Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Magnetic Resonance 161 (2003) 77–90
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Magnetically aligned phospholipid bilayers in weak magnetic fields: optimization, mechanism, and advantages for X-band EPR studies

Thomas B. Cardon, Elvis K. Tiburu, and Gary A. Lorigan*

Department of Chemistry and Biochemistry, Miami University, Oxford, OH 45056, USA

Received 26 July 2002; revised 28 October 2002

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 Tm^{3+}-doped bicelle disks was thermodynamically and kinetically very sensitive to experimental conditions. Tm^{3+}-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-cholestane (cholestane) 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).

Keywords: Magnetic alignment; Phospholipid membrane; Spin-labeled EPR; EPR spectroscopy; Solid-state NMR spectroscopy

1. 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 [2–5], which aligns in the presence of a magnetic field, has great promise in NMR spectroscopic studies of membrane and globular proteins [3,4,6–13]. 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 [2]. The normal to the bicelle disk is perpendicular to the plane of the bilayered region and is parallel with the director, **n**. The dimensions of the bicelle disks are dependent on the molar ratio (*q*-ratio) of the long-chain to short-chain phospholipids [14]. Bicelle disks are stable over a large range of *q*-ratios (0.2–6) and hydration (40–95%, wt%) [15–18]. This study used 1,2-dimyristoyl-*sn*-glycerol phosphatidylcholine (DMPC) and 1,2-dihexanoyl-*sn*-glycerol phosphatidylcholine (DHPC) as the long-chain and short-chain phospholip-

* Corresponding author. Fax: 1-513-529-5715.
E-mail address: lorigag@muohio.edu (G.A. Lorigan).

1090-7807/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1090-7807(02)00109-X

ids, respectively, with a mole ratio of DMPC:DHPC of 3.5 (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 [19–21]. The spin probe 3β-doxyl-5α-cholestane (cholestane 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 [22–24]. Previously, we showed three cholestane 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 [24]. Also, a detailed discussion on the anisotropic hyperfine splitting tensoral values for the cholestane spin label was previously given [24]. When the bicelle disks are randomly dispersed and motionally averaged, the observed hyperfine splitting, A_{obs}, for the cholestane 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 cholestane are nearly perpendicular to the static magnetic field. Because cholestane rapidly rotates around the long molecular axis, the observed hyperfine splitting is $A_{\text{obs}} = (A_{xx} + A_{zz})/2$ 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_{obs} for randomly dispersed, perpendicular-aligned, and parallel-aligned bicelle disks were, respectively, 16.0, 18.1, and 9.8 G [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 [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 [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_α) phase [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 318 K [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+}) [24].

The preferred orientation of the bicelle disk director **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:

$$\Delta\chi^{\text{bicelle}} = \chi_{\parallel}^{\text{bicelle}} - \chi_{\perp}^{\text{bicelle}}, \tag{1}$$

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 **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+}) [26], which bind to the phosphatidylcholine headgroups [27,28]. 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 [29]. Mironov and co-workers [29] and Prosser and co-workers [30,31] 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 histeresis effects [32–36].

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 [18,20,29,32,33,35,37–40]. 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 [29,32] or upon heating from the hexagonal phase to the smectic phase [34]. 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.) [22,25,27,28,41]. 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 [42–49] to determine the orientation of a variety of membrane-associated spin-labeled peptides or proteins. For example, a cysteine specific nitroxide 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) [42,45,48] or by incorporating TOAC or POAC spin-labeled amino acids into the sequence of synthesized peptides [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 [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 [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.

2. 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.

2.1. 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 Fig. 1, the minimum temperature-ramping time required to obtain 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 ob-

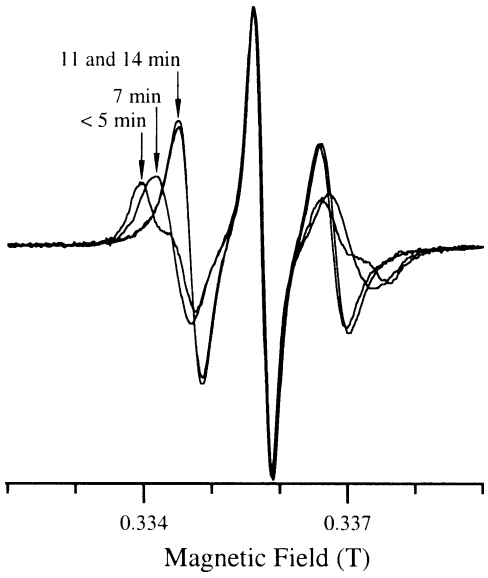

Fig. 1. A stacked plot of normalized cholestane spin-labeled EPR spectra of Tm^{3+}-doped bicelle disks showing the time dependence of the macroscopic magnetic alignment procedure. The bicelle sample composition consisted of DMPC/DHPC/cholesterol/PEG 2000 PE/cholestane/Tm^{3+} in molar ratios of 3.5/1.0/0.35/0.035/0.0056/0.70 in 100 mM 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 318 K 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.

tained at 318 K and are denoted according to the temperature-ramping time used and the spectra are displayed in Fig. 1. The hyperfine splitting was measured for each EPR spectrum shown in Fig. 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 Fig. 3), but significantly larger than that obtained from bicelle disks oriented in the parallel alignment at 318 K (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 [23]. 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.

2.2. 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 [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^{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 Fig. 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^{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.

Fig. 2 shows a select few of the spin-labeled EPR spectra from the experiment described above. All

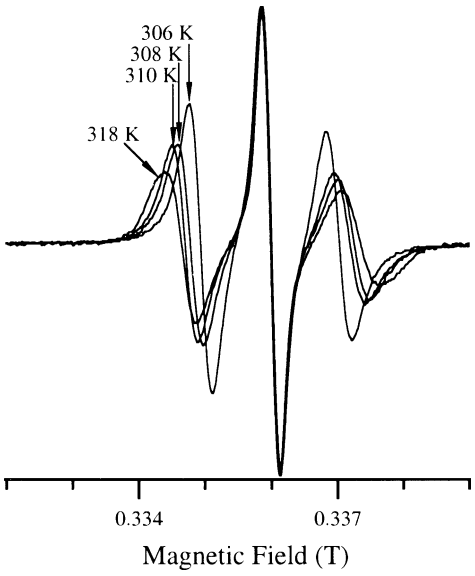

Fig. 2. A stacked plot of normalized cholestane spin-labeled EPR spectra of Tm^{3+}-doped bicelle disks showing that the magnetic alignment process of the Tm^{3+}-doped bicelle sample is dependent upon the initial temperature of the magnetic alignment procedure. The magnetic alignment procedure was performed several times on a Tm^{3+}-doped bicelle sample (same sample composition as in Fig. 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.

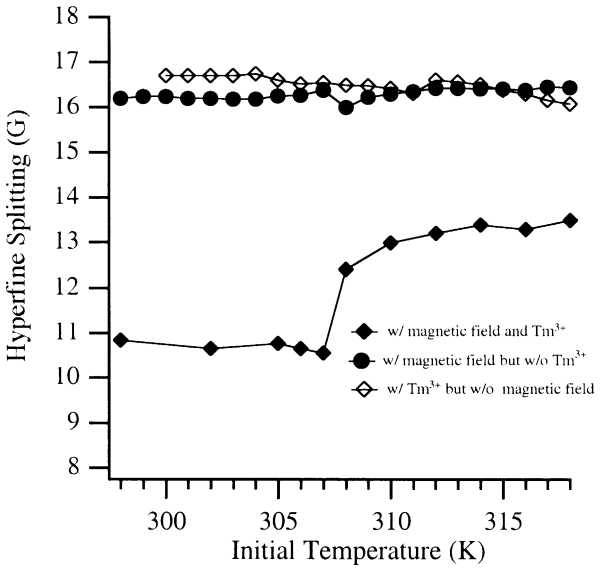

Fig. 3. A graph showing the initial temperature dependence on the magnetic alignment process of Tm^{3+}-doped bicelle disks using the magnetic alignment procedure described in Fig. 2. The hyperfine splitting of the cholestane spin-labeled EPR spectra from Fig. 2 (measured between the down-field and center 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 Fig. 1) was subjected several times to the same magnetic alignment procedure described in Fig. 2, but in the absence of a magnetic field. In the second method (*solid circles*), the same magnetic alignment procedure described in Fig. 2 was performed several times on a bicelle sample with the same sample composition as described in Fig. 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*).

experiments performed using the magnetic alignment procedure with an initial temperature between 298 and 307 K were identical in their hyperfine splitting ($\sim$10.6 G) and lineshape as the 11 min EPR spectrum in Fig. 1 and is typical of cholestane spin-labeled EPR spectra for parallel-aligned bicelle disks at 318 K [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 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.

Fig. 3 shows a more convenient way of looking at the results of the EPR spectra obtained in Fig. 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 (Fig. 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 Fig. 1) was subjected several times to the same magnetic alignment procedure described in Fig. 2, but in the absence of a static magnetic field. In the second method (*solid circles*), the same magnetic alignment procedure described in Fig. 2 was performed several times on a bicelle sample with the same sample composition as described in Fig. 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 Fig. 3 as a function of the corresponding

initial temperature used in the magnetic alignment procedure (*open-faced diamonds* and *solid circles*).

Fig. 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, Fig. 3 shows more clearly than Fig. 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 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 [23]. 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 [23].

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 Fig. 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 Fig. 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 Fig. 4 as a function of the temperature at which the spectra were acquired (*solid circles*). For the cholestane 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 [23]. 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-

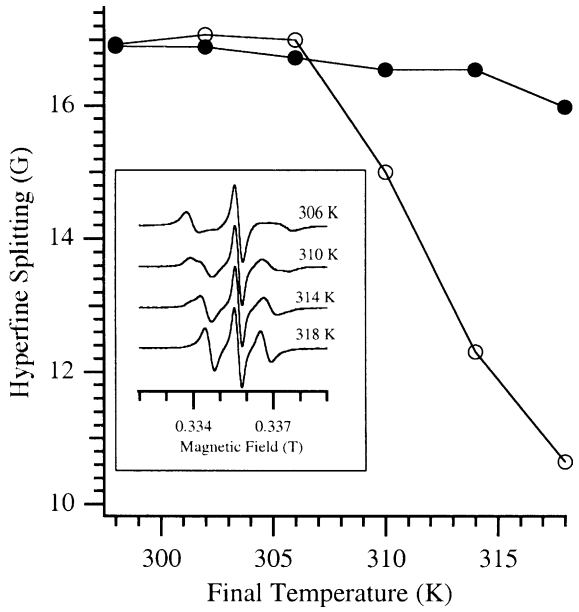

Fig. 4. A graph showing the final temperature dependence on the magnetic alignment process of Tm^{3+}-doped bicelle disks. The magnetic alignment procedure was performed several times on a Tm^{3+}-doped bicelle sample (same sample composition as described in Fig. 1). The initial temperature of the magnetic 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 Fig. 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 Fig. 1 except the alignment reagent Tm^{3+} was omitted.

dependent hyperfine splitting curve for randomly dispersed bicelle disks at these same temperatures [23]. Combining these results with the results in Figs. 2 and 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 (Fig. 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 (Fig. 4,inset). At a final temperature of 318 K the cholestane spin-labeled EPR spectrum (Fig. 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 (Fig. 4,inset), which is in agreement of well-aligned Tm^{3+}-doped bicelle disks in the parallel alignment at a temperature of 318 K (Figs. 1–3) [23]. Therefore, it was determined that the minimum final temperature of the optimized magnetic alignment procedure was 318 K.

2.3. Determination of the phase changes for Tm^{3+}-doped bicelle disks using solid-state ^2H 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 metallomesogen [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 histeresis effects [29,32]. Firestone and co-workers [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 ^2H NMR study [31] and a neutron diffraction study [35] observed a lanthanide-induced nematic-to-smectic phase transition for parallel-aligned Tm^{3+}-doped bicelle disks.

Fig. 5 shows several solid-state ^2H 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 ^2H NMR spectrum was acquired. The solid-state ^2H NMR spectra taken between 298 and 302 K exhibit an isotropic phase (only the spectra 298 K is shown in Fig. 5), which has been described as either rapidly tumbling bicelle disks accompanied by higher fluidity of the sample or a mixed micelle phase [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 [15,17]. Solid-state ^2H 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_α phase at these temperatures. This main phase transition temperature is greater than that observed for pure DMPC without added Tm^{3+} [28], which may be explained by the Tm^{3+} cations invoking greater order and packing of the phospholipid acyl chains [28,31]. ^2H 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_α phase. ^2H NMR spectra taken at a temperature of 313 K or above showed increased resolution and order and was ascribed by Prosser and co-workers [31] and Katsaras and

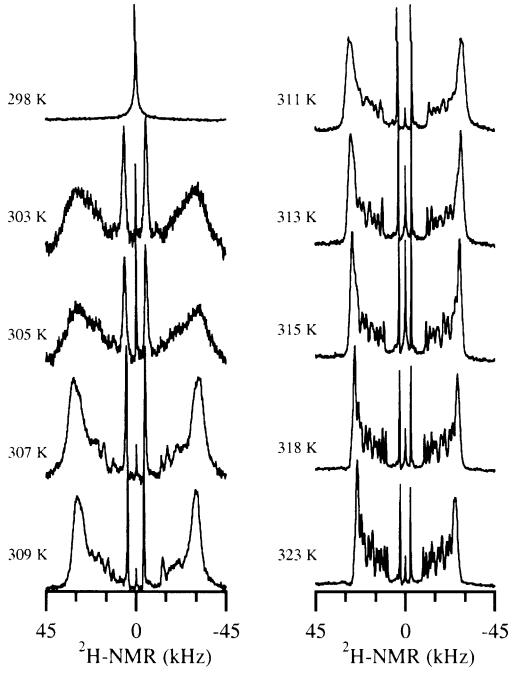

Fig. 5. Normalized solid-state ^2H NMR spectra of a Tm^{3+}-doped bicelle sample showing the various temperature-dependent phases of the Tm^{3+}-doped bicelle system. The sample composition was the same as in Fig. 1 except cholestane is omitted and the isotopic label $DMPC_{d54}$ was added (8% $DMPC_{d54}$/DMPC molar ratio). ^2H 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.

co-workers [35] as the formation of a lanthanide-induced lamellar L_α smectic phase.

2.4. Magnetic alignment of the smectic phase near the isotropic phase

Prosser and co-workers [31] showed that a lanthanide-induced lamellar L_α smectic phase could be cooled slowly to a temperature close to the isotropic phase without the recovery of the L_α nematic phase and retain its high degree of order and alignment. If a smectic phase had formed for the experiments described in Figs. 2–4, 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, Fig. 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, Figs. 4 and 5). Therefore, the magnetic alignment procedure was performed on a Tm^{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. Fig. 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 Fig. 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 [23]. This EPR spectrum exhibited a broad down-field peak as compared to the cholestane spin-labeled EPR spectrum of a well-aligned sample at 318 K (Figs. 1–4). 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 Fig. 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 Fig. 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 Fig. 5.

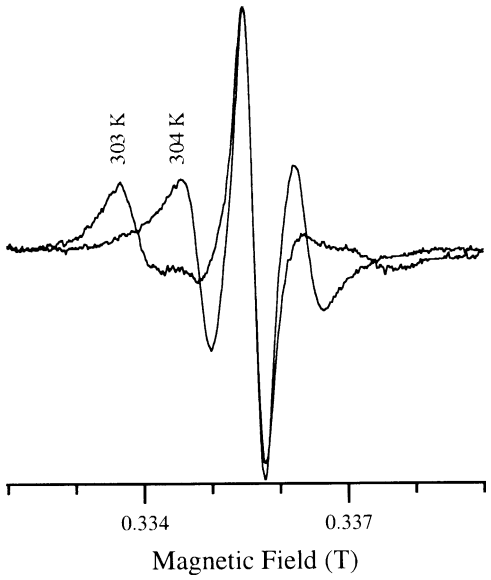

Fig. 6. A stacked plot of cholestane spin-labeled EPR spectra showing the retention of well-aligned phospholipid bilayers upon lowering the sample temperature close to the main liquid crystalline phase–gel phase transition temperature (303 K). The sample composition and magnetic alignment procedure were the same as described for the 11 min EPR spectrum in Fig. 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.

3. 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 [23]. From the solid-state ^{2}H NMR spectra in Fig. 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 [2,15–18,40]. 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 [23]. In the latter case, small, rapidly tumbling bicelle disks accompanied by low viscosity similar to that of pure water [2] 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 [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 [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) [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^{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 (Figs. 2 and 3) [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 [15,17,31,35]. Struppe and Vold [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 [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) [17], then small polydomains of stacked lamellar sheets may have formed in the smectic phase [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 [17]. 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 [29]. This is supported by a study [32] which observed a low effective magnetic moment μ_{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 [29] and Binnemans and co-workers [32] 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_α phase, and the smectic L_α 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 [31]. 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 [29] 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 [29].

Binnemans and co-workers [32] showed that for their liquid crystalline sample there was a large increase in

effective magnetic moment μ_{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 (Figs. 2 and 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 [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 [18,39]. Also, the presence of lanthanide cations may also aid in increasing the hydration attraction forces between bicelle disks [39], and may act to stabilize the formation of the bicelle disks [18]. Gaemers and Bax [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 [29]. This was supported by the results in Fig. 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 [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 [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 Fig. 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 (Fig. 5) after partial or complete alignment of the Tm^{3+}-doped bicelle disks would serve to stabilize macroscopic alignment. Partial alignment of Tm^{3+}-doped bicelle disks is observed in the EPR spectra in Fig. 4 (310 and 314 K, 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 [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 [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 (Fig. 5) to the final temperature of 318 K (Fig. 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^{3+}-doped bicelle disks (Figs. 2–4) coincide very well with the various phases observed in the ^2H NMR spectra in Fig. 5. Therefore, we make the assumption that at low magnetic field strengths Tm^{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 [23]. Stabilization of the magnetic alignment of the bicelle system in the smectic phase is supported in a study by Firestone and coworkers [34]. 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 [34].

3.1. 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 fields 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.

4. Experimental

4.1. Materials

1,2-Dihexanoyl-*sn*-glycerol phosphatidylcholine (DHPC), 1,2-dimyristoyl-*sn*-glycerol phosphatidylcholine (DMPC), deuterated 1,2-dimyristoyl-*sn*-glycerol phosphatidylcholine ($DMPC_{d54}$), 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-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).

4.2. 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 Tm^{3+} 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.

4.3. 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 2.5 mW.

4.4. Solid-state ^2H NMR studies

The sample composition was the same as that for the EPR studies except cholestane was omitted from the sample and $DMPCd_{54}$ (8% molar ratio of $DMPCd_{54}$ 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 ^2H NMR spectra were recorded using a quadecho pulse sequence (4-μs 90° pulses, 45-μs delay, 5.12-ms acquisition time, 1024 scans) at a ^2H resonance frequency of 46.07 MHz. Solid-state ^2H 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.

4.5. 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).

Acknowledgments

This work was supported by a National Institutes of Health Grant (GM60259-01) and a National Science Foundation CAREER Award (CHE-0133433). The X-band EPR spectrometer was obtained from a National Science Foundation Grant (CHE-97,24192).

References

[1] A. Arora, L.K. Tamm, Biophysical approaches to membrane protein structure determination, Curr. Opin. Struct. Biol. 11 (2001) 540–547.
[2] C.R. Sanders, J.P. Schwonek, Characterization of magnetically orientable bilayers in mixtures of dihexanoylphosphatidylcholine and dimyristoylphosphatidylcholine by solid-state NMR, Biochemistry 31 (1992) 8898–8905.
[3] C.R. Sanders, B.J. Hare, K.P. Howard, J.H. Prestegard, Magnetically-oriented phospholipid micelles as a tool for the study of membrane-associated molecules, Prog. NMR Spectrosc. 26 (1994) 421–444.
[4] C.R. Sanders, G.C. Landis, Reconstitution of membrane-proteins into lipid-rich bilayered mixed micelles for NMR-studies, Biochemistry 34 (1995) 4030–4040.
[5] C.R. Sanders, R.S. Prosser, Bicelles: a model membrane system for all seasons?, Structure 6 (1998) 1227–1234.
[6] J. Struppe, R.R. Vold, Dilute bicellar solutions for structural NMR work, J. Magn. Reson. 135 (1998) 541–546.
[7] M. Zweckstetter, A. Bax, Single-step determination of protein substructures using dipolar couplings: aid to structural genomics, J. Am. Chem. Soc. 123 (2001) 9490–9491.
[8] N. Tjandra, A. Bax, Direct measurement of distances and angles in biomolecules by NMR in a dilute liquid crystalline medium, Science 278 (1997) 1111–1114.
[9] R.S. Prosser, V.B. Volkov, I.V. Shiyanovskaya, Solid-state NMR studies of magnetically aligned phospholipid membranes: taming lanthanides for membrane protein studies, Biochem. Cell Biol. 76 (1998) 443–451.
[10] M. Ottiger, A. Bax, Characterization of magnetically oriented phospholipid micelles for measurement of dipolar couplings in macromolecules, J. Biomol. NMR 12 (1998) 361–372.
[11] K.P. Howard, S.J. Opella, High-resolution solid-state NMR spectra of integral membrane proteins reconstituted into magnetically oriented phospholipid bilayers, J. Magn. Reson. B 112 (1996) 91–94.
[12] G. Kontaxis, A. Bax, Multiplet component separation for measurement of methyl C-13–H-1 dipolar couplings in weakly aligned proteins, J. Biomol. NMR 20 (2001) 77–82.
[13] G. Cornilescu, A. Bax, Measurement of proton, nitrogen, and carbonyl chemical shielding anisotropies in a protein dissolved in a dilute liquid crystalline phase, J. Am. Chem. Soc. 122 (2000) 10143–10154.
[14] R.R. Vold, R.S. Prosser, Magnetically oriented phospholipid bilayered micelles for structural studies of polypeptides. Does the ideal bicelle exist?, J. Magn. Reson. B 113 (1996) 267–271.

[15] S. Gaemers, A. Bax, Morphology of three lyotropic liquid crystalline biological NMR media studied by translational diffusion anisotropy, J. Am. Chem. Soc. 123 (2001) 12343–12352.
[16] P.A. Luchette, T.N. Vetman, R.S. Prosser, R.E.W. Hancock, M.P. Nieh, C.J. Glinka, S. Krueger, J. Katsaras, Morphology of fast-tumbling bicelles: a small angle neutron scattering and NMR study, Biochim. Biophys. Acta 1513 (2001) 83–94.
[17] M.P. Nieh, C.J. Glinka, S. Krueger, R.S. Prosser, J. Katsaras, SANS study of the structural phases of magnetically alignable lanthanide-doped phospholipid mixtures, Langmuir 17 (2001) 2629–2638.
[18] G. Raffard, S. Steinbruckner, A. Arnold, J.H. Davis, E.J. Dufourc, Temperature-composition diagram of dimyristoylphosphatidylcholine–dicaproylphosphatidylcholine "bicelles" self-orienting in the magnetic field. A solid state H-2 and P-31 NMR study, Langmuir 16 (2000) 7655–7662.
[19] I.C.P. Smith, K.W. Butler, Oriented lipid systems as model membranes, in: L.J. Berliner (Ed.), Spin Labeling Theory and Applications, Academic Press, New York, 1976, pp. 411–453.
[20] J. Seelig, Anisotropic motion in liquid crystalline structures, in: L.J. Berliner (Ed.), Spin Labeling Theory and Applications, Academic Press, New York, 1976, pp. 373–410.
[21] S. Schreier, C.F. Polnaszek, I.C.P. Smith, Spin labels in membranes problems in practice, Biochem. Biophys. Acta 515 (1978) 375–436.
[22] R.D. Lapper, S.J. Paterson, I.C.P. Smith, Spin label study of the influence of cholesterol on egg lecithin multibilayers, Can. J. Biochem. 50 (1972) 969–981.
[23] M.L. Mangels, T.B. Cardon, A.C. Harper, K.P. Howard, G.A. Lorigan, Spectroscopic characterization of spin-labeled magnetically oriented phospholipid bilayers by EPR spectroscopy, J. Am. Chem. Soc. 122 (2000) 7052–7058.
[24] T.B. Cardon, E.K. Tiburu, A. Padmanabhan, K.P. Howard, G.A. Lorigan, Magnetically aligned phospholipid bilayers at the parallel and perpendicular orientations for X-band spin-label EPR studies, J. Am. Chem. Soc. 123 (2001) 2913–2914.
[25] C. Mailer, C.P.S. Taylor, S. Schreier-Muccillo, I.C.P. Smith, Influence of cholesterol on molecular motion in egg lecithin bilayers. Variable-frequency electron spin resonance study of a cholestane spin probe, Arch. Biochem. Biophys. 163 (1974) 671–678.
[26] R.S. Prosser, S.A. Hunt, J.A. DiNatale, R.R. Vold, Magnetically aligned membrane model systems with positive order parameter: switching the sign of S-zz with paramagnetic ions, J. Am. Chem. Soc. 118 (1996) 269–270.
[27] H. Hauser, C.C. Hinckley, J. Krebs, B.A. Levine, M.C. Philips, R.J.P. Williams, The interaction of ions with phosphatidylcholine bilayers, Biochim. Biophy. Acta 468 (1977) 364–377.
[28] J. Conti, H.N. Halladay, M. Petersheim, An ionotropic phase transition in phosphatidylcholine: cation and anion cooperativity, Biochim. Biohys. Acta 902 (1987) 53–64.
[29] V.S. Mironov, Y.G. Galyametdinov, A. Ceulemans, K. Binnemans, On the magnetic anisotropy of lanthanide-containing metallomesogens, J. Chem. Phys. 113 (2000) 10293–10303.
[30] R.S. Prosser, I.V. Shiyanovskaya, Lanthanide ion assisted magnetic alignment of model membranes and macromolecules, Concepts Magn. Reson. 13 (2001) 19–31.
[31] R.S. Prosser, J.S. Hwang, R.R. Vold, Magnetically aligned phospholipid bilayers with positive ordering: A new model membrane system, Biophys. J. 74 (1998) 2405–2418.
[32] K. Binnemans, Y.G. Galyametdinov, R. Van Deun, D.W. Bruce, S.R. Collinson, A.P. Polishchuk, I. Bikchantaev, W. Haase, A.V. Prosvirin, L. Tinchurina, I. Litvinov, A. Gubajdullin, A. Rakhmatullin, K. Uytterhoeven, L. Van Meervelt, Rare-earth-containing magnetic liquid crystals, J. Am. Chem. Soc. 122 (2000) 4335–4344.
[33] M.A. Firestone, P. Thiyagarajan, D.M. Tiede, Structure and optical properties of a thermoresponsive polymer-grafted, lipid-based complex fluid, Langmuir 14 (1998) 4688–4698.
[34] M.A. Firestone, D.M. Tiede, S. Seifert, Magnetic field-induced ordering of a polymer-grafted biomembrane-mimetic hydrogel, J. Phys. Chem. B 104 (2000) 2433–2438.
[35] J. Katsaras, R.L. Donaberger, I.P. Swainson, D.C. Tennant, Z. Tun, R.R. Vold, R.S. Prosser, Rarely observed phase transitions in a novel lyotropic liquid crystal system, Phys. Rev. Lett. 78 (1997) 899–902.
[36] V.S. Mironov, Y.G. Galyametdinov, A. Ceulemans, C. Gorller-Walrand, K. Binnemans, Influence of crystal-field perturbations on the room-temperature magnetic anisotropy of lanthanide complexes, Chem. Phys. Lett. 345 (2001) 132–140.
[37] L. Bennett, S. Hess, Nonequilibrium-molecular-dynamics investigation of the presmectic behavior of the viscosities of a Gay– Berne nematic-liquid crystal, Phys. Rev. E 60 (1999) 5561–5567.
[38] C. Faure, L. Bonakdar, E.J. Dufourc, Determination of DMPC hydration in the L(α) and L(β′) phases by H-2 solid state NMR of D_2O, FEBS Lett. 405 (1997) 263–266.
[39] R.P. Rand, N. Fuller, V.A. Parsegian, D.C. Rau, Variation in hydration forces between neutral phospholipid-bilayers—evidence for hydration attraction, Biochemistry 27 (1988) 7711–7722.
[40] E. Sternin, D. Nizza, K. Gawrisch, Temperature dependence of DMPC/DHPC mixing in a bicellar solution and its structural implications, Langmuir 17 (2001) 2610–2616.
[41] S. Schreier-Muccillo, D. Marsh, H. Duas, H. Schneider, I.C.P. Smith, A spin probe study of the influence of cholesterol on motion and orientation of phospholipids in oriented multibilayers and vesicles, Chem. Phys. Lipids 10 (1973) 11–27.
[42] C. Altenbach, S.L. Flitsch, H.G. Khorana, W.L. Hubbell, Structural studies on transmembrane proteins. 2. Spin labeling of bacteriorhodopsin mutants at unique cysteines, Biochemistry 28 (1989) 7806–7812.
[43] C. Altenbach, T. Marti, H.G. Khorana, W.L. Hubbell, Transmembrane protein-structure—spin labeling of bacteriorhodopsin mutants, Science 248 (1990) 1088–1092.
[44] C. Altenbach, D.A. Greenhalgh, H.G. Khorana, W.L. Hubbell, A collision gradient method to determine the immersion depth of nitroxides in lipid bilayers: application to spin-labeled mutants of bacteriorhodopsin, Proc. Natl. Acad. Sci. USA 91 (1994) 1667–1671.
[45] C. Altenbach, K. Yang, D.L. Farrens, Z.T. Farahbakhsh, H.G. Khorana, W.L. Hubbell, Structural features and light-dependent changes in the cytoplasmic interhelical E–F loop region of rhodopsin: a site-directed spin-labeling study, Biochemistry 35 (1996) 12470–12478.
[46] M. Bennati, G.J. Gerfen, G.V. Martinez, R.G. Griffin, D.J. Singel, G.L. Millhauser, Nitroxide side-chain dynamics in a spin-labeled helix-forming peptide revealed by high-frequency (139.5-GHz) EPR spectroscopy, J. Magn. Reson. 139 (1999) 281–286.
[47] P.P. Borbat, A.J. Costa-Filho, K.A. Earle, J.K. Moscicki, J.H. Freed, Electron spin resonance in studies of membranes and proteins, Science 291 (2001) 266–269.
[48] H.S. Mchaourab, T. Kalai, K. Hideg, W.L. Hubbell, Motion of spin-labeled side chains in T4 lysozyme: effect of side chain structure, Biochemistry 38 (1999) 2947–2955.
[49] M.D. Rabenstein, Y.K. Shin, Determination of the distance between 2 spin labels attached to a macromolecule, Proc. Natl. Acad. Sci. USA 92 (1995) 8239–8243.
[50] K.G. Victor, D.S. Cafiso, Location and dynamics of basic peptides at the membrane interface: electron paramagnetic resonance spectroscopy of tetramethyl-piperidine-*N*-oxyl-4-amino-4-carboxylic acid-labeled peptides, Biophys. J. 81 (2001) 2241–2250.

[51] M. Tominaga, S.R. Barbosa, E.F. Poletti, J. Zukerman-Schpector, R. Marchetto, S. Schreier, A. Paiva, C.R. Nakaie, Fmoc-POAC: [(9-fluorenylmethyloxycarbonyl)-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl-3-amino-4-carboxylic acid]: a novel protected spin labeled β-amino acid for peptide and protein chemistry, Chem. Pharm. Bull. 49 (2001) 1027–1029.

[52] M.L. Mangels, A.C. Harper, A.I. Smirnov, K.P. Howard, G.A. Lorigan, Investigating magnetically aligned phospholipid bilayers with EPR spectroscopy at 94 GHz, J. Magn. Reson. 151 (2001) 253–259.

[53] E.K. Tiburu, D.M. Moton, G.A. Lorigan, Development of magnetically aligned phospholipid bilayers in mixtures of palmitoylstearoylphosphatidylcholine and dihexanoylphosphatidylcholine by solid-state NMR spectroscopy, Biochim. Biophys. Acta 1512 (2001) 206–214.

[54] E.K. Tiburu, G.A. Lorigan, Development of new magnetically oriented phospholipid bilayer systems with solid-state NMR spectroscopy, Biophys. J. 80 (2001) 2335.

[55] S.M. Garber, G.A. Lorigan, K.P. Howard, Magnetically oriented phospholipid bilayers for spin label EPR studies, J. Am. Chem. Soc. 121 (1999) 3240–3241.

[56] M.J. Janiak, D.M. Small, G.G. Shipley, Nature of the thermal pretransition of synthetic phospholipids: dimyristoyl- and dipalmitoyllecithin, Biochemistry 15 (1976) 4575–7580.

[57] A. Tardieu, V. Luzzati, F.C. Reman, Structure and polymorphism of the hydrocarbon chains of lipids. Lecithin–water phases, J. Mol. Biol. 75 (1973) 711–733.

[58] R.S. Prosser, V.B. Volkov, I.V. Shiyanovskaya, Novel chelate-induced magnetic alignment of biological membranes, Biophys. J. 75 (1998) 2163–2169.