Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1714 (2005) 141 – 151
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Electron paramagnetic resonance studies of magnetically aligned phospholipid bilayers utilizing a phospholipid spin label: The effect of cholesterol

Paresh C. Dave, Nisreen A. Nusair, Johnson J. Inbaraj, Gary A. Lorigan*

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

Received 21 April 2005; received in revised form 21 June 2005; accepted 21 June 2005
Available online 11 July 2005

Abstract

X-band EPR spectroscopy has been employed to study the dynamic properties of magnetically aligned phospholipid bilayers (bicelles) utilizing a variety of phosphocholine spin labels (n-PCSL) as a function of cholesterol content. The utilization of both perpendicular and parallel aligned bicelles in EPR spectroscopy provides a more detailed structural and orientational picture of the phospholipid bilayers. The magnetically aligned EPR spectra of the bicelles and the hyperfine splitting values reveal that the addition of cholesterol increases the phase transition temperature and alignment temperature of the DMPC/DHPC bicelles. The corresponding molecular order parameter, S_{mol}, of the DMPC/DHPC bicelles increased upon addition of cholesterol. Cholesterol also decreased the rotational motion and increased the degree of anisotropy in the interior region of the bicelles. This report reveals that the dynamic properties of DMPC/DHPC bicelles agree well with other model membrane systems and that the magnetically aligned bicelles are an excellent model membrane system.

Keywords: Magnetically aligned bilayers; Order parameter; Spin-label; Bicelle; EPR

1. Introduction

There are an extraordinary number of investigations of cholesterol in lipid membrane studies that started at least 80 years ago [1–9]. Surprisingly, a complete understanding of the function of cholesterol in membranes at the molecular level has still not been reached. In most of the investigations, the lipid component is phosphatidylcholines (PCs), mainly 1,2-dipalmitoyl-*sn*-glyecrol phosphatidylcholine (DPPC) or 1,2-dimyristoyl-*sn*-glyecrol phosphatidylcholine (DMPC), i.e., a lipid with saturated acyl chains has been used [10–17]. Cholesterol is a major component of membrane systems and is unique in its ability to cause a wide variety of effects on the physical properties of membranes such as phase transitions and ordering effects [15,18–20]. Therefore, its structural role has been the subject of much speculation [15]. Concentration of cholesterol in living cell membranes can be as high as 50% in some cases [15,21,22]. Besides phospholipid molecules, cholesterol can also be considered as one of the main constituents of eukaryotic membranes. Therefore, investigating the properties of phospholipid/cholesterol mixed membranes is of great interest, as it is an essential step toward a deeper understanding of the structure and biological function of real biological membranes. X-ray, neutron diffraction, and ^{2}H NMR spectroscopic studies indicate that cholesterol is embedded into the phospholipid bilayers in such a way that its polar hydroxyl group is located in the aqueous phase and the hydrophobic steroid ring system is buried in the hydrocarbon chains of the phospholipids and oriented parallel to the phospholipid bilayer normal [23,24].

Electron paramagnetic resonance (EPR) spectroscopy has proven to be a very useful technique in providing insight

* Corresponding author. Tel.: +1 513 529 3338; fax: +1 513 529 5715.
E-mail address: lorigag@muohio.edu (G.A. Lorigan).

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.06.009

into the dynamics and molecular structure of phospholipid membranes by utilizing a variety of different spin labels [22,25–30]. The dynamic properties of phospholipid membranes can be extensively investigated by carefully analyzing the hyperfine splittings and the corresponding EPR line shapes. Spin-label EPR spectroscopy has become a standard technique for studying not only lipid-dynamics, but also lipid–protein interactions in membrane systems [31–33]. EPR spectral investigations have been carried out extensively on multi-lamellar phospholipid systems composed of either DMPC/cholesterol or DPPC/cholesterol [3,7,13,14,16,17,34,35]. Interestingly, the effects of cholesterol incorporated into magnetically aligned phospholipid bilayers (bicelles) have not been fully investigated.

Bicelles have emerged as an important model membrane system [36–40]. An early work with phospholipids and bile salt derivatives eventually led to the use of long chain phospholipids, 1,2-dimyristoyl-*sn*-glyecrol phosphatidylcholine (DMPC), and short chain phospholipids, 1,2-dihexanoyl-*sn*-glyecrol phosphatidylcholine (DHPC) to form bicelles [41–44]. The most intriguing aspect of bicelle behavior is that they become aligned in the presence of a magnetic field [19,41,42,45–49]. This discovery spawned intense interest within the solid-state NMR community to probe the structure of integral membranes proteins [49–51]. Recently, our group reported for the first time the magnetic alignment of bicelles using an X-band EPR spectrometer (9.5 GHz) at low magnetic fields [5,52]. The dynamic properties of the magnetically aligned bicelles can be investigated by analyzing the hyperfine splittings and the corresponding EPR line shapes directly from the perpendicular and parallel aligned spectra exclusive of further simulations [8,52–55].

The magnetic alignment of bicelles depends upon the magnetic susceptibility anisotropy tensor ($\Delta\chi$) of the phospholipid bilayers. At low magnetic fields, the bilayers cannot spontaneously align at either the perpendicular or the parallel alignment. The association of paramagnetic lanthanide ions with the bicelles changes the sign and the value of the magnetic susceptibility anisotropy tensor ($\Delta\chi$) of the bicelles [54]. The negative value of $\Delta\chi$ is increased by the addition of Dy^{3+} to the sample and causes the bicelles to align with their bilayer normal (n) oriented perpendicular to the direction of the static magnetic field. Tm^{3+} with a large positive magnetic susceptibility anisotropy $\Delta\chi$ can cause the bicelles to flip 90° and align with their bilayer normal (n) oriented parallel to the direction of the static magnetic field [5,6,52,56].

The presence of lanthanides could affect the electron spin-lattice relaxation rate and cause significant paramagnetic line broadening and complicate detailed analysis of spin-label EPR spectra of magnetically aligned bicelle samples. Power saturation studies have been performed by Caporini et al. to explore the possibility of a potential drawback of the magnetically aligned bicelle system. The report demonstrated that the addition of Tm^{3+} or Dy^{3+} to the bicelle system has a very small effect on the spin-lattice relaxation rate [55]. In addition, Dave et al. confirmed that the addition of Tm^{3+} or Dy^{3+} to DMPC/DHPC bicelles does not significantly alter the EPR spectral line widths [57]. Thus, the presence of Ln^{3+} ions in the bicelle samples will not change the hyperfine tensor values.

Previously, Dave et al. discussed extensively the effects of temperature variation on the dynamic properties of the acyl chain bicelles containing cholesterol and the effect of different spin labels, n-PCSL ($n=5$, 7, 12, and 14) where the doxyl group is attached to different positions on the acyl chain [57]. Thus, spectral parameters of oriented n-PCSL, plotted as a function of position (n) of the nitroxide group, were used to assay the profiles of ordering and molecular mobility of the acyl chains of the membrane lipids. In the present work, the experimental EPR spectra have been analyzed systematically to investigate the structural and dynamic properties of magnetically aligned DMPC/DHPC bicelles containing phospholipid spin labels, 1-Palmitoyl-2-[n-(4,4-dimethyloxazolidine-*N*-oxyl) stearoyl]-*sn*-glycero-3-phosphocholine (n-PCSL). In this paper, we are focusing on the effects of cholesterol, embedded inside DMPC/DHPC magnetically aligned bicelles, on the phase transition temperature, alignment temperature, degree of anisotropy, molecular order parameters, and rotational correlation times.

2. Materials and methods

1,2-dimyristoyl-*sn*-glyecrol phosphatidylcholine (DMPC), 1,2-dihexanoyl-*sn*-glyecrol phosphatidylcholine (DHPC), and 1-Palmitoyl-2-[n-(4,4-dimethyloxazolidine-*N*-oxyl) stearoyl]-*sn*-glycero-3-phosphocholine (phosphocholine spin labels, n-PCSL) were purchased from Avanti Polar Lipids (Alabaster, AL). All phospholipids were dissolved in chloroform and stored at 253 K prior to use. Thulium (III) chloride hexahydrate ($TmCl_3{\cdot}6H_2O$), Dysprosium (III) chloride hexahydrate ($DyCl_3{\cdot}6H_2O$) and HEPES (*N*-[2-hydroxyethyl] piperazine-N′-2-ethanesulfonic acid) were obtained from Sigma-Aldrich (St. Louis, MO). Deuterium-depleted water was obtained from Isotec (Miamisburg, OH). Cholesterol powder was purchased from Alfa Aesar (Ward Hill, MA). Aqueous solutions of HEPES buffer and lanthanide ions were prepared on the day of sample preparation and were adjusted to pH 7. All aqueous solutions were prepared fresh with deuterium-depleted water. Several bicelle samples were prepared with the cholesterol content varied from 0 up to 20 mol% with respect to the long chain DMPC phospholipid. Bicelle samples containing more than 20 mol% cholesterol were opaque and viscous, which made it difficult to homogenize and align for the X-band EPR spectroscopic studies. Specific details on the preparation of the bicelle sample and magnetic alignment procedure for the bicelle samples in the weak magnetic fields used in X-band EPR measurements can be found in previous papers [52–55,57].

2.1. EPR spectroscopy

All EPR experiments were carried out on a Bruker EMX X-band 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 3350 G (0.3350 T), sweep width of 140 G, a microwave frequency of approx. 9.39 GHz, modulation frequency 100 kHz, modulation amplitude of 1.0 G, and at a power of 2.0 mW.

2.2. Molecular order parameter calculations

The chemical structures and the magnetic principal axes of the phospholipid spin labels, n-PCSL, used in the present study as EPR probes, have been clearly revealed in the literature [57]. The magnetic principal axes have the x-axis along the nitroxide N–O bond, the z-axis is along the 2p π orbital of the nitrogen, and the y-axis is perpendicular to the other two. The S_{mol} molecular order parameter corresponding to the long molecular axis can be calculated from the following equation [56]:

$$S_{mol} = S_{33}\left[\left(3\cos^2\theta - 1\right)/2\right]^{-1} \quad (1)$$

where θ denotes the angle between the long molecular axis and the corresponding z-axis. The order parameter S_{33} can be determined by measuring the resultant hyperfine splittings of the aligned spectra using the following equation [56]:

$$S_{33} = \left[\left(A_{||} - A_{\perp}\right)/\left(A_{ZZ} - A_{XX}\right)\right]\left(a_N/a'_N\right) \quad (2)$$

where, $a_N = 1/3\ (A_{XX}+A_{YY}+A_{ZZ})$, is the isotropic hyperfine splitting constants in a rigid molecular frame and is sensitive to the solvent polarity, $a'_N = 1/3\ (A_{||}+2A_{\perp})$ is the solvent polarity correction factor for the hyperfine splitting [56]. The hyperfine tensoral components $A_{XX}=A_{YY}=5.0$ G and $A_{ZZ}=33.0$ G were taken from a previously reported analysis in the rigid limit [58,59]. The hyperfine splittings $A_{||}$ and $A_{\perp}$ were measured between the $m_I=+1$ and 0 spectral lines from the parallel and perpendicular oriented EPR spectra, respectively. In our case, the z-axis is always collinear to the long molecular axis ($\theta=0°$); thus, $S_{mol} \approx S_{33}$ [56].

2.3. Rotational correlation time

In the rapid motional regime, the rotational correlation time (τ_r) can be obtained from the corresponding spectral linewidths using the following equation [60]:

$$\tau_r = 6.5 \times 10^{-10}\Delta H(0)\left[\left(h_0/h_{-1}\right)^{1/2} - 1\right] sec \quad (3)$$

where, h_0 and h_{-1} is the peak-to-peak heights of the central and high field line, respectively and ΔH(0) is the peak-to-peak linewidth of the central line ($M_I=0$). The above equation is used to calculate the τ_r value for 12 and 14-PCSL based on the assumption that 12-PCSL and 14-PCSL are in an isotropic medium. In order to verify the anisotropy of the motion of 12-PCSL and 14-PCSL inside the DMPC/DHPC bicelle, rotational correlation times were calculated using two different formulas based upon linear and quadratic terms of the spectral linewidth. The following equations were used:

$$\tau_B = 6.5 \times 10^{-10}\Delta H(0)\left[\left(h_0/h_{+1}\right)^{1/2} - \left(h_0/h_{-1}\right)^{1/2}\right] \text{sec} \quad (4)$$

$$\tau_C = 6.5 \times 10^{-10}\Delta H(0) \times \left[\left(h_0/h_{+1}\right)^{1/2} + \left(h_0/h_{-1}\right)^{1/2} - 2\right] \text{sec} \quad (5)$$

Where, h_{+1} is the peak-to-peak height of the low field line. If $\tau_B=\tau_C$, then this is an indication of isotropic motion of the spin label. In the case of anisotropic motion, $\tau_B \neq \tau_C$. The difference between τ_B and τ_C can be used to evaluate the degree of anisotropy [60].

3. Results and discussion

The bicelle system under investigation is composed of a long chain phospholipid (DMPC) and short chain phospholipid (DHPC) at a molar ratio of 3.5:1 (q ratio=3.5, DMPC/DHPC) [53,61]. The EPR spectral investigations are carried out for the bicelle system containing different amounts of cholesterol (0, 5, 10, 15, and 20 mol% cholesterol with respect to DMPC) as a function of temperature (308 K to 348 K). The goal is to systematically analyze the EPR spectral data in terms of line shapes of the EPR spectra, hyperfine splitting values, molecular order parameters, and rotational correlation times utilizing n-PCSL as an EPR probe incorporated into magnetically aligned DMPC/DHPC bicelles.

Fig. 1 illustrates a series of X-band EPR spectra of the spin labels 7-PCSL and 12-PCSL, respectively, incorporated into DMPC/DHPC bicelle samples containing 5 mol% cholesterol (with respect to DMPC) as a function of temperature. The black solid line spectra represent the randomly dispersed DMPC/DHPC bicelles in the absence of lanthanide ions. The dotted line and the gray solid line spectra represent the Dy^{3+}-doped perpendicular aligned and Tm^{3+}-doped parallel aligned bicelles, respectively. The EPR spectra of 7-PCSL and 12-PCSL in the presence of lanthanide ions, either Tm^{3+} or Dy^{3+}, are all characteristic of well-aligned bicelles. The addition of Dy^{3+} helps to achieve perpendicular alignment in the low magnetic fields (0.64 T) used in EPR studies (dotted line spectra) [52]. Conversely, the addition of Tm^{3+} causes the bicelles to flip

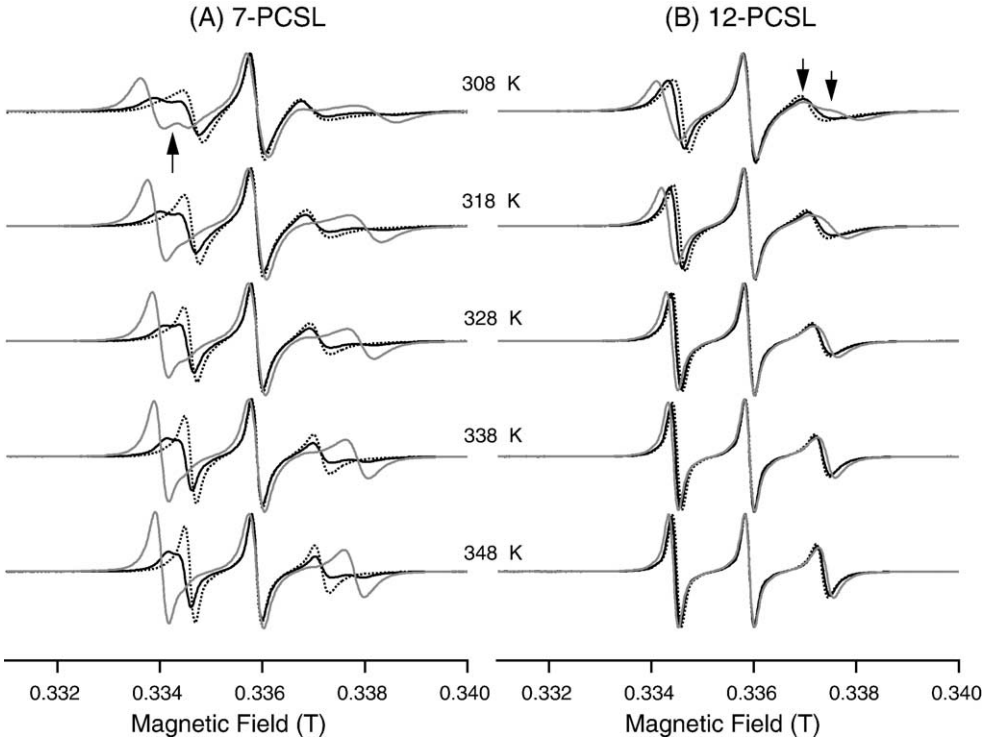

Fig. 1. EPR spectra of (A) 7-PCSL and (B) 12-PCSL incorporated into DMPC/DHPC bicelle samples containing 5 mol% cholesterol with respect to DMPC as a function of temperature. The black solid line spectra represent unoriented bicelles in the absence of lanthanide ions. The dotted line spectra and the gray solid line spectra represent the perpendicular and parallel aligned bicelles in the presence of Dy^{3+} and Tm^{3+}, respectively.

90° such that the average bilayer normal is collinear with the direction of the magnetic field (gray solid line spectra) [52]. In the absence of lanthanide ions, the bicelle system has no orientational characteristics in the weak magnetic field (0.64 T) used for this X-band EPR study (solid line spectra). Similar EPR spectral line shape analysis has also been observed previously upon magnetic alignment of bicelles when cholestane and n-doxylstearic acid spin labels are incorporated into bicelles [5,6,52,54,56].

Inspection of Fig. 1 indicates that the hyperfine splittings of the parallel and perpendicular spectra approach the hyperfine splitting of the randomly dispersed spectra as the nitroxide moiety is transferred from position 7 (7-PCSL) at the lipid polar head group region toward position 12 (12-PCSL) near the bottom of the acyl chains in the interior part of the bicelles. EPR data for 5-PCSL and 14-PCSL incorporated into DMPC/DHPC bicelles have also been carried out in the presence and absence of lanthanide ions (data have not shown). The EPR spectra for 5-PCSL and 14-PCSL spin labels incorporated into DMPC/DHPC bicelle system reveal similar spectral line shapes as the 7-PCSL and 12-PCSL data, respectively. Analysis of the EPR spectral line shapes of the 5-PCSL and 7-PCSL spin labels incorporated into the bicelles indicates more anisotropic motion. In this case, the nitroxide group attached to positions 5 and 7 (5-PCSL and 7-PCSL) of the acyl chain of the phospholipid spin labels incorporated into the bicelles are situated closer to the lipid polar head group region where molecular motions are more restricted. In contrast, the EPR spectral line shapes corresponding to the nitroxide groups attached to positions 12 and 14 (12-PCSL and 14-PCSL) are less anisotropic [5,57]. This is a result of the nitroxide group that is attached near the end of the acyl chains and experiences considerable motional freedom.

The parallel oriented EPR spectrum (gray solid line spectra) of 7-PCSL spin label has a small shoulder located between the low (m_I=+1) and the center (m_I=0) field peaks at 308 K in Fig. 1A as pointed out with the arrow. In addition, the EPR spectral line of the parallel oriented spectrum (gray solid line spectra) of 12-PCSL spin label splits into two lines in the high field (m_I=−1) at 308 K in Fig. 1B as pointed out with the arrows. Similar spectral features have also been observed previously for mechanically oriented multibilayers at temperature ranges from 293 to 296 K [4,62]. These characteristic EPR line shapes suggested the presence of an intermediate transition phase from the fluid to liquid crystalline phase. In the present study, the bicelles are in the nematic phase at 308 K and may not be in the perfectly aligned smectic phase [54]. The intensity of the three EPR spectral lines at 318 K and above increases and the mobility of the acyl chains increases as the temperature increases. These results clearly reveal that the magnetically aligned bicelles are in the smectic liquid crystalline phase, which has been described as perforated lamellar sheets with DHPC lining the edges of the sheets and pores [54].

The principle magnetic tensor values were obtained from spectral analysis of aligned membranes in the rigid limit [58,59]. These values, A_{xx}, A_{yy}, A_{zz}=5, 5, 33 G, are obtained from the literature at 138 K [58,59]. Thus, the isotropic hyperfine splitting (A_{iso}) is equal to (A_{xx}+A_{yy}+A_{zz})/3=14.33 G. The dynamics of the n-PCSL is complicated because of the rapid segmental motion that occurs due to a large number of the *gauche-trans* inter conversions of the acyl chain of the phospholipid spin label as well as rotational motion along the lipid long axis. This motion will partially average the hyperfine tensor anisotropy differently for a perpendicular or parallel bicelle alignment with respect to the direction of the applied magnetic field. The hyperfine splitting values of the perpendicular aligned bicelles with respect to the direction of the static magnetic field are less than A_{iso} (14.33 G). In contrast, the hyperfine splitting values of the parallel aligned bicelles with respect to the direction of the static magnetic field are greater than the A_{iso} (14.33 G), irrespective of the positions of the spin labels to the acyl chains. Interestingly, the *z*-axis is co-linear with the bilayer normal (*n*) when the bicelles are perpendicular or parallel aligned with respect to the direction of the static magnetic field. This clearly reveals that the contribution from the z-tensoral component is higher for the parallel aligned bicelles and lower for the perpendicular aligned bicelles [52,57].

Fig. 2 summarizes the X-band EPR results from Fig. 1 by plotting the hyperfine splittings for the 7-PCSL and 12-PCSL spin labels, respectively, incorporated into the aligned DMPC/DHPC bicelle system versus temperature. The data indicate that as the temperature increases from 308 to 348 K, the hyperfine splittings of the Tm^{3+}-doped parallel aligned spectra decrease and the hyperfine splittings of the Dy^{3+}-doped perpendicular aligned spectra increase. These data indicate that increasing the temperature of the bicelle system

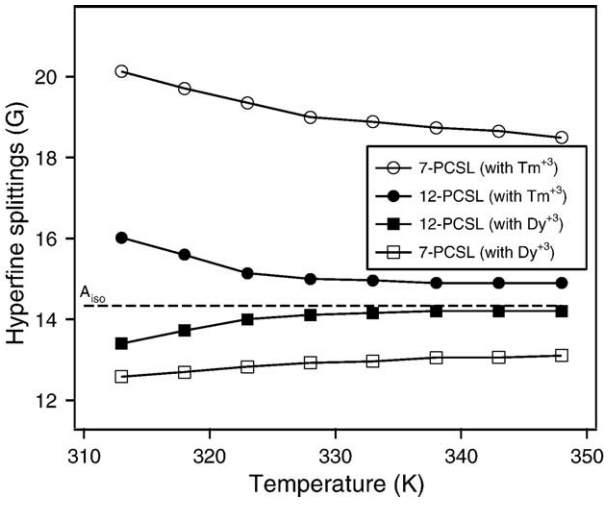

Fig. 2. Temperature dependence of the hyperfine splittings gleaned from the EPR spectra of 7-PCSL and 12-PCSL (Fig. 1) incorporated into the perpendicular aligned and parallel aligned bicelles with respect to the static magnetic field in the presence of Dy^{3+} and Tm^{3+}, respectively.

increases the rotational motion of the nitroxide group as a result of increasing the motion of the phospholipid acyl chains. The EPR spectral line shapes and the hyperfine splittings, as illustrated in Figs. 1 and 2, approach the isotropic value (A_{iso}) as the temperature increases from 308 K to 348 K. The hyperfine splittings for the parallel oriented bicelles decreases in the order of 5-PCSL>7-PCSL>12-PCSL>14-PCSL, respectively. Conversely, the hyperfine splittings for the perpendicular oriented bicelles increases in the order of 5-PCSL<7-PCSL<12-PCSL<14-PCSL. This observation confirms that the molecular motion of the phospholipid acyl chains in the bicelle system increases as the nitroxide group is transferred from the rigid polar head group region towards the flexible hydrophobic acyl chain region [57].

The behavior and physical properties of bicelles in the presence of cholesterol are of significant importance in the biophysical research [20]. Therefore, we have investigated the effect of cholesterol incorporated into magnetically aligned bicelles. The cholesterol concentration varies for different types of membrane systems. For example, plasma membranes contain 45–50 mol% cholesterol, endoplasmic reticulum membranes contain 10–12 mol% cholesterol, while sarcoplasmic reticulum membranes contain 6–7 mol% cholesterol [20,63]. In the present study, up to 20 mol% cholesterol with respect to the DMPC has been incorporated into the bicelle membrane system to better mimic a natural membrane system.

Fig. 3 represents the X-band EPR spectra of 7-PCSL and 12-PCSL incorporated into DMPC/DHPC bicelles at 318 K as a function of cholesterol concentration. The cholesterol concentration ranges from 0% to 20 mol% with respect to the long chain phospholipid DMPC. The black solid line spectra represent the randomly dispersed DMPC/DHPC bicelles in the absence of lanthanide ions. The dotted line and the gray solid line spectra demonstrate the Dy^{3+}-doped perpendicular and Tm^{3+}-doped parallel aligned bicelles, respectively. As the mol% of cholesterol with respect to DMPC increases, the EPR line shapes and the hyperfine splittings of the parallel and perpendicular aligned spectra gradually deviate from the line shapes and the hyperfine splittings of the randomly dispersed spectra. In addition, the deviation of the line shapes and the hyperfine splittings of the parallel and perpendicular aligned spectra from the randomly dispersed spectra is more pronounced in the 7-PCSL spectra when compared to the 12-PCSL spectra at a particular mol% of cholesterol.

The EPR line shapes displayed in Fig. 3 for the bicelle samples containing 5%, 10%, and 15 mol% cholesterol at 318 K indicate that the bicelles are well aligned in the smectic liquid crystalline phase in the presence of lanthanide ions. The presence of one small shoulder between the first (m_I=+1) and the center (m_I=0) EPR spectral lines as pointed out with the arrow in the Tm^{3+}-doped parallel oriented bicelles containing 0 mol% cholesterol indicate that it is not perfectly aligned at 318 K as

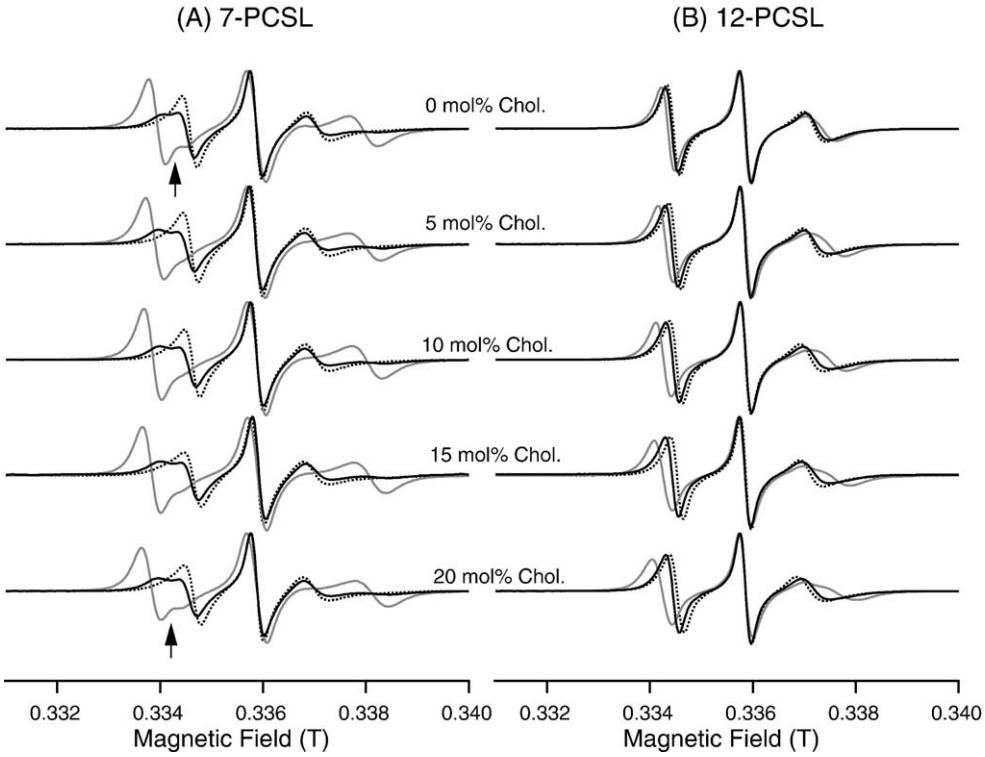

Fig. 3. EPR spectra of (A) 7-PCSL and (B) 12-PCSL incorporated into DMPC/DHPC bicelles at 318 K as a function of cholesterol concentration with respect to DMPC. The black solid line spectra represent unoriented bicelles in the absence of lanthanide ions. The dotted line spectra and the gray solid line spectra represent the perpendicular and parallel alignment of the bilayer normal in the presence of Dy^{3+} and Tm^{3+}, respectively.

shown in (Fig. 3A gray solid line spectra). The spectrum indicates that the 7-PCSL spin-label is not completely aligned in the bicelles due to the fact that the lipid system experiences a lower degree of ordering at low cholesterol concentrations. Additionally, the changes in the relative intensity and the presence of one small shoulder between the first (m_I=+1) and the center (m_I=0) EPR spectral lines as pointed out with the arrow in the Tm^{3+}-doped parallel oriented bicelles containing 20 mol% cholesterol at 318 K confirms that the bicelles are not perfectly aligned (Fig. 3A gray solid line spectra).

Fig. 4 reveals the hyperfine splitting changes as a function of cholesterol concentration for the EPR spectra shown in Fig. 3. The observed hyperfine splittings increase from 0 to 15 mol% cholesterol for the Tm^{3+}-doped parallel oriented bicelle samples with respect to the direction of the static magnetic field. In contrast, the observed hyperfine splittings decrease from 0% to 15 mol% cholesterol for the Dy^{3+}-doped perpendicular oriented bicelle samples with respect to the direction of the static magnetic field. The hyperfine splittings displayed in Fig. 4 for the bicelle samples containing 5, 10, and 15 mol% cholesterol at 318 K indicate that the bicelles are well aligned in the liquid crystalline phase in the presence of lanthanide ions. The slight deviation in the hyperfine splitting trends for all bicelle samples containing 20 mol% cholesterol with respect to DMPC reveal that the bicelles may not be perfectly aligned at 318 K.

The EPR spectra and hyperfine splitting values of n-PCSL as a function of 20 mol% cholesterol at 318 K shown in Figs. 3 and 4 reveal that cholesterol plays an important role in increasing the phase transition temperature of the DMPC/DHPC bicelles. The bicelle sample is in the nematic phase at 318 K and may not be in the perfectly aligned smectic phase [54]. The data suggest that the lipid gel-to-

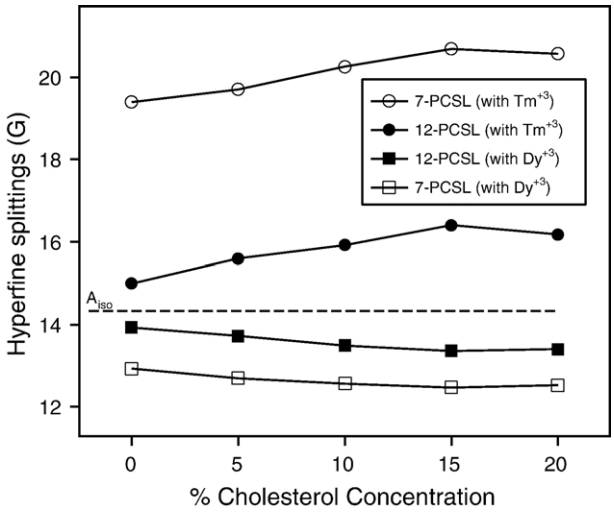

Fig. 4. Cholesterol dependence of the hyperfine splitting of 7-PCSL and 12-PCSL incorporated into the perpendicular aligned and parallel aligned bicelles with respect to the static magnetic field in the presence of Dy^{3+} and Tm^{3+}, respectively, at 318 K.

liquid phase transition temperature is raised by increasing the cholesterol concentration, and the bicelle alignment temperature increases as well. The spectra represent bicelle samples containing 20 mol% cholesterol were perfectly aligned at temperature ranges from 323 K to 348 K (data not shown). This implies that this bicelle system is in the smectic liquid crystalline phase at temperature higher than 318 K. The EPR experiments of the 5-PCSL and 14-PCSL have also been carried out for the bicelle samples in the absence and in the presence of lanthanide ions with different cholesterol concentrations (0, 5, 10, 15, and 20 mol%) as a function of temperature (308 K to 348 K) (data not shown). The EPR spectral features for the 5-PCSL and 14-PCSL are similar to the 7-PCSL and 12-PCSL, respectively.

EPR studies of the different spin labels incorporated into liposomes or multi-lamellar vesicles in the presence of cholesterol have been reported previously in the literature [4,35,64]. The data clearly indicate that the physical properties of cholesterol can disrupt the homogeneity of the lipid bilayers and lead to the formation of different micro domains and alter the phase transition temperature [4,35,64]. Differential scanning calorimetry (DSC) studies on cholesterol/phosphatidylcholine mixtures have showed that cholesterol progressively decreases the phase transition temperature (T_m) of phosphatidylcholine bilayers with saturated acyl chains of 18 or more carbon atoms [65,66]. However, cholesterol increases the corresponding T_m if the chain length is equal to or less than 16 carbons. Magnetic resonance studies on cholesterol containing lipid bilayers have suggested that cholesterol increases the overall ordering and decreases fluidity of the phospholipids in the liquid crystalline phase. This property is often referred to as the condensing effect of cholesterol, which occurs at a temperature above the gel-to-liquid crystalline phase transition [19]. Solid-state NMR spectroscopic studies on magnetically aligned bicelles have indicated that the addition of 10 mol% cholesterol increases the phase transition temperature [18,19]. The low field X-band EPR results presented in this study agree with the solid-state NMR results.

The molecular order parameter, S_{mol}, describes the local orientational and/or dynamic perturbations of the doxyl group from its standard state due to the perturbations of n-PCSL conformations or dynamics as a result of the incorporation of the spin labels into the bicelle samples. Fig. 5 depicts the molecular orientational order parameter profiles derived from the EPR spectra of the n-PCSL

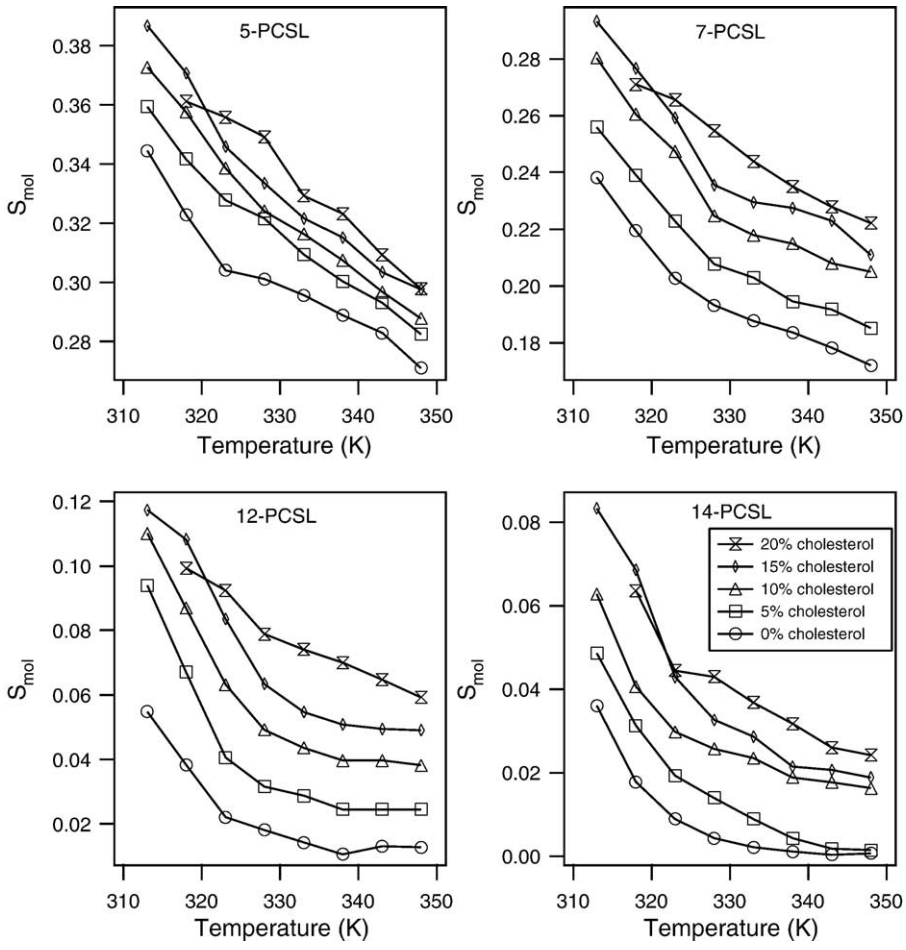

Fig. 5. Cholesterol concentration dependence of the S_{mol} order parameter profiles for the 5-, 7-, 12- and 14-PCSL spin-labels embedded into magnetically aligned DMPC/DHPC bicelles as a function of temperature.

embedded into DMPC/DHPC bicelles oriented parallel or perpendicular to the static magnetic field as function of temperature. S_{mol} has been calculated using Eq. (1) as described in Materials and methods. Fig. 5 shows the S_{mol} order profiles of the spin labels 5, 7, 12, and 14-PCSL embedded into the DMPC/DHPC bicelles in the presence of 0, 5, 10, 15, and 20 mol% cholesterol with respect to the DMPC. The S_{mol} profile clearly indicates that the molecular ordering of the spin labels decreases as the nitroxide spin-label is moved further from the polar head group region along the hydrocarbon chain (from positions 5, 7, 12, and finally to 14) [57]. These results confirm that the acyl chain motion increases from the more rigid head group region to the more flexible central bilayer region. Interestingly, one can clearly see in Fig. 5 that the values of the order parameter for all the spin labels (n-PCSL) decrease as the temperature increases from 308 K to 348 K due to an increase in the motion of the phospholipid acyl chains. In addition, the values of S_{mol} increase for the bicelle samples by increasing the amount of cholesterol with respect to the phospholipids. Cholesterol decreases fluidity and increases the overall motional order by decreasing the number of *gauche* conformations for DMPC acyl chains located near the cholesterol molecules in the bicelles. Analysis of EPR spectra of 5- and 14-PCSL incorporated into sphingomyelin/cholesterol mixture have been studied by Collado et al., who described a phase diagram for bilayers and proposed that cholesterol would induce formation of a "liquid-ordered" phase that coexists with the "liquid-disordered" phase at 323 K and a given cholesterol concentration (~20 mol%) [67]. The "liquid-ordered" phase has been described as a liquid-crystalline phase with a high acyl-chain order. This study implies that the "liquid-ordered" phase may coexist with the liquid-crystalline phase in the magnetically aligned bicelle system containing 20 mol% cholesterol.

S_{mol} order parameters on cholestane, n-doxylstearic acids, cholesterol-d_6, and phospholipid molecules, DMPC-d_{54} and n-PCSL from both spin-label EPR and ^{2}H NMR spectroscopic studies presented in this report and previous reports on DMPC/DHPC bicelles are consistent with each other [5,6,56,57]. The degree of ordering increases as the amount of cholesterol in the bicelle samples increases. Cholesterol molecules have a higher degree of ordering and slower motion than the corresponding phospholipid molecules in the same sample. The dynamics of the phospholipid membrane can be characterized by three correlation times corresponding to the rotation about the principal diffusion axis of the molecule (chain rotation), rotation about this axis (chain fluctuation or wobbling), and a *trans-gauche* isomerization of the acyl chain. The flat cholesterol molecule with cylindrical symmetry probably has relatively higher activation energy for molecular rotation about its molecular axis than the corresponding phospholipid molecule. The molecular rotation of phospholipid molecules will slow down because of the close contact of cholesterol molecules with slower motions. At the same time, the rigidity of the cholesterol molecules can also restrict the *trans-gauche* isomerization of the acyl chains of the phospholipid molecules next to it. As the temperature increases, the molecules gain more energy; thus, both the intermolecular (chain rotation and fluctuation) and intramolecular motion will increase. Therefore, the molecular ordering decreases when the temperature increases.

The effect of cholesterol on the degree of anisotropy can be discussed more clearly based upon the difference in the rotational correlation time, which is obtained from the EPR spectral line width of the spin label at different cholesterol concentrations. The estimated effective rotational correlation times, τ_r, (using Eq. (3)) for 12-PCSL and 14-PCSL from EPR spectra of unoriented DMPC/DHPC bicelles containing 0% and 20 mol% cholesterol at 318 K are given in Table 1. The τ_r values are larger for 12-PCSL when compared to 14-PCSL at 318 K indicating that 12-PCSL is undergoing additional anisotropic motion when compared to the 14-PCSL. Additionally, the τ_r values increase for 12-PCSL and 14-PCSL when the cholesterol concentration increases from 0 to 20 mol% cholesterol in the bicelle samples. This clearly indicates that bicelle samples containing higher cholesterol content undergoes a higher degree of anisotropic motion. If the motion is not truly isotropic, then the τ_r values obtained are not true correlation times. Thus, in order to evaluate the degree of anisotropy, the effective rotational correlation times, τ_B and τ_C (calculated using Eqs. (4) and (5)) and the difference between these two values are presented in Table 1. The small discrepancy in the τ_B and τ_C values may arise from the anisotropic motion of the spin label. The smaller difference between the two τ values ($\tau_B - \tau_C$) (Table 1) indicates that the spectra look more isotropic in nature. Larger differences between the two τ values ($\tau_B - \tau_C$) have been observed in the bicelle samples containing 20 mol% cholesterol (0.54 and 0.34) when compared to bicelle samples containing 0 mol% cholesterol (0.44 and

Table 1
Rotational correlation time for spin-labeled phosphatidylcholines, n-PCSL, in DMPC/DHPC bicelle sample containing 0 mol% and 20 mol% cholesterol at 318 K

Spin label	0 mol% cholesterol				20 mol% cholesterol			
	τ_r (ns)	τ_B (ns)	τ_C (ns)	$\tau_B - \tau_C$ (ns)	τ_r (ns)	τ_B (ns)	τ_C (ns)	$\tau_B - \tau_C$ (ns)
12-PCSL	1.54±0.04	1.76±0.05	1.32±0.03	0.44±0.02	1.88±0.01	2.15±0.01	1.61±0.01	0.54±0.00
14-PCSL	0.90±0.02	0.97±0.02	0.83±0.01	0.14±0.01	1.31±0.02	1.48±0.08	1.14±0.04	0.34±0.12

0.14) for 12-PCSL and 14-PCSL, respectively. This result clearly suggests a systematic increase in the spectral anisotropy of the spin label based upon the increase in cholesterol content in the bicelle membrane system.

4. Conclusion

In this work, we have presented spin-labeled EPR spectroscopic studies that document pertinent structural and dynamic information about n-PCSL ($n=5$, 7, 12, and 14) incorporated into DMPC/DHPC bicelles. The utilization of Dy^{3+}-doped perpendicular and Tm^{3+}-doped parallel aligned bicelles in EPR spectroscopy provides a more detailed structural and orientational picture of the bicelles when compared to unoriented bicelles. We have shown that the hydrocarbon chains of the phospholipids are restricted in motion in the region close to the polar head groups and able to move more freely in the hydrophobic region towards the terminal methyl group. Also, increasing the temperature enhances the extent of this motion. Furthermore, the addition of cholesterol increases the phase transition temperatures, the alignment temperature, the degree of anisotropy, and the degree of ordering of the phospholipid chains. The bicelle data presented in this study compare well with previous studies and indicate that magnetically aligned bicelles represent an excellent model membrane system that can be used for EPR as well as solid-state NMR spectroscopic techniques.

Acknowledgements

This work was supported by an NSF CAREER award (CHE-0133433) and a National Institutes of Health Grant (GM60259-01).

References

[1] J.B. Leathes, Role of fats in vital phenomena, Lancet 208 (1925) 853–856.
[2] R.D. Lapper, S.J. Paterson, I.C.P. Smith, A spin label study of the influence of cholesterol on egg lecithin multibilayers, Can. J. Biochem. 50 (1972) 969–981.
[3] M.B. Sankaram, T.E. Thompson, Modulation of phospholipid acyl chain order by cholesterol. A solid-state ^2H nuclear magnetic resonance study, Biochemistry 29 (1990) 10676–10684.
[4] S. Schreier-Muccillo, D. Marsh, H. Dugas, 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.
[5] N.A. Nusair, G.A. Lorigan, Investigating the structural and dynamic properties of n-doxylstearic acid in magnetically-aligned phospholipid bilayers by X-band EPR spectroscopy, Chem. Phys. Lipids 133 (2005) 151–164.
[6] J. Lu, M.A. Caporini, G.A. Lorigan, The effects of cholesterol on magnetically aligned phospholipid biayers: a solid-state NMR and EPR spectroscopy study, J. Magn. Reson. 168 (2004) 18–30.
[7] D. Kurad, G. Jeschke, D. Marsh, Lateral ordering of lipid chains in cholesterol-containing membranes: high-field spin-label EPR, Biophys. J. 86 (2004) 264–271.
[8] 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.
[9] W.L. Hubbell, H.M. McConnell, Molecular motion in spin-labeled phospholipids and membranes, J. Am. Chem. Soc. 93 (1971) 314–326.
[10] A. Fillipov, G. Oradd, G. Lindblom, Influence of cholesterol and water content on phospholipid lateral diffusion in bilayers, Langmuir 19 (2003) 6397–6400.
[11] A. Fillipov, G. Oradd, G. Lindblom, The effect of cholesterol on the lateral diffusion of phospholipids in oriented bilayers, Biophys. J. 84 (2003) 3079–3086.
[12] P. Jedlovszky, M. Mezei, Effect of cholesterol on the properties of phospholipid membranes: 1. Structural features, J. Phys. Chem., B 107 (2003) 5311–5321.
[13] D. Kurad, G. Jeschke, D. Marsh, Spin-label HF-EPR of lipid ordering in cholesterol-containing membranes, Appl. Magn. Reson. 21 (2001) 469–481.
[14] D. Kurad, G. Jeschke, D. Marsh, Lipid membrane polarity profiles by high-field EPR, Biophys. J. 85 (2003) 1025–1033.
[15] H. Ohvo-Rekila, B. Ramstedt, P. Leppimaki, J.P. Slotte, Cholesterol interactions with phospholipids in membranes, Prog. Lipid Res. 41 (2002) 66–97.
[16] M.B. Sankaram, T.E. Thompson, Interaction of cholesterol with various glycerophospholipids and sphingomyelin, Biochemistry 29 (1990) 10670–10675.
[17] M.B. Sankaram, T.E. Thompson, Cholesterol-induced fluid-phase immiscibility in membranes, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8686–8690.
[18] P.C. Dave, E.K. Tiburu, N.A. Nusair, G.A. Lorigan, Calculating order parameter profiles utilizing magnetically aligned phospholipid bilayers for ^2H solid-state NMr studies, Solid State Nucl. Magn. Reson. 24 (2003) 137–149.
[19] E.K. Tiburu, P.C. Dave, G.A. Lorigan, Solid-state ^2H NMR studies of the effects of cholesterol on the acyl chain dynamics of magnetically aligned phospholipid bilayers, Magn. Reson. Chem. 42 (2004) 132–138.
[20] P.L. Yeagle, Cholesterol and the cell membrane, Biochim. Biophys. Acta 82 (1985) 267–287.
[21] O.G. Mouritsen, K. Jorgensen, Dynamical order and disorder in lipid bilayers, Chem. Phys. Lipids 73 (1994) 3–25.
[22] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[23] E.J. Dufourc, E.J. Parish, S. Chitrakorn, I.C.P. Smith, Structural and dynamical details of cholesterol–lipid interaction as revealed by deuterium NMR, Biochemistry 23 (1984) 6062–6071.
[24] P.A. Luchette, T.N. Vetman, R.S. Prosser, R.E.W. Hancock, M.-P. Nieh, C.J. Glinka, S. Krueger, J. Katasaras, Morphology of fast-tumbling bicelles: a small angle scattering and NMR study, Biochim. Biophys. Acta 1513 (2001) 83–94.
[25] R.L. Cornea, L.R. Jones, J.M. Autry, D.D. Thomas, Mutation and phosphorylation change the oligomeric structure of phospholamban in lipid bilayers, Biochemistry 36 (1997) 2960–2967.
[26] D. Marsh, L.I. Horvath, M.J. Swamy, S. Mantripragada, J.H. Kleinschmidt, Interaction of membrane-spanning proteins with peripheral and lipid-anchored membrane proteins: perspectives from protein–lipid interactions, Mol. Membr. Biol. 19 (2002) 247–255.
[27] D. Mihailescu, L.I. Horvath, Molecular dynamics of lipid association at the hydrophobic interface of gramicidin S, Eur. Biophys. J. 28 (1999) 216–221.
[28] M. Persson, J.R. Harbridge, P. Hammarstrom, R. Mitri, L.G. Martensson, U. Carlsson, G.R. Eaton, S.S. Eaton, Comparison of

electron paramagnetic resonance methods to determine distances between spin labels on human carbonic anhydrase II, Biophys. J. 80 (2001) 2886–2897.
[29] J. Seelig, in: L.J. Berliner (Ed.), Spin Labeling: Theory and Applications, Academic Press, New York, 1976, pp. 373–409.
[30] J.H. Freed, in: L.J. Berliner (Ed.), Spin Labeling: Theory and Applications, Academic Press, New York, 1976, pp. 53–132.
[31] T.L. Kirby, C.B. Karim, D.D. Thomas, Electron paramagnetic resonance reveals a large-scale conformational change in the cytoplasmic domain of phospholamban upon binding to the sarcoplasmic reticulum Ca-ATPase, Biochemistry 43 (2004) 5842–5852.
[32] R.D. Nielsen, S. Canaan, J.A. Gladden, M.H. Gelb, C. Mailer, B.H. Robinson, Comparing continuous wave progressive saturation EPR and time domain saturation recovery EPR over the entire motional range of nitroxide spin labels, J. Magn. Reson. 169 (2004) 129–163.
[33] W.L. Hubbell, C. Altenbach, Investigation of structure and dynamics in membrane proteins using site-directed spin labeling, Curr. Opin. Struct. Biol. 4 (1994) 566–573.
[34] P.F.F. Almeida, W.L.C. Vaz, T.E. Thompson, Lateral diffusion in the liquid phases of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis, Biochemistry 31 (1992) 6739–6747.
[35] M.J. Swamy, D. Marsh, Spin-label paramagnetic resonance studies on the interaction of avidin with dimyristoyl-phosphatidylglycerol membranes, Biochim. Biophys. Acta 1513 (2001) 122–130.
[36] J.A. Whiles, K.J. Glover, R.R. Vold, E.A. Komives, Methods for studying transmembrane peptides in bicelles: consequences mismatch and peptide sequence, J. Magn. Reson. 158 (2002) 149–156.
[37] E. Sternin, D. Nizza, K. Garwisch, Temperature dependence of DMPC/DHPC mixing in a bicellar solution and its structural implications, Langmuir 17 (2001) 2610–2616.
[38] 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 ^{2}H and ^{31}P NMR study, Langmuir 16 (2000) 7655–7662.
[39] 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.
[40] I. Marcotte, E.J. Dufourc, M. Ouellet, M. Auger, Interaction of the neuropeptide Met-Enkephalin with zqitterionic and negatively charged bicelles as viewed by ^{31}P and ^{2}H solid-state NMR, Biophys. J. 85 (2003) 328–339.
[41] 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.
[42] 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.
[43] B.A. Rowe, S.L. Neal, Fluorescence probe study of bicelle structure as a function of temperature: developing a practical bicelle structure model, Langmuir 19 (2003) 2039–2048.
[44] 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.
[45] C.R. Sanders, G.C. Landis, Reconstitution of membrane-proteins into lipid-rich bilayered mixed micelles for NMR-studies, Biochemistry 34 (1994) 4030–4040.
[46] C.R. Sanders, J.H. Prestegard, Magnetically orientable phospholipid bilayers containing small amounts of a bile salt analogue, CHAPSO, Biophys. J. 58 (1990) 447–460.
[47] J. Struppe, R.R. Vold, Dilute bicellar solutions for structural NMR work, J. Magn. Reson. 135 (1998) 541–546.
[48] E. Oldfield, M. Meadows, D. Rice, R. Jacobs, Spectroscopic studies of specifically deuterium labeled membrane systems. Nuclear magnetic resonance investigation of the effects of cholesterol in model systems, Biochemistry 17 (1978) 2727–2740.
[49] A.D. Angelis, A.A. Nevzorov, S.H. Park, S.C. Howell, A.A. Mrse, S.J. Opella, High-resolution NMR spectroscopy of membrane proteins in aligned bicelles, J. Am. Chem. Soc. 126 (2004) 15340–15341.
[50] E.K. Tiburu, E.S. Karp, P.C. Dave, K. Damodaran, G.A. Lorigan, Investigating the dynamic properties of the transmembrane segment of phospholamban incorporated into phospholipid bilayers utilizing ^{2}H and 15N solid-state NMR spectroscopy, Biochemistry 43 (2004) 13899–13909.
[51] P.C. Dave, E.K. Tiburu, K. Damodaran, G.A. Lorigan, Investigating structural changes in the lipid bilayer upon insertion of the transmembrane domain of the membrane-bound protein phospholamban utilizing P-31 and H-2 solid-state NMR spectroscopy, Biophys. J. 86 (2004) 1564–1573.
[52] 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.
[53] 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.
[54] T.B. Cardon, E.K. Tiburu, G.A. Lorigan, Magnetically aligned phospholipid bilayers in weak magnetic fields: optimization, mechanism, and advantages for X-band EPR studies, J. Magn. Reson. 161 (2003) 77–90.
[55] M.A. Caporini, A. Padmanabhan, T.B. Cardon, G.A. Lorigan, Investigating magnetically aligned phospholipid bilayers with various lanthanide ions for X-band spin-label EPR studies, Biochim. Biophys. Acta 1612 (2003) 52–58.
[56] N.A. Nusair, E.K. Tiburu, P.C. Dave, G.A. Lorigan, Investigating fatty acids inserted into magnetically aligned phospholipid bilayers using EPR and solid-state NMR spectroscopy, J. Magn. Reson. 168 (2004) 228–237.
[57] P.C. Dave, J.J. Inbaraj, G.A. Lorigan, Electron paramagnetic resonance studies of magnetically aligned phospholipid bilayers utilizing a phospholipid spin label, Langmuir 20 (2004) 5801–5808.
[58] R. Cassol, M.T. Ge, A. Ferrarini, J.H. Freed, Chain dynamics and the simulation of electron spin resonance spectra from oriented phospholipid membranes, J. Phys. Chem., B 101 (1997) 8782–8789.
[59] M. Ge, K.A. Field, R. Aneja, D. Holowka, B. Baird, J.H. Freed, Electron spin resonance characterization of liquid ordered phase of detergent-resistant membranes from RBL-2H3 cells, Biophys. J. 77 (1999) 925–933.
[60] L.J. Berliner, Spin labeling in enzymology: spin-labeled enzymes and proteins, Methods Enzymol. 49 (1978) 466–470.
[61] 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.
[62] K.A. Riske, R.M. Fernandez, O.R. Nascimento, B.L. Bales, M. Teresa Lamy-Freund, DMPG gel-fluid transition monitored by a phospholipid spin labeled at the acyl chain end, Chem. Phys. Lipids 124 (2003) 69–80.
[63] M. Rosemblatt, C. Hidalgo, C. Vergara, N. Ikemoto, Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle, J. Biol. Chem. 256 (1981) 8140–8148.
[64] M.J. Swamy, M. Ramakrishnan, B. Angerstein, D. Marsh, Spin-label electron spin resonance studies on the mode of anchoring and vertical location of the N-acyl chain in *N*-acylphosphatidylethanolamines, Biochemistry 39 (2000) 12476–12484.
[65] T.P.W. McMullen, N.A.H.R. Lewis, R.N. McElhaney, Differ-

ential scanning calorimetric study of the effect of cholesterol on the thermotropic phase—Behavior of a homologous's series of linear saturated phosphatidylcholines, Biochemistry 32 (1993) 516–522.
[66] P. Mattjus, G. Hedstrom, J.P. Slotte, Monolayer interaction of cholesterol with phosphatidylcholines—Effects of phospholipid acyl-chain length, Chem. Phys. Lipids 74 (1994) 195–203.
[67] M.I. Collado, F.M. Goni, A. Alonso, D. Marsh, Domain formation in sphingomyelin/cholesterol mixed membranes studied by spin-label electron spin resonance spectroscopy, Biochemistry 44 (2005) 4911–4918.