligned bicelle disks increases as the final temperature is increased from 310 to 314 K. Prosser and co-workers\textsuperscript{31} observed an increase in the order of the bicelle disks upon going from the nematic phase to the lamellar smectic phase. An increase in the order of the bicelle system may result in an increase in the macroscopic magnetic susceptibility anisotropy for the lamellar sheets as compared to bicelle disks. Partial alignment of the bicelle system in the smectic phase would suffer less from polydomain and wall effects than for the situation described above for the formation of the smectic phase from randomly dispersed bicelle disks. The lower degree of macroscopic disorder in the former case would result in greater macroscopic magnetic susceptibility anisotropy when compared to the latter case.\textsuperscript{29} Although reorientation of liquid crystalline materials in the smectic phase is more difficult than in the nematic phase, the magnitude of the macroscopic $\Delta \chi$ may be large enough and the viscosity low enough to allow further magnetic alignment upon increasing the final temperature from the nematic-to-smectic phase transition temperature between 311 and 313 K (Figure 4.4) to the final temperature of 318 K (Figure 4.4, inset, 314 and 318 K).

In summary, the optimized maximum initial temperature (307 K) and minimum final temperature (318 K) of the magnetic alignment procedure for X-band EPR studies of Tm\textsuperscript{3+}-doped bicelle disks (Figures 4.2–4.4) coincide very well with the various phases observed in the $^2$H NMR spectra in Figure 4.5. Therefore, we make the assumption that at low magnetic field strengths Tm\textsuperscript{3+}-doped bicelle disks start to align once the nematic liquid crystalline phase stabilizes at 307 K. At 307 K, it is assumed that the macroscopic
magnetic susceptibility anisotropy and the viscosity of the Tm$^{3+}$-doped bicelle sample is optimal for the reorientation of the bicelle disks in the presence of an applied magnetic field. The macroscopic alignment of the bicelle system gradually increased as the temperature was raised slowly from 307 to 318 K in the presence of an applied static magnetic field. This occurs presumably because of the decrease in viscosity with increasing temperature and an increase in the order of the bicelle system upon going from the nematic phase to the smectic phase. The magnetic alignment of the bicelle system was well aligned and stabilized by the smectic phase at 318 K because the system is thermodynamically (large enough macroscopic magnetic susceptibility anisotropy) and kinetically (high enough viscosity) optimal for retaining macroscopic magnetic alignment. This is supported by a significant retention in the magnetic alignment of the bicelle system over time when the magnetic field is turned off after magnetic alignment is achieved. Stabilization of the magnetic alignment of the bicelle system in the smectic phase is supported in a study by Firestone and coworkers. They showed that to magnetically align their model membrane system it was necessary to place their sample into a magnetic field at a temperature corresponding to a hexagonal phase and increase the temperature slowly to a smectic phase. The hexagonal phase allowed for the mesogens to align in the magnetic field, but it was necessary to undergo a phase transition to a smectic phase to “lock in” the alignment.

**Impact and future of magnetically aligned phospholipid bilayers for EPR studies.** Magnetically aligned model membrane samples can provide a wealth of structural and dynamical information for X-band EPR studies as well as for Electron Nuclear Double Resonance (ENDOR) and Electron Spin Echo Envelope Modulation (ESEEM) studies. Anisotropic hyperfine splitting and anisotropic $g$ values are better resolved and can be directly measured with greater precision, and may also resolve peaks that are inhomogenously broadened. This methodology can be complementary with existing solid-state NMR techniques to extend the time scale over which the dynamics of the system can be studied especially if dynamic motions are too fast for solid-state NMR studies. Also, magnetically aligned bicelle disks do not suffer from biologically irrelevant low hydration that occurs in mechanically aligned phospholipid membrane systems and are relatively easy to construct.
The goal of this study was to obtain a better understanding of the magnetic alignment process of bicelle disks at low magnetic field so that the ease and degree of magnetic alignment may be optimized. An increase in the degree of bicelle disk alignment would increase the resolution of the EPR spectra. Optimizing the $q$-ratio, wt% hydration, salt concentration, and mol% cholesterol may optimize the viscosity, cooperativity, and kinetics of the magnetic alignment process of the bicelle disks and increase the degree of macroscopic alignment. Also, another way to increase the degree of alignment is to increase the binding affinity of lanthanide cations to the bicelle disk, which would increase the magnetic susceptibility anisotropy of the bicelle disks. The use of the phospholipid DMPE-DTPA as a lanthanide chelator should greatly enhance the binding affinity of lanthanides to the bicelle disk and minimize the unwanted interactions between lanthanides and integral membrane proteins.$^{9,58}$ Bicelle disks offer an incredibly valuable way to obtain an intimate knowledge of how a sample component interacts and perturbs the membrane system, because the kinetics, dynamics, and degree of magnetic alignment of bicelle disks is highly dependent upon the magnetic field strength used to align the bicelle disks.

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CHAPTER 5

Magnetically Aligned Phospholipid Bilayers with Large q-ratios Stabilize Magnetic Alignment with High Order in the Gel and L\(_{\alpha}\) Phases

5.1 Abstract

The magnetic alignment behavior of bicelles (magnetically alignable phospholipid bilayered membranes) as a function of the q-ratio (DMPC/DHPC mole ratio) and temperature was studied by spin-labeled X-band EPR spectroscopy and solid-state $^2$H and $^{31}$P NMR spectroscopy. Well-aligned bicelle samples were obtained at 45 °C for q-ratios between 2.5 and 9.5 in both the EPR and NMR spectroscopic studies. The molecular order of the system, $S_{mol}$, increased as the q-ratio increased and as the temperature decreased. For higher q-ratios ($\geq 5.5$), bicelles maintained magnetic alignment when cooled below the main phase transition temperature ($\sim 30$ °C when in the presence of lanthanide cations), which is the first time, to our knowledge, that bicelles were magnetically aligned in the gel phase. For the 9.5 q-ratio sample at 25 °C, $S_{mol}$ was calculated to be 0.83 (from $^2$H NMR spectra, utilizing the isotopic label DMPC$_{54}$) and 0.911 (from EPR spectra utilizing the spin probe 3β-doxyl-5α-cholestan). The molecular ordering of the high q-ratio bicelles is comparable to literature values of $S_{mol}$ for both multilamellar vesicles and macroscopically aligned phospholipid bilayers on glass plates. The order parameter $S_{bicelle}$ revealed that the greatest degree of bicelle alignment was found at higher temperatures and larger q-ratios ($S_{bicelle} = -0.92$ for q-ratio 8.5 at 50 °C).

5.2 Introduction

A magnetically alignable phospholipid bilayered membrane mimetic called a bicelle$^1$ has shown great promise in the study of the structure, orientation, and dynamics of integral membrane proteins, peptides, and other membrane biomolecules. Bicelles are a binary mixture of long-chain and short-chain phospholipids that are highly hydrated (40% to 98% w/w). This study uses dimyristoyl phosphatidylcholine (DMPC) and dihexanoyl phosphatidylcholine (DHPC) as the long-chain and short-chain phospholipids, respectively. The mole ratio of DMPC/DHPC is referred to as the q-ratio.
Typical q-ratios for solid-state NMR spectroscopic studies are between 2.5 and 3.5. Several studies have shown their application in solution NMR, solid-state NMR, spin-labeled EPR, and fluorescence spectroscopic studies, small angle neutron scattering (SANS), polarized optical microscopy (POM), and X-ray crystallography.

In the past, micelles, multilamellar vesicles (MLV), and macroscopically aligned phospholipids bilayers on glass plates were predominantly used as membrane mimetics for NMR and EPR spectroscopic studies. Bicelles combine all of the favorable characteristics of these membrane mimetics (i.e., high hydration, exhibits a bilayered morphology, alignable with respect to the magnetic field, no curvature strain on incorporated biomolecules) and, in addition, are easily prepared and the sample composition can be changed by titration: whereas, micelles confer curvature strain on incorporated biomolecules, micelles and MLV are not alignable due to their spherical morphologies, bilayers on glass plates have low hydration and suffer from sample heating due to the low filling factor. One disadvantage of bicelles, when compared with aligned bilayers on glass plates, is their lower degree of alignment. Also, bicelle alignment is completely disrupted upon lowering the sample to the gel phase and exhibits isotropic motion in solid-state $^2\text{H}$ and $^{31}\text{P}$ NMR studies and randomly dispersed spectra in spin-labeled EPR studies. The ideal bicelle for studying the orientation and structure of incorporated biomolecules into phospholipid bilayered membranes would need to achieve the same degree of alignment of macroscopically aligned bilayers on glass plates and be able to maintain a high degree of alignment at gel phase temperatures.

Past models of the magnetically alignable phase for bicelles was thought to be disk-shaped at temperatures above the main phase transition temperature, $T_m$. Recent studies have shown the morphology and phase diagram of DMPC/DHPC bicelles to be very complex and under some debate. Nieh and coworkers proposed the following model for bicelles with and without lanthanide cations ($\text{Ln}^{3+}$), which will be utilized in this study to compare and interpret the results. At temperatures below the main phase transition temperature, $T_m$, of DMPC, bicelles are disk-shaped. As the temperature is raised from the gel phase to the liquid crystalline phase in the presence of $\text{Ln}^{3+}$ the bicelles fuse together in an end-to-end manner to form lamellar sheets with perforated holes that are lined with DHPC. Further increases in temperature may cause phase separation with
the formation of DHPC-rich mixed micelles and DMPC-rich oriented lamellae, and the DHPC-rich mixed micelles may become incorporated into the oriented bilayers at even higher temperatures.\textsuperscript{13} In the absence of Ln\textsuperscript{3+}, bicelle samples are disk-shaped in the gel phase, chiral nematic in the liquid crystalline phase, which are described as worm-like micelles, and at higher temperatures are multilamellar vesicles.\textsuperscript{9} Hereafter, the use of the term bicelle in this study refers only to the sample composition and not to the disk-shaped morphology.

The magnetic alignment of bicelles for X-band EPR spectroscopic studies is very challenging due to the weak magnetic field strengths used by the EPR spectrometer to induce alignment and are insufficient to induce magnetic alignment under isothermal conditions.\textsuperscript{2} The degree of magnetic alignment is dependent upon the strength of the magnetic field $B_0$ and the magnitude of the magnetic susceptibility anisotropy tensor $\Delta \chi$, which is sensitive to the sample composition.\textsuperscript{2,14-16} $\Delta \chi$ is given by $\Delta \chi = \chi_\parallel - \chi_\perp$ where $\chi_\parallel$ and $\chi_\perp$ are, respectively, the tensorial components that are parallel and perpendicular to the normal of the bilayer. The sign of $\Delta \chi$ determines the preferred orientation that the bicelle normal will make with respect to the static magnetic field ($B_0$).

A standard procedure used to induce the magnetic alignment of bicelles in weak magnetic field strengths consists of slowly raising the temperature from 25 °C to 45 °C in the presence of both an applied magnetic field and paramagnetic lanthanide cations (Ln\textsuperscript{3+}) with large $\Delta \chi$ values (i.e., Tm\textsuperscript{3+} and Dy\textsuperscript{3+}).\textsuperscript{1} Lanthanide cations bind to the phospholipid headgroups with two phospholipids per cation.\textsuperscript{17} The addition of lanthanide cations has been known to increases $\Delta \chi$ by about one to two orders of magnitude in other liquid crystals, thereby, making magnetic alignment possible for X-band EPR spectroscopic studies.\textsuperscript{16} Tm\textsuperscript{3+} has a positive $\Delta \chi$, which induces bicelle alignment with the bicelle normal being parallel to the direction of $B_0$, and Dy\textsuperscript{3+} has a negative $\Delta \chi$, which induces bicelle alignment with the bicelle normal being perpendicular to the direction of $B_0$.\textsuperscript{1} For standard bicelles (i.e., q-ratio of 2.5 to 3.5), the magnetic field strengths used to induce alignment in X-band are larger (i.e., $\geq 4500$ G)\textsuperscript{15} than that used to acquire the EPR spectra (i.e., $g = 2$ with resonance at a center field of $\approx 3300$ G).

Because the magnetic field strengths utilized by X-band EPR spectrometers are much weaker than that used in NMR spectrometers, the degree of order and alignment of
bicelles is expected to be significantly less for spin-labeled EPR spectroscopic studies. This factor combined with the lower degree of alignment when compared with aligned bilayers on glass plates may make it difficult to compare parallel results from solid-state NMR spectroscopic studies. The purpose of this study is to optimize the bicelle composition in order to increase the degree of alignment and order of the system so that these disadvantages may be addressed. In this study, we investigated the range of q-ratios that can achieve a high degree of alignment (q-ratios between 2.5 and 9.5) and assess what is the optimal q-ratio for the bicelle samples for both solid-state NMR and spin-labeled EPR studies. Bicelles with high q-ratios were found to achieve a similar degree of molecular ordering and macroscopic alignment as that found in macroscopically aligned bilayers on glass plates. Also, high q-ratio samples were found to maintain a high degree of magnetic alignment in both the solid-state NMR and spin-labeled EPR studies when the temperature was lowered to the gel phase. This is the first time, in our knowledge, that bicelle alignment has been shown for gel phase temperatures. This work has important applications to both solid-state NMR and spin-labeled EPR spectroscopic studies on membrane systems.

5.3 Experimental

Materials. 1,2-Dihexanoyl-sn-glycerol phosphatidylcholine (DHPC), 1,2-dimyristoyl-sn-glycerol phosphatidylcholine (DMPC), perdeuterated DMPC (DMPC_{d54}), and 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000] (PEG2000-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Thulium (III) chloride hexahydrate (Tm^{3+}), dysprosium (III) chloride hexahydrate (Dy^{3+}), 3β-doxyl-5α-cholestane (cholestane or CLS), and HEPES buffer were obtained from Sigma-Aldrich (St. Louis, MO). Cholesterol was obtained from Avocado Research Chemicals (Ward Hill, MA).

Sample preparation for EPR studies. DMPC, DHPC, PEG2000-PE, CLS, and cholesterol were purchased as stock solutions that were dissolved in chloroform, shipped
in amber colored bottles, and stored at -20 °C. Aqueous solutions of HEPES buffer, Tm³⁺ and Dy³⁺ were prepared fresh on the day of sample preparation.

Bicelle samples were prepared by dispensing the appropriate volumes of the DMPC, DHPC, PEG2000-PE, cholesterol, and CLS stock solutions into a 25 mL pear-shaped flask having mole ratios of q/1.0/0.035/0.35/0.0196, respectively, where q is the moles of DMPC needed to achieve the desired q-ratio between 1.5 and 12.0. The lipids were rotary evaporated at room temperature and vacuum desiccated overnight to remove chloroform. 100 mM HEPES buffer was added to the pear-shaped flask to a final lipid concentration of 25 wt %. The sample was solublized by vortexing, followed by sonication (30 min), and the sample was annealed by performing at least three freeze-thaw cycles using liquid nitrogen. A small volume of Tm³⁺(aq) or Dy³⁺(aq) was added to the bicelle samples (20 mol % with respect to DMPC).

For EPR spectra, the bicelle samples were drawn into 1mm ID capillary tubes and sealed on both ends with Critoseal purchased from Fisher Scientific (Florence, KY). The capillary tubes were placed into standard quartz EPR tubes (707-SQ-250M) purchased from Wilmad Glass (Buena, NY) 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 an ER 4119HS cavity that is coupled with an ER 4131 VT nitrogen gas temperature controller (temperature stability of ±0.2 °C). Each cholestane spin-labeled EPR spectrum was acquired by taking a 42-s field-swept scan with a center field of 3370 G, a sweep width of 100 G, a microwave frequency of 9.434 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 1.0 G, and a microwave power of 2 mW.

The molecular order parameter, \( S_{\text{mol}} \), of cholestane incorporated into magnetically aligned phospholipid bilayers is calculated using the following equations.\(^{18}\)

\[
S_{\text{mol}} = \left[ (A_{||} - A_{\bot})/(A_{zz} - A_{xx}) \right] (a_N/a'_N)[(3\cos^2\theta - 1)/2]^{1/2} \\
a_N = 1/3(A_{xx} + A_{yy} + A_{zz}) \\
a'_N = 1/3(A_{||} + 2A_{\bot})
\]
where $A_\parallel$ and $A_\perp$ are the observed hyperfine splittings when the magnetic field is, respectively, parallel or perpendicular to the long molecular axis of the spin probe; $A_{xx}$, $A_{yy}$, and $A_{zz}$ are the principle hyperfine splitting tensorial values obtained for a single crystal; $a_N$ is the isotropic hyperfine splitting; $\alpha_N$ adjusts for the polarity of the sample; $\theta$ is the angle between the long molecular axis and the corresponding principle z-axis of the nitrooxide spin probe. $A_\parallel$ and $A_\perp$ are measured directly from the observed hyperfine splitting for the parallel (Tm$^{3+}$-doped) and perpendicular (Dy$^{3+}$-doped) samples, respectively. The single crystal values for $A_{xx}$, $A_{yy}$, $A_{zz}$, and $\theta$ were 6.6 G, 5.0 G, 30.8 G, and 85°, respectively.$^{18}$

**Solid-state $^2$H and $^{31}$P NMR studies.** The sample composition was the same as that used for the EPR studies except cholestane was omitted from all bicelle samples and 5 mg of DMPC$_{d54}$ was added as a $^2$H-labeled probe. Also, deuterium-depleted water purchased from Isotec (Miamisburg, OH) was used to make the aqueous solutions. Samples were loaded into a NMR flat-bottom tube with an o.d. of 5 mm purchased from Wilmad Glass (Buena, NJ).

All solid-state NMR experiments were carried out on a 500 MHz WB Bruker solid-state NMR spectrometer. Samples were inserted into a 5 mm round-coil probe purchased from Bruker. Solid-state $^2$H NMR spectra were recorded at 76.8 MHz using a standard quadrupole-echo pulse sequence (3.5 µs 90° pulses, 40 µs echo delay, 0.5 s recycle delay, and a 100 kHz sweep width).$^{19}$ Typically 4096 scans were accumulated. Prior to Fourier transformation, an exponential line broadening of 200 Hz was performed on the spectra. Solid-state $^{31}$P NMR spectra were gathered at 202.5 MHz with high power proton decoupling with the spin echo sequence with a 4 µs 90° pulse, a 5 s recycle delay, and a sweep width of 300 ppm. Typically 256 scans were accumulated. Prior to Fourier transformation, an exponential line broadening of 100 Hz was performed on the spectra.

From the solid-state $^2$H NMR spectroscopic studies, an $S_{mol}$ profile can be calculated for each carbon position along the acyl chains of DMPC$_{d54}$, which is incorporated into magnetically aligned bicelles, using the following equations.$^{20,21}$

$$S'_{CD} = \Delta v_q / 252 \text{ kHz}; \text{ for CD}_2 \text{ groups} \quad (4)$$
$$S'_{CD} = 3(\Delta v_q / 252 \text{ kHz}); \text{ for CD}_3 \text{ groups} \quad (5)$$
\[ S_{\text{mol}}^i = -2S_{\text{CD}}^i \]  

(6)

where \( \Delta \nu_q^i \), \( S_{\text{CD}}^i \), and \( S_{\text{mol}}^i \) are, respectively, the quadrupolar splitting, segmental order parameter, and the molecular order parameter of the \( i \)th carbon of the DMPC acyl chain. The quadrupolar splitting for the deuterons in the plateau region were estimated by integration of the last broad peak according to the literature.\(^{22,23}\)

From the solid-state \(^{31}\)P NMR spectroscopic studies, the order parameter of the bicelles, \( S_{\text{bicelle}} \), can be calculated using the following equation:\(^4\)

\[ \delta = \delta_i + S_{\text{bicelle}} \times (\delta_{\|} - \delta_{\perp})/3 \]  

(7)

where \( \delta \) is the DMPC \(^{31}\)P chemical shift, \( \delta_i \) is the isotropic \(^{31}\)P chemical shift for DMPC, and \( \delta_{\|} - \delta_{\perp} \) is the \(^{31}\)P chemical shift anisotropy for DMPC that is rapidly rotating about its long molecular axis. Approximately, \( \delta_{\|} - \delta_{\perp} = 45 \) ppm.\(^{4,24}\) \( S_{\text{bicelle}} \) incorporates the order of the whole bicelle, molecular, and local orientations and dynamics into its value and highly reflects the degree of bicelle alignment. \( S_{\text{bicelle}} \) is analogous to the product of \( S_{\text{bilayer}} \times S_{\text{mol}} \times S_{\text{loc}} \) except the negative sign is incorporated into \( S_{\text{bicelle}} \).

**5.4 Results and Discussion**

*Spin-labeled EPR Spectroscopic Studies.* The spin probe 3β-doxy1-5α-cholestane (cholestane or CLS) was incorporated into several bicelle samples having different q-ratios from 1.5 to 12.0. It was observed that the viscosity of the samples increased as the q-ratio increased. All the samples were clear in the gel phase and slightly turbid (except for q-ratio 12.0, which was opaque) in the liquid crystalline phase (turbidity increased as the q-ratio increased). Well-aligned bicelle samples were obtained at 45 °C for q-ratios between 2.5 and 9.5. Figures 5.1(A) and 5.1(B) show two representative sets of EPR spectra for q-ratios 2.5 and 9.5, respectively, to illustrate the range of magnetic alignment behavior. EPR spectra were taken at various temperatures as the samples were cooled from 45 °C to 25 °C. Randomly dispersed samples (solid-line spectra) were obtained by
placing a Tm\(^{3+}\)-doped bicelle sample into the spectrometer with the magnetic field off, the
temperature was set to 45 °C and the sample temperature was allowed to equilibrate for at
least 5 minutes. Magnetically aligned samples were obtained by placing a Tm\(^{3+}\)- or Dy\(^{3+}\)-
doped bicelle sample (dotted-line or dashed-line spectra, respectively) into the
spectrometer and allowing the sample temperature to equilibrate at 25 °C for at least 5
minutes, then slowly raising the sample temperature to 45 °C over 11 minutes in the
presence of a static magnetic field set to 1.3 T. For the Tm\(^{3+}\)-doped DMPC/DHPC
samples (dotted-line spectra), the normal to the phospholipid bilayers was aligned
parallel to B\(_o\). Alternatively, for the Dy\(^{3+}\)-doped bicelle samples (dashed-line spectra),
the normal to the phospholipid bilayers was aligned perpendicular with B\(_o\).\(^1\) The
hyperfine splitting for the parallel-aligned spectra decreases (peaks shift in) and the
hyperfine splitting for the perpendicular-aligned spectra increases (peaks shift out), as the
temperature is lowered and as the q-ratio was increased. Magnetic alignment was
retained while cooling the samples until a threshold temperature was reached where
either a randomly dispersed or partially aligned sample was obtained. In general, this
alignment temperature threshold decreased as the q-ratio was increased from 2.5 to 9.5.
In Figure 5.1(B), the 9.5 q-ratio sample retained much of its magnetic alignment as low
as 25 °C, especially in the parallel-aligned spectra (dotted-line spectra).

Ln\(^{3+}\)-doped bicelle samples having a q-ratio of 1.5 or 12.0, achieved (at best) only
partial alignment of the bicelle samples at 45 °C (spectra not shown). For a q-ratio of
1.5, EPR spectra showed that the sample was completely randomly dispersed at
temperatures below 45 °C. For a q-ratio of 12.0, the sample was extremely viscous,
which made it impractical to mix homogenously and draw up into capillary tubes for X-
band EPR spectroscopic studies, and some phase separation could be seen at
temperatures slightly higher than room temperature to 45 °C.

Table 1 displays A\(_{ll}\) and A\(_{\perp}\) values from the EPR spectra for bicelle samples having q-
ratios between 2.5 and 9.5 (Figures 5.1(A) and 5.1(B)) and were used in the calculation
of the molecular order parameter, S\(_{\text{mol}}\), of cholestane (standard deviation of S\(_{\text{mol}} = \pm
0.016\)). Figure 5.2 shows a plot of S\(_{\text{mol}}\) as a function of temperature for each bicelle
Figure 5.1. X-band CW-EPR spectra of the cholestane spin probe incorporated into bicelle samples having either a q-ratio of 2.5 (A) or 9.5 (B). Spectra show the temperature and q-ratio dependence of the magnetic alignment of bicelles. The bicelle samples were randomly dispersed (solid-line spectra) or magnetically aligned parallel (dotted-line spectra) or perpendicular (dashed-line spectra) to the applied magnetic field at 45 °C and EPR spectra were acquired at various temperatures decreasing in 5 °C intervals from 45 °C to 25 °C. The vertical dotted lines are for convenience to compare changes in hyperfine splitting between spectra.
TABLE 5.1

Temperature Dependence of Hyperfine Splitting Values for CLS EPR Spectra of Parallel and Perpendicular Aligned Bicelle Samples

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Q 2.5</th>
<th>Q 4.5</th>
<th>Q 6.5</th>
<th>Q 7.5</th>
<th>Q 9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{</td>
<td></td>
<td>}^{a,b}$</td>
<td>$A_{\perp}^{a,c}$</td>
<td>$A_{</td>
</tr>
<tr>
<td>45</td>
<td>10.50</td>
<td>17.69</td>
<td>9.82</td>
<td>17.59</td>
<td>9.38</td>
</tr>
<tr>
<td>40</td>
<td>9.43</td>
<td>18.02</td>
<td>9.03</td>
<td>17.98</td>
<td>8.78</td>
</tr>
<tr>
<td>35</td>
<td>8.50</td>
<td>18.02</td>
<td>8.00</td>
<td>18.57</td>
<td>7.77</td>
</tr>
<tr>
<td>29</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>6.93</td>
</tr>
<tr>
<td>28</td>
<td>--</td>
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<td>--</td>
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<td>--</td>
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<td>27</td>
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<tr>
<td>25</td>
<td>--</td>
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</table>

*a.* Units in Gauss.

*b.* Standard deviation = $± 0.18$ G

*c.* Standard deviation = $± 0.06$ G
Figure 5.2. $S_{mol}$ order parameters for cholestane incorporated into bicelles were measured as a function of temperature from the X-band EPR spectra described in Figure 5.1. $S_{mol}$ was calculated using Equation 1 and the hyperfine splitting values in Table 5.1.
sample having a q-ratio from 2.5 to 9.5. The general trend in Figure 5.2 shows an increase in the molecular ordering as the q-ratio increases and as the temperature decreases.

**Solid-State ²H-NMR Spectroscopic Studies.** Figure 5.3(A) shows the solid-state ²H NMR spectra for various DMPC/DHPC/DMPC₅₄ q-ratio bicelle samples taken at 45 °C. All spectra are typical of well-aligned bicelle samples with the normal of the bicelles being parallel to the applied magnetic field. The quadrupolar splitting increases as the q-ratio increases, which may be due to increases in the cooperative aggregate size, viscosity, and/or dampening of the thermal undulations (i.e., membrane rigidity increases). The temperature dependence of the magnetic alignment behavior of bicelles was studied by comparing ²H NMR spectra between parallel-aligned bicelle samples (q-ratios between 2.5 to 9.5) and a randomly dispersed sample (same sample composition as the 8.5 q-ratio bicelle sample except DHPC was omitted) as the samples were cooled in 5 °C intervals from 45 °C to 25 °C. The ²H NMR spectra of aligned bicelle samples with a q-ratio of 2.5 (Figure 5.3(B), solid-line spectra) and 8.5 (Figure 5.3(C), solid-line spectra) are representative of the two general types of magnetic alignment behavior over the range of q-ratios that were probed. The 2.5 q-ratio sample is representative of q-ratios 2.5 - 4.5 and the 8.5 q-ratio sample is representative of q-ratios 5.5 - 9.5. In Figures 5.3(A) and 5.3(B), ²H NMR spectra for the randomly dispersed sample (dashed-line spectra) are superimposed onto both sets of ²H NMR spectra of aligned bicelles. For all samples, the quadrupolar splitting monotonically increased as the temperature decreased from 45 °C to 35 °C. Upon cooling the samples from 35 °C to 25 °C there is a significant departure in the temperature dependence between the two sets of bicelle spectra. For lower q-ratios (Figure 5.3(B), solid-line spectra), the reduction in resolution and quadrupolar splitting at 30 °C and finally collapse at 25 °C to an isotropic peak is due to the disruption of the magnetic alignment of bicelles and is an indication of the L₅-to-gel phase transition (Tₘ ~ 30 °C). Similar behavior is observed in the literature for bicelles in the gel phase except they report Tₘ to be ~ 23 °C. In contrast, the higher q-ratios (Figure 3(C), solid-line spectra) exhibit a very dramatic increase in the quadrupolar
Figure 5.3. The magnetic alignment behavior of bicelles as a function of q-ratio (A) and temperature (B and C) was studied utilizing solid-state $^2$H NMR spectroscopy. (A) Solid-state $^2$H NMR spectra were taken at 45 °C for parallel-aligned bicelle samples that were doped with DMPC$_{ds}$ and Tm$^{3+}$ having various q-ratios from 2.5 (bottom spectra) to 9.5 (top spectra) that were doped with DMPC$_{ds}$ and Tm$^{3+}$. (B and C) Solid-state $^2$H NMR spectra were taken at 5 °C intervals when the samples in (A) were cooled from 45 °C to 25 °C. Solid-state $^2$H NMR spectra for a randomly dispersed sample (dashed-line spectra) were superimposed onto the spectra for bicelle samples with a q-ratio of (B) 2.5 (solid-line spectra) or (C) 8.5 (solid-line spectra). The randomly dispersed sample had the same sample composition as the 8.5 q-ratio bicelle sample except DHPC was excluded. The vertical dotted lines in Figures 3(A) – 3(C) are for convenience to compare changes in quadrupolar splittings between spectra.
splitting (outer maxima increase by ~ 41 kHz when the temperature is lowered from 35 °C to 30 °C) accompanied by a loss in spectral resolution but differs markedly from the powder spectra (Figure 5.3(C), dashed-line spectra).

Figure 5.4 shows the molecular order parameter profile ($S_{\text{mol}}^i$) of the DMPC$_{d54}$ acyl chains at 45 °C for the DMPC/DHPC bicelle samples described in Figure 5.3(A). For comparison, additional points are included in this figure, which show $S_{\text{mol}}^i$ at various temperatures for the C2 and C14 positions of the 9.5 q-ratio sample. The standard deviation of $S_{\text{mol}}^i$ values is ± 0.002, which is less than the size of the symbols in Figure 5.4.

The temperature-dependent order parameter profiles of DMPC in lipid dispersions and macroscopically aligned bilayers on glass plates are well known.$^{26-31}$ The ideal bicelle would need to have a comparable degree of alignment and ordering as macroscopically aligned bilayers on glass plates over a range of temperatures spanning the gel phase and L$_\alpha$ phases. Therefore, a comparison of the order parameter values found in this study and in the literature may help understand the physical behavior of bicelle samples and determine the optimized sample composition.

From the literature, solid-state $^2$H NMR studies of pure DMPC lipid dispersions show a dramatic, large increase in the phospholipid acyl chain order parameter ($S_{zz}$) when lowering the temperature below the main phase transition temperature.$^{30,31}$ The quadrupolar splitting is proportional to $S_{zz}$; therefore a dramatic, large increase in quadrupolar splitting would be expected when cooling the bicelle samples below $T_m$. Similarly, the high q-ratio samples in Figures 5.3 and 5.4 also exhibit a very dramatic, large increase in quadrupolar splitting and $S_{\text{mol}}^i$ accompanied by a loss in resolution between 30 to 35 °C. A loss in resolution is also observed near the $T_m$ of DPPC bilayers that are macroscopically aligned on glass plates.$^{32}$ The increase in the acyl chain order parameter profile when lowering the temperature to the gel phase is a result of the nearly all-trans conformation accompanied by tighter packing of the phospholipids. The loss in resolution results from line broadening and from the ordering of each carbon position along the phospholipid acyl chain being nearly identical. Therefore, the main phase
**Figure 5.4.** A graph showing the segmental molecular order parameters, $S_{mol}$, of DMPC$_{d54}$ as a function of carbon position on the acyl chains, which were measured from the solid-state $^2$H NMR spectra of parallel-aligned bicelle samples (Tm$^{3+}$-doped) described in Figure 5.3.
transition temperature for the high q-ratio samples is ~ 30 °C and the samples are highly oriented below this temperature.

Other studies in the literature have shown that for bicelles with low q-ratios between 2.7 to 4.8 the main phase transition is governed by the $T_m$ of pure DMPC (i.e., ~ 23 °C), and for temperatures below $T_m$ the anisotropic motion collapses to an isotropic peak. Similar results are obtained for the present bicelle samples with a q-ratio of 3.5 and in the absence of Ln$^{3+}$ cations (spectra not shown), whereas upon adding 20 mol% Tm$^{3+}$, the spectra become isotropic at ~ 30 °C, which is due to the condensing effect on lipid membranes from the binding of two phospholipid headgroups per Ln$^{3+}$ cation.

Therefore, we speculate that near 30 °C there may be a threshold for $|\Delta \chi|$ between q-ratios 4.5 and 5.5 where $|\Delta \chi|$ is large enough for high q-ratio samples and is able to maintain magnetic alignment in the gel phase but is too small for low q-ratio samples and is not able to maintain magnetic alignment. $|\Delta \chi|$ is dependent on the viscosity, morphology, and cooperative size of the mesogenic units. Viscosity may play a very important role in the magnetic alignment process. An increase in viscosity may cause an increase in the cooperativity of the magnetic alignment process (i.e., increasing cooperativity increases $|\Delta \chi|$), but increasing the viscosity too high may severely slow down the magnetic alignment process, which may be impractical for our purposes. This may partially explain why we were unsuccessful in our attempts to magnetically align the bicelle samples with a q-ratio of 12.0.

Because of the difference in $T_m$ between samples with (~ 30 °C) and without (~ 23 °C) added Ln$^{3+}$, all comparisons of $S_{mol}$ to literature values are scaled relative to the corresponding phase transition temperature. For the 9.5 q-ratio sample at carbon position 2, $S_{mol}$ increases from 0.50 to 0.54 when the temperature is cooled from 45 °C to 35 °C (Figure 4). Similarly, literature values of $S_{mol}$ increased from approximately 0.43 to 0.50 when the temperature is cooled from 35 °C to 25 °C. The difference in $S_{mol}$ of 0.06 relative to the literature values may be accounted for by the addition of 10 mol% cholesterol in the present sample. Thus, the high q-ratio bicelle samples compare very well to the order and temperature-dependent behavior and approximate the lipid environment of multilamellar and macroscopically aligned bilayers.
Solid-State $^{31}$P-NMR Spectroscopic Studies. Figure 5.5 shows solid-state $^{31}$P NMR powder spectra of a randomly dispersed sample (dashed-line spectra) and three perpendicular-aligned bicelle samples having q-ratios of 4.5, 6.5, and 8.5 (solid-line spectra). The randomly dispersed sample has the same sample composition as the 8.5 q-ratio sample except DHPC was omitted. Also, Ln$^{3+}$ cations were omitted from the samples because it would broaden the $^{31}$P NMR spectra. In the absence of Ln$^{3+}$ cations, the main phase transition temperature $T_m$ is $\sim 23$ °C. All spectra were taken in the L$_\alpha$ phase at 35 °C. All aligned spectra exhibit two peaks corresponding to DMPC (upfield) and DHPC (downfield) and are characteristic of perpendicular-aligned bicelles. As the q-ratio was increased, both the DHPC and DMPC peaks shift upfield. The degree of bicelle order, or $S_{bicelle}$, is greatly influenced by the degree of alignment of the system. $S_{bicelle}$ increased from -0.71 to -0.88 when the q-ratio was increased from 4.5 to 8.5. In the absence of Ln$^{3+}$, Nieh and coworkers showed that the structure of the magnetically aligned bicelles in the liquid crystalline phase are worm-like micelles or ribbons. The upfield shift of the DMPC and DHPC peaks may result from increases in the average size the aggregates. Sanders and coworkers also showed changes in the DMPC peak as a function of the DHPC concentration and was due to changes in the bulk properties of the DMPC-rich bilayers and not from conformational changes in the phospholipid headgroups. An increase in the average size of the mesogenic units as the q-ratio increased agrees with the $^2$H NMR results in Figure 3(A), which showed increased quadrupolar splitting as the q-ratio increased. The spectra also suggest that DHPC becomes more DMPC-like in conformation as the q-ratio is increased, shown by the shift in the $^{31}$P DHPC peak upfield and the decrease in the difference between the chemical shift values for DHPC and DMPC decreased. This may result from increased DHPC interdigitation into the DMPC-rich bilayers. $^{31}$P spectra for both the 6.5 and 8.5 q-ratio samples have highly symmetrical spectra, which reflect the high degree of bicelle alignment. The 4.5 q-ratio sample exhibits slight asymmetry in both the DHPC and DMPC peaks, suggesting that the bicelles are aligned with a slight distribution of orientations about the ideal 90°. The broad peaks in the 8.5 q-ratio sample are not very well understood, but may be due to either a distribution in the size of the aggregates,
Figure 5.5. The magnetic alignment behavior of bicelles as a function of q-ratio was studied utilizing $^{31}$P NMR spectroscopy. $^{31}$P NMR spectra were taken at 35 °C for perpendicular-aligned bicelle samples (solid-line spectra) having a q-ratio of 4.5, 6.5, or 8.5 and were compared with a randomly dispersed sample (dashed-line spectra). The randomly dispersed sample had the same sample composition as the 8.5 q-ratio bicelle sample except DHPC was excluded. The vertical dotted line is for convenience to compare changes in chemical shifts between the spectra.
reduced frequency of thermal undulations (which may stiffen the membrane and cause insufficient motional averaging), and/or other defects in the membrane. Line broadening was also observed in the $^2$H NMR spectra in Figure 3(A) for q-ratios $\geq 6.5$. This may also explain why the perpendicular-aligned EPR spectra in the gel phase (Figure 1(B), dashed-line spectra, $25 \, ^\circ\mathrm{C}$ and $30 \, ^\circ\mathrm{C}$) also exhibit broad, non-Lorentzian lineshapes.

Figure 6 shows the temperature dependence of the solid-state $^{31}\text{P}$ NMR spectra. As the temperature is increased for the aligned samples, both the DHPC and DMPC peaks gradually shift upfield and the difference between the chemical shift values for DHPC and DMPC decreases. This is consistent with a similar study done by Ottiger and coworkers, where increasing the temperature increases the aggregate size. They attributed the increase in aggregate size to an increase in DHPC solubility with increasing temperature, which increases the effective q-ratio ($q_{\text{eff}}$). Also, Nieh and coworkers showed that for DMPC/DHPC bicelle sample without lanthanide cations and with a q-ratio of 3.2 there was a gradual transition from bicelle disks to lamellae as the temperature was increased up to $45 \, ^\circ\mathrm{C}$. The temperature at which the two peaks are indistinguishable decreased as the q-ratio was increased. In other words, increasing the q-ratio stabilizes the incorporation of DHPC into the magnetically aligned DMPC-rich bilayers. For the 8.5 q-ratio sample (Figure 6, right column, solid-line spectra), $S_{\text{bicelle}}$ increases from -0.82 to -0.92 when the temperature is increased from $25 \, ^\circ\mathrm{C}$ to $50 \, ^\circ\mathrm{C}$. In contrast, the $^{31}\text{P}$ powder spectra of DMPC-rich randomly dispersed samples (dashed-line spectra) shows a slight shift in the edges of the powder spectra toward the isotropic component as the temperature is increased. When increasing the temperature, the randomly dispersed sample is presumably multilamellar vesicles over all temperatures, whereas the bicelle samples undergo transitions from small aggregates (like bicelle disks) to larger aggregates (like worm-like micelles or perforated lamellar sheets). It is assumed that the size of the multilamellar vesicles for the randomly dispersed sample does not increase in size as the temperature is raised; therefore, the changes in the powder spectra as a function of temperature are due to increased molecular and local motions only. This also explains why the aligned 8.5 q-ratio sample has a chemical shift value that is slightly
Figure 5.6. The magnetic alignment behavior of bicelles as a function of temperature was studied utilizing $^{31}$P NMR spectroscopy. $^{31}$P NMR spectra taken at 5 °C intervals as the samples were cooled from 50 °C to 25 °C for perpendicular-aligned bicelle samples having a q-ratio of 4.5 (left column, solid-line spectra), 6.5 (center column, solid-line spectra), or 8.5 (right column, solid-line spectra), and a randomly dispersed sample (dashed-line spectra). The randomly dispersed sample had the same sample composition as the 8.5 q-ratio bicelle sample of except DHPC was excluded. The vertical dotted line is for convenience to compare changes in the chemical shifts between the spectra.
greater than the $\alpha_i$ component of the powder spectra at 50 °C (right column, dashed-line spectra). This suggests that DHPC plays an important role in the formation of extended lamellar sheets for DMPC/DHPC bicelle samples. The 4.5 q-ratio sample (left column, solid-line spectra) shows phase separation at 25 °C with the DHPC forming an isotropic mixed micelle phase and DMPC forming DMPC-rich bilayers with a powder-like distribution of orientations. The edges of the DMPC peak (left column, solid-line spectra, 25 °C) are shifted in relative to the randomly dispersed sample (dashed-line spectra), which may be a result of DMPC undergoing fast exchange between the DHPC-rich mixed micelles (isotropic peak) and the DMPC-rich bilayers. This is consistent with the increased broadening in both the DHPC and DMPC peaks. The q-ratio 6.5 (center column, solid-line spectra) and 8.5 (right column, solid-line spectra) spectra also exhibit an isotropic DHPC peak at 25 °C, but the DMPC peaks are still very well aligned with only slight asymmetry and very little or no line broadening near the phase transition temperature of pure DMPC (~23 °C).

5.5 Conclusion

We obtained well-aligned spin-labeled EPR and solid-state $^2$H and $^{31}$P NMR spectra of bicelle samples having a q-ratio from 2.5 to 9.5. One very striking result from this study is that the higher q-ratio samples maintain a significant degree of magnetic alignment in the gel phase. The $^2$H and $^{31}$P NMR spectra (Figures 3 and 6, respectively) show that magnetic alignment in the gel phase was observed for q-ratios ≥ 5.5. Similarly, the EPR spectra (Figure 1) show that the magnetic alignment was obtained below the gel-to-liquid crystalline phase transition temperature (~30 °C) for q-ratios ≥ 5.5, but magnetic alignment at 25 °C was only observed for a q-ratio of 9.5. Previously, bicelles in the gel phase were thought to only exist as completely unaligned disks.$^{2,3}$ It is important to emphasize that those studies only used a q-ratio between 2.5 and 3.5. To our knowledge, this is the first example of magnetically aligned bicelles at gel-phase temperatures. Potential advantages for utilizing high q-ratio samples have been discussed in the literature.$^{41-44}$
When utilizing different sample compositions, it is imperative that the optimal q-ratio be determined for that system. Sanders and coworkers reported that a q-ratio of 2.8 was optimal for their bicelle system, which gave highly resolved spectra free of any residual asymmetry. Based upon the criteria posed by Sanders and coworkers, a q-ratio of ~ 6.5 would be optimal for our bicelle system for solid-state NMR studies because the spectra have the narrowest linewidths (Figure 3(A)) and highest symmetry (Figures 5 and 6). For EPR studies, the q-ratio 9.5 would be optimal for determining the tilt orientation of a spin-labeled transmembrane peptide because the 9.5 q-ratio sample (Figures 1 and 2) had the highest molecular order and was the only q-ratio to be magnetically aligned in the gel phase at 25 °C.

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5.6 References


CHAPTER 6

Orientation of a TOAC-labeled Model Membrane Peptide Determined by Spin-labeled EPR Spectroscopy

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6.1 Abstract.

This research details an exciting new development for structural biology and spin-labeled EPR spectroscopy of integral membrane proteins. For the first time, this study demonstrates the feasibility of determining the topology of a spin-labeled integral membrane protein incorporated into magnetically aligned phospholipids bilayers in the relatively low magnetic field of an EPR spectrometer. The nitroxide spin probe, 2,2,6,6-tetramethylpiperidine-1-oxyl-14-amino-4-carboxylic acid (TOAC) was attached to the pore-lining transmembrane domain (M2δ) of the nicotinic acetylcholine receptor (AChR) and the corresponding EPR spectra revealed hyperfine splittings that were highly dependent on the macroscopic orientation of the membranes with respect to the magnetic field. The orientational dependent EPR spectra were used to determine the orientation of the α-helix with respect to the bilayer normal of the membrane. This new structural biology EPR technique is much more efficient and inexpensive than comparable solid-state NMR methods, because EPR spectroscopy is approximately a 1000 times more sensitive than NMR.

6.2 Introduction.

The nicotinic acetylcholine receptor (nAChR) is the most studied of all the neurotransmitter receptors and its structure-function has been well characterized by biochemical, biophysical, and mutagenesis techniques.1-6 These studies revealed that nAChR from mammalian muscle cells and the electric organs of Torpedo californica (electric ray) is a heterogeneous pentameric complex with a stoichiometry of α2β1γδ.7,8 Each subunit has four α-helical transmembrane spanning domains (M1, M2, M3 and M4), where the M2 transmembrane domain from each subunit forms the ion-channel pore. nAChR exhibits high sequence homology between the subunits and across several different species.7-10

A synthetic peptide based on the δ subunit M2 domain from Torpedo californica nicotinic acetylcholine receptor (AChR M2, Figure 6.2A), was shown to self assemble
within phospholipid bilayers to form functional ion-channel pores. A 2-D solid-state NMR PISEMA (Polarized Inversion with Spin Exchange at the Magic Angle) spectrum, which correlates the backbone amide $^{15}$N chemical shift and $^1$H—$^{15}$N dipolar coupling frequencies, revealed that AChR M2 forms a single confirmation in phospholipid bilayers—a linear $\alpha$-helical transmembrane peptide with no kinks, a helical tilt of 12°, and is rotated about the helical axis $\mathbf{h}$ so that the polar residues lining the pore point to the N-terminus (Figure 6.2C).

Therefore, AChR M2 was utilized as a model membrane peptide to develop a new spin-labeled EPR spectroscopic technique to study the orientation of integral membrane proteins with respect to phospholipid bilayers. The magnetic interactions of nitoxide spin labels used to label peptides and proteins show a high degree of spatial anisotropy. The EPR spectra of spin-labeled peptides have axially symmetric hyperfine interactions, where the observed values are dependent upon the time-averaged orientation the spin label makes with respect to the direction of the applied magnetic field, $B_0$. The orientation of the spin-labeled peptide with respect to the phospholipid bilayer (i.e., transmembrane or surface-binding) can be directly measured from the observed hyperfine splitting values ($A_{obs}$) of aligned EPR spectra only if the following criteria are met: (1) the unique axis of motion of the spin label (i.e., director axis, $z^2$) is restricted within the limits of a narrow cone, (2) the director axis is collinear with one of the principal axes of the spin label, and (3) the director axis makes a well-defined angle to the helical axis $\mathbf{h}$. The spin label 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) is a tetrasubstituted unnatural amino acid (see Figure 6.1A) and exhibits highly restricted motion due to the constrained structural conformations. TOAC can be functionalized with the 9-fluorenymethyl-oxycarbonyl (Fmoc) moiety (Figure 6.1B) for solid-phase peptide synthesis (SPPS) using an Fmoc-protected strategy. The TOAC spin label is known to stabilize $\alpha$-helical structures. Therefore, the $\alpha$-helical structure of a TOAC-labeled AChR M2 analog should be similar to AChR M2. Based on the X-ray structure of an $\alpha$-helical TOAC-labeled peptide, the spin label is structurally constrained and adopts only the twist-boat conformation, which causes the $z$-axis to make a 21° angle with $\mathbf{h}$, the $x$-axis is perpendicular to $\mathbf{h}$, and $z^2$ is expected to be collinear with $\mathbf{h}$ (Figures 6.2B). Therefore, TOAC-labeled EPR spectroscopy may potentially be a
powerful structural biology tool to determine the orientation of integral membrane peptides reconstituted into magnetically aligned membranes. This could lead to a greater understanding of how changes to the orientation of transmembrane domains through various biologically important mechanisms (hydrophobic mismatch, protein-protein and protein-lipid interactions, ligand-gated and voltage-gated events, etc.) regulate the activity of integral membrane proteins.

In this study, a TOAC-labeled AChR M2 analog was synthesized with Leu18 being replaced with the TOAC spin label (TOAC18 AChR M2, Figures 6.2A and B). From the solid-state PISEMA NMR study discussed above, the $^{15}$N chemical shift anisotropy, $\delta$, for Leu18 was 198.2 ppm, which is near the limit of the chemical shift tensor, $\sigma_{33}$ (217 ppm). This reflects a high degree of ordering at Leu18 due to highly restricted local backbone motions with $\sigma_{33}$ oriented nearly parallel to the applied magnetic field, $B_0$. Because of the high order at residue 18 and the restricted motion of TOAC, the hyperfine splitting values obtained from the spin-labeled EPR spectra of TOAC18 AChR M2 reconstituted into magnetically-aligned phospholipid bilayers, called bicelles, were strongly orientation-dependent and TOAC18 AChR M2 was found to be transmembrane in bicelles. This work represents the first time a magnetically aligned spin-labeled membrane peptide has been achieved for spin-labeled EPR spectroscopic studies. The focus of this work is to develop a technique that is complimentary to other structural biology techniques and to probe dynamics that are not available on the NMR timescale, thereby adding to and refining our understanding of the structure-function of membrane proteins.
**Figure 6.1.** A reaction schematic showing the synthesis of Fmoc-TOAC by reacting the 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) spin label with N-(9-fluorenylmethyloxycarbonyloxy)succinimide (Fmoc-OSu) for at least 24 hrs while maintaining the pH between 8.5 – 9.0 with triethylamine (TEA).
Figure 6.2  (A) Schematic showing the amino acid sequences of the channel-forming transmembrane domain (M2 domain, δ subunit) of *T.californica* nicotinic acetylcholine receptor (AChR M2) and the TOAC18 AChR M2 peptide, where τ is the TOAC spin label. (B) Schematic showing TOAC18 AChR M2 as a linear α-helical peptide with the principal axes of the TOAC spin label moiety being labeled as follows: the z-axis is directed along the π-orbital of nitrogen, the x-axis is directed along the N-O bond, and the y-axis is perpendicular to the other axes. Based on X-ray crystal structures, the z-axis should be nearly collinear to the helical axis *h* (21°). Also, the motion about the z-axis is expected to be restricted to the limits of a narrow cone due the highly restricted structural conformations of the TOAC label in α-helical peptides. (C) Based on solid-state NMR studies of AChR M2, the schematic shows the proposed transmembrane orientation of TOAC18 AChR M2 with respect to the phospholipids bilayers (transmembrane, helical tilt = 12°).
6.3 Experimental.

**Materials.** 1,2-dihexanoyl-sn-glycero-3-phosphatidylcholine (DHPC), 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000] (PEG2000-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Triethylamine (TEA), 30% ammonium hydroxide (NH$_3$(aq)), triisopylsilane (TIS), anisole, hexafluoroisopropanol (HFIP), thulium (III) chloride hexahydrate (Tm$^{3+}$), dysprosium (III) chloride hexahydrate (Dy$^{3+}$), and HEPES buffer were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol was purchased from Avocado Research Chemicals (Ward Hill, MA). 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) was purchased from Acros Organics (Pittsburgh, PA). N-(9-fluorenylmethyloxycarbonyloxy)succinimide (Fmoc-OSu), Fmoc-protected amino acids, 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyleuronium hexafluorophosphate (HCTU), and Fmoc-Arginine(Pbf)-NovaSyn TGA resin were purchased from Novabiochem (La Jolla, CA). Trifluoroacetic acid (TFA), 1-hydroxybenzo-triazole (HOBt), diisopropylethylamine (DIEA), N-methylpyrolidone (NMP), dichloromethane (DCM), and acetic anhydride (Ac$_2$O) were purchased from Applied Biosystems Inc. (Foster City, CA).

**Fmoc-TOAC synthesis.** The synthesis was done according to published procedures.$^{18,27}$ TOAC (1 equiv in 20 mL H$_2$O) was reacted with Fmoc-OSu (1.1 equiv in 20 mL acetonitrile), the pH was adjusted between 8.5 – 9.0 with TEA, and the reaction was allowed to proceed for at least 24 hrs. Unreacted Fmoc-OSu was filtered from the reaction mixture and discarded. The reaction mixture was rotary evaporated to remove acetonitrile. The reaction mixture was acidified with ice cold 10% acetic acid (10 mL), and transferred to a separatory funnel. Several organic extractions were performed with ethyl acetate until the organic layer was clear. The ethyl acetate fractions were pooled, concentrated by rotary evaporation, and placed into a separatory funnel. The ethyl acetate mixture was washed twice with H$_2$O, twice with aqueous NaCl (saturated), and dried with Na$_2$SO$_4$ overnight. The Na$_2$SO$_4$ was filtered from the ethyl acetate mixture and discarded. The final product was recrystallized from the ethyl acetate mixture with hexane, filtered, dried with dry N$_2$ gas, and vacuum desiccated overnight. The synthesis
was monitored on TLC plates using a 1:3 mixture of ethyl acetate/methanol as the mobile phase. The TLC plates were developed with 50% H$_2$SO$_4$ (aq) and heated in an oven at 50 °C.

**TOAC18 AChR M2 peptide synthesis.** Solid-phase peptide synthesis using an Fmoc-protection strategy$^{19,28}$ was performed on a 433A Peptide Synthesizer from Applied Biosystems Inc. (Foster City, CA) to synthesize the model membrane peptide TOAC18 AChR M2 using the channel-forming transmembrane domain (M2 domain, δ subunit) of *T.californica* nicotinic acetylcholine receptor (AChR M2) as a template. The amino acid sequences of TOAC18 AChR M2 and AchR M2 are shown in Figure 6.2A. The peptide synthesizer was equipped with a UV detector (wavelength set to 301 nm) to monitor the Fmoc removal from the N-terminus of the growing peptide. The FastFmoc chemistry-0.1 mmol protocol provided in the SynthAssist 2.0 software (Applied Biosystems Inc., Foster City, CA) was modified to optimize the yield of the synthesis and allow for customized functionality (i.e., double coupling, increased coupling times, incorporating unnatural amino acids, etc.).$^{28}$ An appropriate amount of Fmoc-Arginine(Pbf)-NovaSyn TGA resin (substitution number = 0.22 g resin/mmol peptide) was used to synthesize 0.1 mmol of peptide (based on a theoretical yield of 100%). All amino acids were purchased as the Fmoc-protected form and with chemically modified side-chain residues (side-chain protected) to minimize any side reactions.

**Post TOAC18 AChR M2 peptide synthesis preparation.** A cleavage mixture of TFA/TIS/anisole/H$_2$O (8.5 mL/0.5 mL/ 0.5 mL/0.5 mL) was added to an appropriate amount of peptide-resin and allowed to react for 3 hrs to remove the side-chain protecting groups (except for the tert-butyl moiety (tBu) on Cys16) and cleave the peptide from the resin. The peptide was filtered to remove the resin beads and precipitated with cold methyl tert-butyl ether. The precipitated peptide was centrifuged, and the pellet was dried under dry N$_2$ gas and vacuum desiccated overnight. Because TFA from the cleavage mixture reduces nitroxides to the hydroxylamine form, the spin-labeled peptides were treated with NH$_3$ (aq) to reoxidize the spin label back to the nitroxide form, then lyophilized and stored at −20 °C.

Spin-labeled peptides were purified on a C$_4$ reverse-phase analytical column (214TP104, 10µm, 300 Å pore size, 0.46 × 25 cm, column volume of 4.15 mL) from
Grace Vyadac, Inc. (Hesperia, CA) operated at a flow rate of 1 ml/min. Solvent A (Sol A) consisted of 0.1% TFA in H$_2$O, and solvent B (Sol B) consisted of 50% n-propanol, 50% H$_2$O, and 0.1% TFA. The elution was performed as follows: column equilibrated at 5% Sol B (95 % Sol A) over two column volumes (CV), gradient increased to 50 % Sol B (50 % Sol A) over 1 CV, then isocratic at 50 % Sol B over 3 CV. The C$_4$ column was interfaced with an AKTA Explorer 10S HPLC controlled by the Unicorn 3.0 software from Amersham Pharmacia Biotech (Piscataway, NJ). The synthesis and purification of the peptide was analyzed with MALDI-TOF and analytical HPLC.

**TOAC18 AChR M2 peptide incorporation into bicelle samples.** Appropriate volumes of the DMPC, PEG2000-PE, and cholesterol were dispensed into a 25 mL pear-shaped flask (Flask 1) and DHPC and TOAC18 AChR M2 peptide were dispensed into a second 25 mL pear-shaped flask (Flask 2). The mole ratios of DMPC/DHPC/PEG2000-PE/cholesterol/spin-labeled peptide were respectively 3.5/1.0/0.035/0.35/0.0035. The samples were dried with N$_2$ gas and vacuum desiccated overnight to remove all organic solvents. The following day, an appropriate amount of HEPES buffer (100 mM) was added to Flask 2, vortexed until contents of Flask 2 were solublized (~15 min), contents from Flask 2 were transferred to Flask 1, vortexed until the contents of Flask 1 were solublized (~1 hr), and then sonicated for an additional 30 min at 4 °C. The final lipid concentration was 25 wt %. The samples were annealed by performing at least three freeze-thaw cycles using liquid nitrogen. A small volume of Tm$^{3+}$aq) or Dy$^{3+}$aq) was added to the bicelle samples (final lanthanide cation concentration was 20 mol % with respect to DMPC).

**Sample preparation for spin-labeled X-band EPR spectroscopy.** Samples were drawn into ID capillary tubes (1mm i.d.) and sealed on both ends with Critoseal (Fisher Scientific, Florence, KY). The capillary tubes were placed into standard quartz EPR tubes (707-SQ-250M, Wilmad Glass, Buena, NY) that were filled with light mineral oil. Magnetic alignment was induced by placing the lanthanide-doped sample into the X-band EPR spectrometer at 298 K and raising the temperature to 318 K over 11 min while in the presence of an applied magnetic field (0.64 T), after which EPR spectra were taken at 318 K.$^{22,29}$ A randomly dispersed sample was obtained by placing a Tm$^{3+}$-doped sample
into the spectrometer that was preheated to 318 K and an EPR spectrum was taken after thermal equilibration for ~ 10 min.\textsuperscript{22,29}

*Spin-labeled X-band 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 an ER 4119HS cavity that is coupled with an ER 4131VT nitrogen gas temperature controller (temperature stability of ± 0.2 °C). Each spin-labeled EPR spectrum was acquired by taking a 42-s field-swept scan (3370 G center field, 100 G sweep width, 9.43 GHz microwave frequency, 100 kHz modulation frequency, 1.0 G modulation amplitude, and a microwave power of 2 mW). All EPR spectra were processed on a 300 MHz G3 Macintosh computer utilizing the Igor software package (Wavemetrics, Lake Oswego, OR).

### 6.4 Results and Discussion

*Theoretical.* The hyperfine splitting tensoral components $A_{xx}$, $A_{yy}$, and $A_{zz}$ (7.455 G, 7.455 G and 33.3 G, respectively) are coincident with the respective principal $x$-, $y$-, and $z$-axes of the TOAC spin label (Figure 6.2B).\textsuperscript{30} The observed hyperfine splitting ($A_{\text{obs}}$) is a time-averaged value of the respective tensoral components in the direction of $B_0$. Because the axis of rotation is expected to be about the helical axis $\mathbf{h}$ and the $z$-axis is expected to be almost parallel to $\mathbf{h}$, the time-averaged value of $A_{\text{obs}}$ will be similar to that observed for the spin probe 5-doxylstearic acid (see Chapter 2), which has a similar spatial behavior as TOAC18 AChR. The following scenarios are the theoretical $A_{\text{obs}}$ values that will be expected for samples having aligned or randomly dispersed TOAC18 AChR helical peptides:

**Case 1.** If the helical axis of TOAC18 AChR ($\mathbf{h}$) is oriented parallel to $B_0$, then the principle $z$-axis will be nearly parallel with $B_0$. Due to the rotation about $\mathbf{h}$, the time-averaged hyperfine splitting interaction will be dominated by the $z$-tensoral component and $A_{\text{obs}} (A_{||})$ will be close to the value of $A_{zz}$ (33.3 G).
Case 2. If $h$ is oriented perpendicular to $B_0$, then the $z$-axis will also be perpendicular to $B_0$ and rotation about $h$ will result in $A_{\text{obs}}$ ($A_\perp$) to be close to the time-averaged value of the $x$- and $y$-tensoral components (i.e., $A_\perp = (A_{xx} + A_{yy})/2 = 7.455$ G).

Case 3. If $h$ has an isotropic distribution accompanied with rapid reorientation of the peptide or the motion of the spin label is not restricted to the limits of a narrow cone (i.e., isotropic motion), then all tensoral components are time averaged and $A_{\text{obs}} \approx a_N = (A_{zz} + A_{yy} + A_{xx})/3 = 16.07$ G ($a_N$ is the isotropic hyperfine splitting constant). Also, the spectrum will have three distinct resonant lines with narrow lineshapes.

Case 4. If the helical axis has an isotropic distribution but the reorientation of the peptide is highly anisotropic and the motion of the spin label is immobilized or slow on the EPR timescale, then the spectrum will not be time-averaged and the lineshape will no longer show three resonant lines. Instead, a broad powder-like spectrum will be obtained, where the lineshape is an envelope of superimposed spectra for every possible orientation of $h$.

If TOAC18 AChR behaves similar to the AChR M2 peptide when incorporated into DMPC phospholipid bilayers, then it will be approximately transmembrane ($h \parallel n$) with a helical tilt of approximately 12° (Figure 6.2B). $A_{\text{obs}}$ will be expected to be close to the theoretical value of 33.3 G or 7.455 G when $n$ is oriented, respectively, parallel or perpendicular to $B_0$ (i.e., Case 1 and Case 2).

Experimental. Figure 6.3A shows the spin-labeled EPR spectra of the TOAC18 AChR M2 peptide reconstituted into bicelle membranes that were magnetically aligned in the presence of added Tm$^{3+}$ (middle spectrum) or Dy$^{3+}$ (bottom spectrum), where the average $n$ is respectively oriented either parallel or perpendicular to the direction of the applied magnetic field $B_0$ (middle and bottom schematics, Figure 6.3B). A randomly dispersed EPR spectrum was obtained for comparison (top spectrum and schematic, Figures 6.3A and 6.3B).

The randomly dispersed spectrum exhibits a broad powder-like spectrum with a typical lineshape and a rotational correlation time ($\tau_\| = 1.8 \times 10^{-8}$ s) that is in the slow
Figure 6.3. Spin-labeled X-band EPR spectra (A) and corresponding schematics (B) of TOAC18 AChR M2 reconstituted into either randomly dispersed (top row) or magnetically aligned (middle and bottom rows) phospholipid bilayers. For magnetically aligned samples, the average normal to the phospholipid bilayers, \( \mathbf{n} \), were oriented either parallel (middle spectrum and schematic) or perpendicular (bottom spectrum and schematic) to the applied magnetic field, \( B_0 \). The outer \( 2A_{\text{max}} \) and inner \( 2A_{\text{min}} \) extrema used to approximate \( A_{\parallel} \) and \( A_{\perp} \) from the randomly dispersed spectrum is indicated in the top spectrum and the measurement of \( A_{\text{obs}} \) from the parallel-aligned spectrum is indicated in the middle spectrum.
motional regime. These spectral features follow the expected results listed in Case 4 above and supports the assumption that the TOAC label will be motionally restricted when incorporated into AChR M2. The hyperfine splitting values for well-aligned samples with \( \mathbf{h} \) either parallel \( (A_\parallel) \) or perpendicular \( (A_\perp) \) to \( B_0 \) can be approximated from the spectral extrema of the randomly dispersed spectrum.\(^{32} \) The maximum extrema \( (2A_{\text{max}}) \) correspond to \( 2A_\parallel \) and the minimum extrema \( (2A_{\text{min}}) \) correspond to \( 2A_\perp \) (indicated in top spectrum, Figure 6.3A). The \( 2A_{\text{max}} \) and \( 2A_{\text{min}} \) values were, respectively, 60.020 G and 17.302 G. Therefore, the approximate hyperfine splitting values for \( A_\parallel \) and \( A_\perp \) are 30.010 G and 8.651 G, respectively. The degree of order about the \( z \)-axis with respect to \( \mathbf{h} \) is given by the anisotropic order parameter \( S_{zz} \) and is obtained directly from the randomly dispersed spectrum and by the following equations

\[
S_{zz} = \frac{(A_\parallel - A_\perp)(a_N')[A_{zz} - (A_{xx} + A_{yy})/2]}{a_N'} \quad (1)
\]

\[
a_N' = \frac{(A_\parallel + 2 A_\perp)}{3} \quad (2)
\]

\[
S_{zz} = \frac{3\langle \cos^2 \theta \rangle - 1}{2} \quad (3)
\]

where \( \theta \) is the time-averaged angle between the \( z \)-axis and \( \mathbf{h} \) and \( a_N' \) corrects for the local polarity about the nitrooxide radical. Incorporating \( A_\parallel \) and \( A_\perp \) from the randomly dispersed spectrum into Equation 1 results in \( S_{zz} = 0.842 \). Incorporating the result from Equation 1 into Equation 3 results in the apparent angle \( \theta = 18.9^\circ \). This is close to the theoretical value of 21° obtained from the crystal structures of TOAC-labeled \( \alpha \)-helical peptides.

The magnetically aligned samples exhibited well-aligned EPR spectra with three highly resolved resonant lines and \( A_{\text{obs}} \) showed strong orientation dependence. \( A_{\text{obs}} \) values were measured between the down-field and center-field peaks as illustrated for the parallel-aligned spectrum (middle spectrum, Figure 6.3A). \( A_{\text{obs}} \) values of 26.6 G and 11.8 G were obtained for the parallel-aligned (middle spectrum) and perpendicular-aligned (bottom spectrum) samples, respectively. These values are close to the approximated values of \( A_\parallel \) (30.010 G) and \( A_\perp \) (8.651 G) obtained from the randomly dispersed spectrum (top spectrum, Figure 6.3A) and the departure in these values (respectively, 3.41 G and 3.15 G) is due to a small degree of disorder in the helical tilt of the peptide.\(^{33} \)
The orientation dependence and narrow lineshapes exhibited in the parallel- and perpendicular-aligned spectra follow the expected results listed, respectively, in Cases 1 and 2 above. The asymmetry in the lineshapes (i.e., linewidths and relative peak heights are not equal for each peak) arise from second order contributions, which become significant when the motion of the spin label approaches the rigid limit on the EPR timescale. This supports the results that the TOAC label is highly constrained and the reorientation about $\mathbf{h}$ is in the slow motional regime.

The angle that the $z$-axis makes with $B_0$ (i.e., $\varphi$) for the parallel-aligned sample can be approximated utilizing the following equation

$$A_{\text{obs}} = (A_\parallel \cos^2 \varphi + A_\perp \sin^2 \varphi)^2$$  \hspace{1cm} (4)$$

where $A_\parallel$ and $A_\perp$ were obtained from the randomly dispersed spectrum (30.010 G and 8.651 G, respectively). Therefore, using $A_{\text{obs}}$ from the parallel-aligned spectrum (26.002 G), we calculate $\varphi = 28.9^\circ$. The angle $\varphi$ includes the helical tilt of the peptide with respect to $B_0$ ($\theta$) and the angle $\mathbf{h} \angle z$-axis. By taking into account $\theta = 18.9^\circ$, then $\varphi = 10.0^\circ$. This is in good agreement with the helical tilt of 12° obtained from PISEMA NMR spectroscopic studies of AChR M2. This result indicates that the angular dependence of the magnetically aligned EPR spectra can be used to determine the orientation of an integral membrane protein relative to the bilayer normal.

### 6.5 Conclusions.

This study details an exciting new development for structural biology. This is the first time a spin-labeled model membrane peptide has been incorporated into magnetically aligned phospholipid bilayers utilizing the weak magnetic field strengths from an X-band EPR spectrometer. Our results revealed aligned EPR spectra with strong orientation-dependent hyperfine splitting interactions ($A_\parallel - A_\perp \sim 14$ G). TOAC18 AChR M2 was found to be transmembrane to the phospholipid bilayers with a helical tilt of 10.0° and the $z$-axis $\angle \mathbf{h} = 18.9^\circ$. These values were directly obtained from the EPR
spectra without the need to simulate the spectra and were comparable to those found in PISEMA NMR and X-ray crystallographic studies. Therefore, TOAC-labeled EPR spectroscopy can be a powerful tool that is complimentary to other biophysical techniques to increase our understanding of the structure-function relationships of integral membrane proteins. There are some additional advantages in utilizing this new technique. EPR spectroscopy is approximately a thousand fold more sensitive than NMR spectroscopy. Even at much lower protein/peptide concentrations (µg scale for EPR versus mg scale for NMR), the high S/N ratio obtainable via EPR spectroscopy (one scan, 1min) when compared to solid-state NMR spectroscopy (10,000 scans, 14 hours) will instantaneously indicate the membrane protein orientation with respect to the lipid bilayer. This shorter experimental time will allow for an array of experimental conditions to be performed on the same sample. Because of the higher sensitivity, EPR spectroscopy requires a much lower peptide-to-lipid ratio than solid-state NMR (1:1000 for EPR versus 1:50 to 1:25 for NMR), which would minimize any perturbation to the membrane system upon incorporation of the peptide. Thus, the data presented here indicate that this new structural biology technique is inexpensive and more efficient than comparable solid-state NMR methods to determine the topology/orientation of integral membrane proteins within the lipid bilayer.

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6.6 References.


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