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The effects of cholesterol on magnetically aligned phospholipid bilayers: a solid-state NMR and EPR spectroscopy study

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Abstract

This paper presents the first time that both solid-state NMR spectroscopy and EPR spectroscopy are used to study the effects of cholesterol on magnetically aligned phospholipid bilayers (bicelles). Solid-state deuterium NMR spectroscopy was carried out using both chain perdeuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC-d_{54}) and a partially deuterated β-[2,2,3,4,4,6-$^2H_6$]cholesterol (cholesterol-d_6). Also, EPR spectroscopy was carried out utilizing a 3β-doxyl-5α-cholestane (cholestane) spin probe incorporated into magnetically aligned bilayers to provide a more complete picture about the ordering and dynamics of the phospholipid and cholesterol molecules in the bicelle membrane system. The results demonstrate that cholesterol was successfully incorporated into the phospholipid bilayers. The molecular order parameters extracted directly from the ^2H NMR spectra of both DMPC-d_{54} and cholesterol-d_6 were compared to that from the EPR study of cholestane. The order parameters indicate that the sterol was motionally restricted, and that the DMPC had high order and low motion for the hydrocarbon segments close to the head groups of the phospholipids and less order and more rapid motion toward the terminal methyl groups. Both methods clearly indicate an overall increase in the degree of ordering of the molecules in the presence of cholesterol and a decrease in the degree of ordering at higher temperatures. However, EPR spectroscopy and ^2H NMR spectroscopy exhibit different degrees of sensitivity in detecting the phospholipid molecular motions in the membrane. Finally, cholesterol increases the minimum alignment temperature necessary to magnetically align the phospholipid bilayers.

Keywords: Molecular order parameter; Phospholipid bilayers (bicelles); Cholesterol; EPR spectroscopy; Solid-state NMR spectroscopy

1. Introduction

Cholesterol is a major constituent of eukaryotic cell membranes. The distribution of cholesterol varies among the membranes depending upon different locations and stages of cell development, suggesting that cholesterol plays an integral role in cell biology and metabolism [1]. Cholesterol is implicated in a lot of diseases, such as heart disease, stroke, and Alzheimer's disease [2,3]. The effects of cholesterol on model membranes have been studied extensively by a variety of techniques including molecular dynamics simulations, NMR spectroscopy, EPR spectroscopy, X-ray diffraction, neutron diffraction, differential scanning calorimetric spectroscopy, and Fourier transform infrared spectroscopy [4–11]. It is believed that cholesterol acts as a regulator by modulating the fluidity of cellular membranes. Cholesterol can enhance the mechanical strength of the membrane and alter the gel-to-liquid crystalline (L_α) phase transition temperature of the lipid [12]. Molecular dynamics simulations of phospholipid bilayers as a function of cholesterol concentration indicate a significant increase in ordering of the phospholipid chains, and a reduced fraction of *gauche* conformations as well as a reduction in lateral diffusion of the phospholipids [13,14]. Deuterium NMR relaxation studies on phospholipid bilayers containing cholesterol suggest that axial rotations of the phospholipid molecules occur at a higher rate than in pure phospholipid bilayers. Additionally, the rigid cholesterol molecule appears to undergo slower axial rotation than the corresponding phospholipid molecules [15]. However, the effects of cholesterol on magnetically aligned phospholipid

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bilayers (bicelle) have never been studied in such detail from both the sides of phospholipids and cholesterol molecules [16,17].

Solid-state ^{2}H NMR and EPR spectroscopic techniques have been used to study the structural and dynamic properties of phospholipid bilayers [15,18–20]. However, the intrinsic difference between NMR and EPR spectroscopy has to be understood in order to correctly apply these techniques to a variety of different systems. Because of the low natural abundance of ^{2}H, no background signals from unlabeled components are observed in ^{2}H NMR studies [19,21]. Alternatively, EPR spectroscopy exhibits a high sensitivity to the rate of motion and the degree of organization of the phospholipids, since the characteristic time scale for nitroxide spin-label EPR matches very closely the rates of molecular rotation of the lipids within the membrane [18]. Both techniques are carried out and compared in this study in order to better understand the effect of cholesterol on bicelle model membrane systems. The differences between these two magnetic resonance techniques are discussed.

In this work, magnetically aligned phospholipid bilayers have been investigated using solid-state NMR and spin-label EPR spectroscopic techniques in the presence of cholesterol. Previously, most cholesterol studies were based on unoriented or mechanically aligned membrane samples [9,22]. The use of multilamellar vesicles in solid-state NMR spectroscopic studies gives rise to broad powder type spectra. To obtain pertinent structural and dynamic information requires deconvoluting the spectra through the so-called ''dePakeing'' process [23]. Alternatively, uniaxially bilayer membranes aligned in both EPR and solid-state NMR studies reveal high-resolution spectra and pertinent structural and dynamic information. Traditionally, macroscopic uniaxial orientation of membrane bilayers can be accomplished mechanically by stacking phospholipids bilayers between glass plates [24]. The ^{2}H NMR quadrupolar splittings can yield the dynamic information on deuterium-labeled segments of the phospholipid acyl chains. Also the EPR spectra reveal orientational information derived from the hyperfine splitting of the aligned spectra. The anisotropic hyperfine splitting of an aligned spin probe can provide a more detailed structural picture of the probe with respect to the membrane, when compared to randomly dispersed phospholipid bilayer samples [25].

The magnetically aligned bicelles are a promising technique that is well suited for a wide variety of spectroscopic studies, such as, NMR spectroscopy and EPR spectroscopy [24,26,27]. Bicelles are formed upon mixing long-chain phospholipid molecules, such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) with short lipid molecules, such as 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC). The magnetic alignment of bicelles depends on the magnetic susceptibility anisotropy tensor ($\Delta\chi$) of the phospholipid bilayers. The negative sign of $\Delta\chi$ for bicelles dictates that they align with their bilayer normal oriented perpendicular to the direction of the static magnetic field (B_0) [28–30]. The degree of ordering of bicelles depends upon several factors, including the strength of the magnetic field, the sign and magnitude of the phospholipid bilayers magnetic susceptibility anisotropy tensor, the viscosity of the sample, the types of lanthanide ions used, and the temperature [3,31–33].

In NMR experiments at high magnetic fields, the bicelles can spontaneously align above the gel-to-liquid crystalline phase transition temperature, T_m [29]. When placed into weak magnetic fields typically used in an X-band EPR spectrometer, the bilayers cannot spontaneously align at either the perpendicular or the parallel alignment [27,30]. Paramagnetic lanthanide ions (i.e., Dy^{3+}, Tm^{3+}) can be used as alignment reagents to help the bilayers align either in the perpendicular or parallel orientations [24,33]. 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 [30]. The negative value of $\Delta\chi$ is increased by the addition of Dy^{3+} to the sample. 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. For solid-state ^{2}H NMR studies on bicelles, the spectral resolution is increased in the parallel orientation because the quadrupolar splittings are doubled. The parallel alignment offers advantages in studying membrane-associated peptides with rapid reorientations about the long molecular axis [26]. In this paper, both orientations are studied by adding either Dy^{3+} or Tm^{3+} to the bicelle membrane system.

In order to understand the interaction between the cholesterol and the phospholipids, molecular order parameters (S_{mol}) are used to define the structural and dynamical properties of the corresponding molecules in the membrane. An ensemble of molecules give rise to $S_{mol} = 0$ for unrestricted motions of every individual molecule. A S_{mol} value of 1 indicates that all the molecules are perfectly aligned and motionally restricted in one direction [16,29,34,35]. The order parameter (S_{mol}) calculations concerning the analysis of the EPR spectra of the cholestane spin probe and NMR spectra of deuterium-labeled cholesterol or deuterium-labeled phospholipid molecules are presented in this paper. The results indicate that magnetically aligned DMPC/DHPC bicelles are an excellent model membrane system for both EPR and solid-state NMR studies. Also, the bicelle samples are easy to prepare and avoid using dePakeing, altogether making it easier to calculate the molecular order parameters directly from the quadrupolar splittings of each deuteron from NMR spectra.

2. Results

2.1. EPR study of magnetically aligned phospholipid bilayers using the cholestane spin probe

The EPR spectra of cholestane inserted into DMPC/DHPC bicelles of varying cholesterol concentrations are displayed in Fig. 1 at 318 K (solid line, unaligned bicelles; dotted line, parallel-aligned bicelles; and dashed line, perpendicular-aligned bicelles). The addition of small amounts of lanthanide ions Tm^{3+} and Dy^{3+} enables the DMPC/DHPC bicelles to align at the two different orientations in the magnetic field [25,29]. The lineshapes and hyperfine splittings indicate that the phospholipid bilayers are well aligned. The hyperfine splittings (measured between the $m_I = +1$ and 0 peaks) for parallel-aligned bicelles decrease from 10.6 to 8.6 G as the cholesterol concentration increases, while the hyperfine splittings for the perpendicular-aligned bicelles increase from 17.2 to 18.4 G.

Fig. 2 shows the EPR spectra of cholestane inserted into DMPC/DHPC bicelle samples with 15 mol% cholesterol with respect to DMPC as a function of temperature. The spectra taken at 308 K indicate that the phospholipid bilayers are not well aligned in the magnetic field, because the hyperfine splitting and lineshape

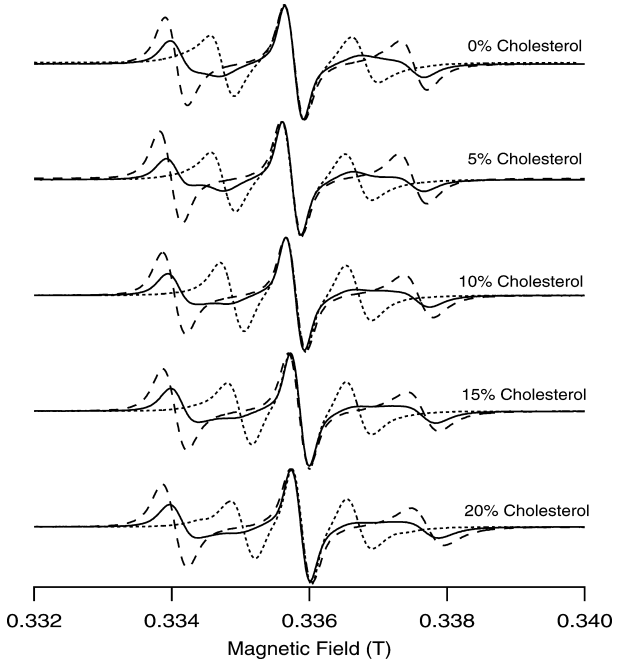

Fig. 1. EPR spectra taken at 318 K of the cholestane spin probe incorporated into oriented and randomly dispersed DMPC/DHPC/cholesterol bicelles at cholesterol concentrations of 0, 5, 10, 15, and 20 mol% with respect to DMPC. The *solid-line* spectra represent the randomly dispersed bicelles without added lanthanide ions. The *dashed-line* spectra represent the Dy^{3+}-doped bicelle sample with the normal of the bilayers being perpendicular to the static magnetic field. The *dotted-line* spectra represent the Tm^{3+}-doped bicelle sample with the normal of the bilayers being parallel to the static magnetic field.

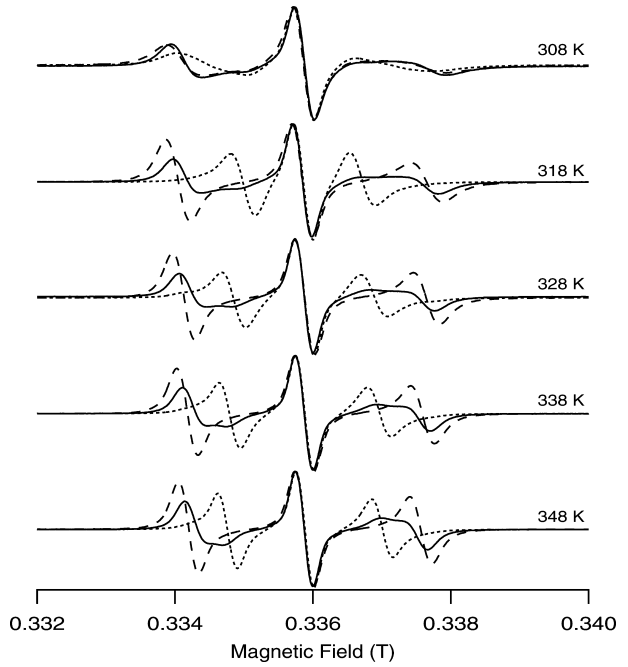

Fig. 2. EPR spectra of the cholestane spin probe incorporated into oriented and randomly dispersed DMPC/DHPC/cholesterol bicelles at 15 mol% cholesterol with respect to DMPC over the temperature range from 308 to 348 K. The *solid-line* spectra represent the randomly dispersed bicelles without added lanthanide ions. The *dashed-line* spectra represent the Dy^{3+}-doped bicelle sample with the normal of the bilayers being perpendicular to the static magnetic field. The *dotted-line* spectra represent the Tm^{3+}-doped bicelle sample with the normal of the bilayers being parallel to the static magnetic field.

of the spectra with either Tm^{3+} or Dy^{3+} are nearly the same as the randomly aligned spectrum. At 313 K, the spectra indicate that the bicelles with 15 mol% cholesterol with respect to DMPC are aligned (data not shown) and look similar to the spectra taken at higher temperatures (318–348 K) shown in Fig. 2. The hyperfine splitting decreases from 18.0 to 16.8 G for the bicelles in the perpendicular orientation and increases from 8.8 to 11.3 G in the parallel orientation as the temperature increases. At 20 mol% cholesterol with respect to DMPC, the bicelles are not well aligned at 308 and 313 K, but are aligned from 318 to 348 K (data not shown). However, magnetically aligned phospholipid bilayers prepared with 0, 5, and 10 mol% cholesterol with respect to DMPC are aligned at both the parallel and perpendicular orientations at 308 K. Therefore, the minimum temperature required for alignment of the phospholipid bilayers increases as the cholesterol concentration increases.

The method for calculating the molecular order parameters (S_{mol}) for the 3β-doxyl-5α-cholestane (cholestane) is described in Section 5. Fig. 3 shows the changes in S_{mol} in the presence of cholesterol as a function of temperature. The concentration of cholesterol ranges from 0 to 20 mol% with respect to DMPC. The ordering of the cholestane in the membrane decreases as the

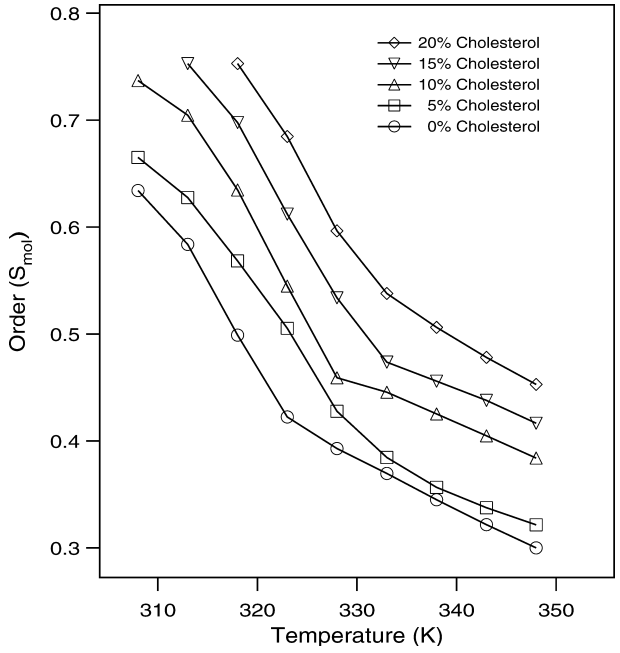

Fig. 3. The molecular order parameters (S_{mol}) calculated from the spin-labeled cholestane EPR spectra as a function of temperature in magnetically aligned DMPC/DHPC/cholesterol bicelles at cholesterol concentrations of 0, 5, 10, 15, and 20 mol% with respect to DMPC.

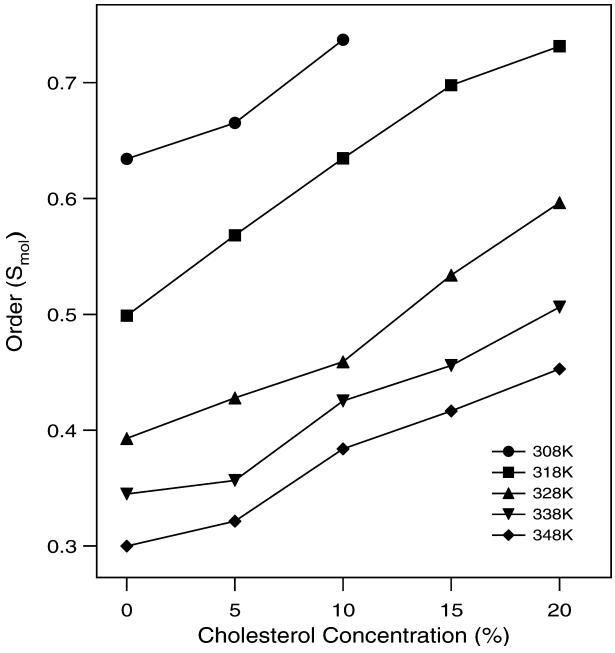

Fig. 4. The molecular order parameters calculated from the cholestane spin probe EPR spectra as a function of cholesterol concentration in magnetically aligned DMPC/DHPC/cholesterol bicelles. The sample temperature of the spectra ranges from 308 to 348 K.

temperature increases. The degree of ordering decreases gradually in the low temperature region. At higher temperatures (above 323–333 K), the ordering shows a slower decrease.

For comparison, Fig. 4 displays the ordering profile of cholestane as a function of cholesterol concentration at different temperatures. At a given temperature, the ordering of the membrane increases as the cholesterol concentration increases, and the slope of the increase of ordering is higher for the bicelle samples at lower temperatures. At 318 K, the ordering increases from 0.5 to more than 0.7. In contrast, the ordering changes from 0.3 to about 0.4 at 348 K. The spin-labeled cholestane is used to probe the motions and the dynamics of the phospholipid membranes. The increase in ordering shows that the rigid sterol ring of cholesterol limits the motion of lipid molecules in the membrane and the restriction effect is more pronounced at low temperatures (308, 318 K).

2.2. Solid-state NMR study of magnetically aligned phospholipid bilayers using deuterium-labeled cholesterol

Deuterium-labeled cholesterol-d_6 NMR studies have also been carried out on the same compositions of DMPC/DHPC/cholesterol bicelle samples. Cholesterol has been shown to orient in phospholipids bilayers with its polar hydroxyl group in close vicinity to the head groups of phospholipid molecules and its alkyl side chain extending towards the bilayer center [36]. The amount of cholesterol-d_6 in the bicelle samples is titrated from 5 to 20 mol% with respect to DMPC. The ^{2}H NMR spectra of 10 mol% cholesterol-d_6 at 318 K is displayed in Fig. 5A. The resolved peaks are simulated using the DMFIT simulation program and are displayed as dotted lines in Fig. 5B [37]. The peak assignments are based upon previous work [22]. The inner doublets with the smallest quadrupolar splitting (4.2 kHz) represent the 6-^{2}H deuteron. The second smallest quadrupolar splittings (48.1 and 50.9 kHz) represent the 4-$^2H_{eq}$ and 2-$^2H_{eq}$ deuterons on the fused ring of cholesterol, and the last set of doublets (73.4, 76.1, and 79.2 kHz) correspond to the quadrupolar splitting of the 4,2-$^2H_{ax}$ deuterons and the deuteron at the C_3–^{2}H position, respectively. The molecular order parameters are calculated as described in Section 5 and the results are shown in Fig. 9. The six quadrupolar splittings from the different C–^{2}H bonds give almost the same molecular order parameter (S_{mol}) values within a small standard deviation. The final result is the average of the six values of S_{mol}. The quadrupolar splittings and S_{mol} values confirm that the cholesterol ring is rather rigid in the phospholipid bilayer under these conditions.

2.3. Solid-state NMR study of magnetically aligned phospholipid bilayers using deuterium-labeled DMPC-d_{54}

The molecular order parameters of deuterium-labeled cholesterol will only give information about the flexibility of the lipid chains indirectly. However, by

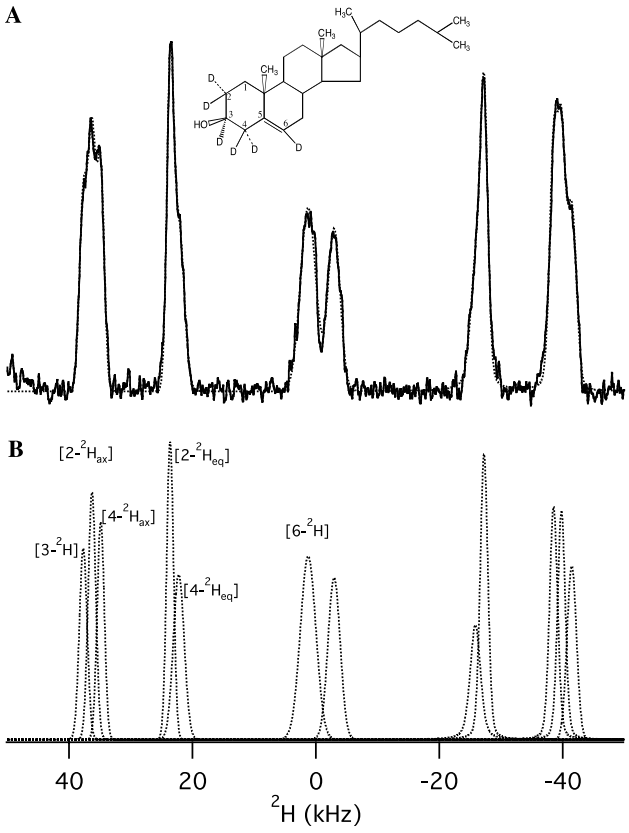

Fig. 5. ^{2}H NMR spectra of Tm^{3+}-doped magnetically aligned DMPC/DHPC/ cholesterol-d_6 phospholipids bilayers at 318 K. Cholesterol-d_6 is incorporated into the magnetically aligned bilayers as the deuterium label. The concentration of cholesterol-d_6 is 10 mol% with respect to DMPC. The structure of cholesterol-d_6 is shown in the inset. The simulations were carried out using the DMFIT simulation program. (A) The *solid-line* spectrum represents the ^{2}H NMR spectrum. The *dotted-line* spectrum is the summed simulated NMR spectrum. (B) The dotted line in the spectrum represents individual peaks that are simulated. The peaks are labeled according to the assignment by Marsan et al. [22].

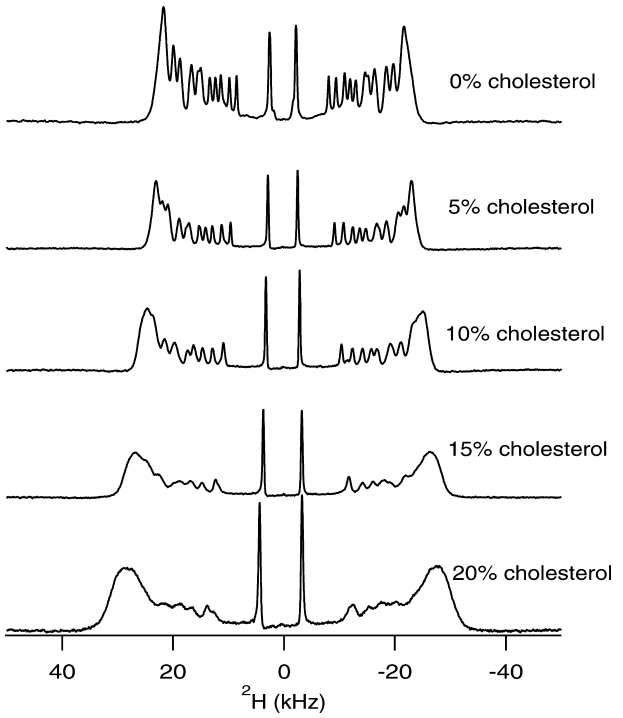

Fig. 6. ^{2}H NMR spectra of Tm^{3+}-doped magnetically aligned DMPC/DHPC/DMPC-d_{54}/cholesterol phospholipid bilayers at 318 K. DMPC-d_{54} is incorporated into the magnetically aligned bicelles as the deuterium label. The cholesterol concentration is 0, 5, 10, 15, and 20 mol% with respect to DMPC.

incorporating DMPC-d_{54} into the bicelle samples, the lipid chain dynamics can be directly investigated with ^{2}H solid-state NMR studies. The bicelle samples are analyzed in both the parallel and perpendicular orientations. The molecular order parameters calculated from both bicelle samples are similar. However, only the parallel-aligned phospholipid bilayer data will be presented due to the increased resolution. Fig. 6 shows a series of ^{2}H NMR spectra of parallel-aligned DMPC/DHPC phospholipid bilayer samples doped with DMPC-d_{54} investigated as a function of cholesterol concentration at 318 K. The ^{2}H NMR spectrum of the bicelle sample with 0 mol% cholesterol with respect to DMPC is well resolved. The good resolution is clearly indicative of well-aligned phospholipid bilayers. As the cholesterol concentration increases, the spectra become broader and start to lose resolution. The bicelle samples prepared in the absence of cholesterol yield well-resolved spectra at 308 K and at a slightly lower temperature. The bicelle samples prepared with 10 mol% cholesterol with respect to DMPC yield well-resolved spectra at a temperature of 318 K or higher. However, for the bicelle samples with 20 mol% cholesterol, well-resolved spectra were obtained only at temperature above 318 K. Thus, the minimum temperature required for alignment of DMPC/DHPC phospholipid bilayers increases as the cholesterol concentration increases from 0 to 20 mol%. This result is consistent with the EPR results.

Fig. 7 displays the molecular order parameter S_{mol} profile versus the carbon atom position along the acyl chains of DMPC-d_{54} placed into magnetically aligned phospholipid bilayers. The S_{mol} for the individual C–^{2}H bonds of the methylene groups and the terminal methyl groups of the acyl chains is directly evaluated from the corresponding quadrupolar splittings of each group. The quadrupolar splittings for the deuterons in the plateau region are estimated by integration of the last broad peak according to the literature [38]. Fig. 7A shows the molecular order parameter S_{mol} profile of varying cholesterol concentrations as a function of the carbon number. The molecular order parameters decrease gradually towards the end of the hydrocarbon chain. Cholesterol enhances the ordering of the entire acyl chain within the phospholipid bilayers as the amount of cholesterol in the bicelle sample increases from 0 to 20 mol% with respect to DMPC. The increase

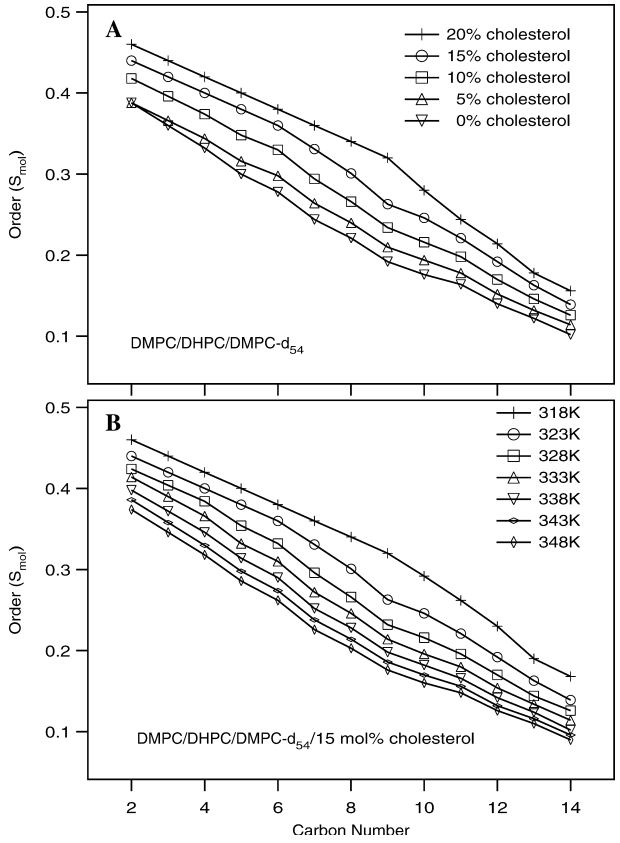

Fig. 7. Molecular order parameter profiles with respect to the position along the acyl chain of deuterium labeled DMPC-d_{54} incorporated into Tm^{3+}-doped DMPC/DHPC/cholesterol bicelle samples. (A) The cholesterol concentrations are 0, 5, 10, 15, and 20 mol% with respect to DMPC. The sample temperature is 323 K. (B) The cholesterol concentration is 15 mol% with respect to DMPC. The temperature range is from 318 to 348 K. The DMPC/DHPC/cholesterol bicelles with 15 mol% cholesterol at 308 and 313 K are not aligned.

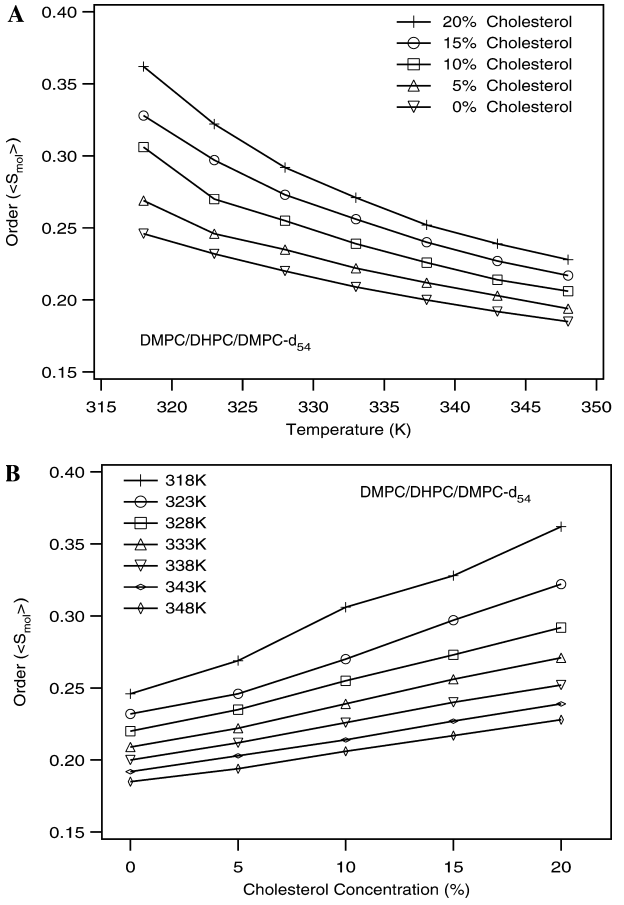

Fig. 8. (A) The average molecular order parameter profiles over the entire length of the acyl chain of DMPC-d_{54} as a function of temperature in magnetically aligned DMPC/DHPC/cholesterol bicelles at cholesterol concentrations of 0, 5, 10, 15, and 20 mol% with respect to DMPC. (B) The average molecular order parameter profiles over the entire length of the acyl chain of DMPC-d_{54} as a function of cholesterol concentration in magnetically aligned DMPC/DHPC/cholesterol bicelles at different temperatures. Both graphs are from bicelle samples that are aligned with their normal being parallel to the static magnetic field.

in ordering of the methylene groups near the top of the hydrocarbon chain is almost the same as the increase in ordering of the methyl groups at the end of the hydrocarbon chain as the cholesterol concentration increases. These results indicate that the ordering effect of cholesterol to the phospholipid molecules is uniform along the entire acyl chains of the phospholipid molecules. Fig. 7B shows the temperature effect on the molecular order parameter of DMPC-d_{54} at 15 mol% cholesterol with respect to DMPC. The molecular order parameters decrease as the temperature increases. The decrease in ordering of the methylene groups and methyl groups at different positions along the acyl chain of the phospholipid molecules are almost identical.

The normalized order parameter over the 13 quadrupolar splittings, calculated using $\langle S_{\mathrm{mol}}\rangle = (1/13)\sum_{i=2}^{14} S_{\mathrm{mol}}^{\mathrm{i}}$, can be used to characterize the ordering of the entire myristoyl chain [29]. Fig. 8A displays the cholesterol concentration dependence of the normalized order parameter profile as a function of temperature. In Fig. 8B, the normalized order parameter is plotted versus cholesterol concentration at various temperatures. $\langle S_{\mathrm{mol}}\rangle$ decreases with increasing temperature, but increases with increasing cholesterol concentration. Both graphs indicate that the ordering effect of cholesterol on the membranes is more significant at low temperatures. In contrast, the disordering effect on the membrane caused by increasing the temperature is more significant at high cholesterol concentrations. These results agree well with the corresponding EPR results (Figs. 3 and 4).

Fig. 9A compares the cholestane EPR data from Fig. 3 with the deuterium-labeled cholesterol-d_6 NMR data. The data show an overall decrease in the S_{mol} order parameters of cholesterol-d_6/cholestane incorporated into the DMPC/DHPC phospholipid bilayers as the sample temperature increases. The cholesterol-d_6/cholestane

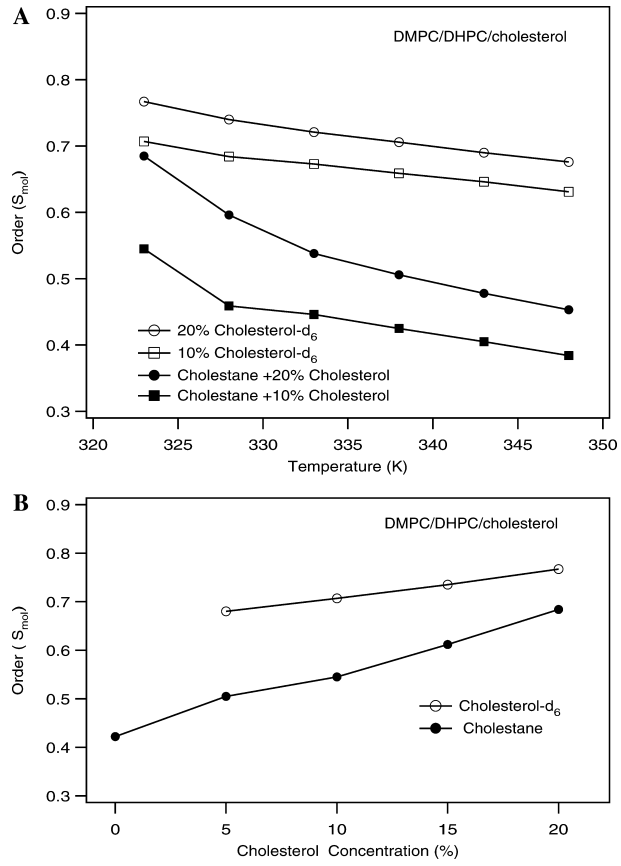

Fig. 9. A comparison of the molecular order parameters (S_{mol}) obtained by spin-labeled cholestane (open symbols) with S_{mol} obtained by [^{2}H]cholesterol-d_6 (closed symbols) incorporated into DMPC/DHPC/cholesterol bicelles as a function of temperature. (A) S_{mol} is displayed and as a function of the cholesterol concentration. (B) S_{mol} at a temperature of 323 K. Tm^{3+}-doped bicelle samples were used to obtain the molecular order parameters from the ^{2}H NMR spectra. Tm^{3+}- and Dy^{3+}-doped bicelle samples were used to obtain the order parameters from cholestane spin probe EPR spectra.

gives high molecular order parameter values while the phospholipids yield lower molecular order parameter values (Figs. 7 and 8). These results indicate that phospholipid molecules undergo faster motions than the corresponding cholesterol molecules. [^{2}H]cholesterol and spin-labeled cholestane give slightly different information with deuterium-labeled cholesterol having larger molecular order parameter values and less dependence upon the temperature. Fig. 9B displays the molecular order parameters as a function of cholesterol concentration at 323 K. The cholesterol-d_6/cholestane molecular order parameters increase as the cholesterol concentration increases. ^{2}H NMR spectra of cholesterol-d_6 incorporated into DMPC/DHPC magnetically aligned bilayers show less dependence on the degree of ordering on cholesterol concentration than the corresponding EPR spectra of cholestane incorporated into magnetically aligned phospholipid bilayers.

3. Discussion

The EPR lineshapes and hyperfine splittings shown in Fig. 1 for magnetically aligned phospholipid bilayers are consistent with previously published spectra of aligned bicelles containing cholestane [30]. The bicelle samples incorporated with cholesterol-d_6 or DMPC-d_{54} yield well-resolved NMR peaks (Figs. 5 and 6). The magnetically aligned spectra demonstrate that cholesterol/cholestane molecules have been successfully incorporated into the bicelle membranes and that the phospholipid bilayers can be well aligned in both EPR and NMR magnetic fields at certain temperatures.

In this paper, we present for the first time the molecular order parameters obtained from both deuterium-labeled cholesterol and deuterium-labeled phospholipid molecules inserted into magnetically aligned phospholipid bilayers. The deuterium-labeled cholesterol-d_6 NMR spectrum yields six different quadrupolar splittings corresponding to C–^{2}H bonds at six different positions of the sterol ring. However, the molecular order parameters calculated from the six quadrupolar splittings are similar to each other in magnitude. The result confirms that cholesterol molecules are fairly rigid inside the DMPC/DHPC phospholipid bilayers. The molecular order parameter profiles of the acyl chains of DMPC-d_{54} incorporated into the bilayers as a function of temperature and cholesterol concentration are also compared in Fig. 7. The magnitude of change of ordering is uniform along the entire acyl chains from carbon 2 to 14. The phospholipid acyl chain carbons at position 2–10 have been estimated to lie close to the sterol ring structure of cholesterol [36] and the carbons at positions 11–14 are close to the side chain tail of cholesterol. Analysis of the data indicates that the cholesterol side chain located at the end of the sterol ring is also rigid and has a similar ordering effect on the phospholipid molecules. The effective length of cholesterol has been estimated to correspond to a 17-carbon all-trans hydrocarbon chain [36], while DMPC has 14 carbons on one of its acyl chains. Therefore, the side chain of cholesterol is probably highly packed and tangled in the middle of the phospholipid bilayers, which induces ordering of the membrane in that region.

Three sets of S_{mol} order parameters on cholestane, cholesterol, and phospholipid molecules from both spin-label EPR and ^{2}H NMR spectroscopic studies are presented in this work for the first time on bicelles and are consistent with each other. The degree of ordering increases as the amount of cholesterol in the DMPC/DHPC bilayer samples increases. Actually cholesterol molecules have a higher degree of ordering and slower motion than the corresponding phospholipids 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 the relatively higher activation energy for molecular rotation about its molecular axis than the corresponding phospholipid molecules. 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 motion (chain rotation and fluctuation) and intramolecular motion will increase. Therefore, the molecular ordering decreases when the temperature increases.

A detailed analysis of the EPR spectra of phospholipid bilayers with the nitroxide spin-label cholestane revealed that the hyperfine anisotropy tensor has the following values $A_{xx} = 5.8\,\mathrm{G}$, $A_{yy} = 5.8\,\mathrm{G}$, and $A_{zz} = 30.8\,\mathrm{G}$ [34]. The observed hyperfine splittings depend upon the orientation of the spin probe with respect to the magnetic field and the motion of the molecules about its three tensor-axes. The long molecular axis of the cholestane molecule and *y*-axis of the nitroxide spin label are reported to lie approximately parallel to the membrane normal of the phospholipid bilayers when cholestane is inserted into the membranes. If the phospholipid bilayers are aligned with their bilayer normal perpendicular to the magnetic field, the hyperfine splitting should equal the average value of A_{zz} and A_{xx} ($(A_{xx} + A_{zz})/2 = 18.3\,\mathrm{G}$). The measured hyperfine splitting ranges from 17 to 18 G, slightly less than the theoretical value of 18.3 G. This variation is attributed to the fluctuation of the long molecular axis of cholestane or the slight difference in the orientation between the long molecule axis and *y*-axis of the nitroxide spin probe. As the cholesterol concentration reaches 20 mol% in the phospholipid bilayers, the hyperfine splitting increases to 18.4 G at 318 K. These results indicate that the addition of cholesterol to the DMPC/DHPC membrane reduces the fluctuation of the molecular axis. Conversely, the hyperfine splitting of cholestane inserted into DMPC/DHPC bilayers in the perpendicular orientation decreases from 18.0 to 16.8 G when the temperature increases. The decrease in the hyperfine splitting is due to the faster molecular axis fluctuations at higher temperatures. The fast molecular motion makes A_{yy} a nonnegligible factor in determining the hyperfine splittings. An ideal motionally averaged isotropic sample yields an isotropic hyperfine value of 14.5 G ($(A_{xx} + A_{yy} + A_{zz})/2 = 14.5\,\mathrm{G}$).

In Fig. 3, the EPR studies indicate that the decrease of ordering displays a steep slope in the low temperature range (308–323 K). At higher temperatures, the decrease of ordering is slower with a smaller slope. There is a noticeable turning point from the steep decrease to the slow decrease around 323–333 K, depending on the cholesterol concentration. This may indicate a morphological change corresponding to the liquid crystalline nematic phase changing to a smectic phase at a temperature around 323–333 K [30]. When the cholesterol concentration is raised, the presumed nematic-to-smetic phase transition temperature is also raised. At this temperature range, the ^{2}H NMR spectra show higher resolution. These results agree with work in the literature and indicates that a conversion from a positively aligned nematic to a positive-aligned smectic phase coexists with an isotropic phase between about 313–328 K [3]. Previously, we have characterized the nematic-to-smectic phase change of DMPC/DHPC bicelle samples with ^{2}H NMR spectroscopy in the absence of cholesterol and cholestane [30]. However, the changes in the molecular order parameters of DMPC-d_{54} and cholesterol-d_6 given by ^{2}H NMR in Figs. 8 and 9 show a gradual decrease as the temperature increases. As mentioned before, EPR spectroscopy is sensitive to molecular motions of phospholipids within the membrane. EPR spectra of nitroxide radicals are particularly sensitive to motions with correlation times between 10^{-10} and 10^{-8} s, which is in the range of correlation times for molecular "long-axis" rotations and wobbling [18]. In this sense, EPR spectroscopy can provide more detailed information about the membrane than NMR spectroscopy.

In the present paper, both deuterium-labeled cholesterol and spin-labeled cholestane are used to study the DMPC/DHPC bicelle membrane system. The data indicates that the behavior of the two probes is similar. The degree of ordering of the membrane increases as the amount of cholesterol increases in the sample and the ordering decreases as the temperature increases. However, the degree of ordering of cholesterol in the membranes is lower for the EPR data than for the corresponding NMR data (Fig. 9). This result can be explained by the different properties of the two probes. The cholesterol OH group is replaced by a bulky nitroxide spin label in cholestane, which will probably slightly distort the close packing of the headgroups of the phospholipids bilayers. For the molecular order parameter calculations, different rotational axes are used for the deuterium-labeled cholesterol and the spin-labeled cholestane. The rotation axis of cholesterol is defined in the OH–C_3–^{2}H reference systems [22], whereas the motion of the cholestane is believed to be about the nitroxide *y*-axis [34]. The small deviation of the rotation axis would also result in the difference observed in the molecular order parameter values.

Inspection of Fig. 9 indicates that the cholesterol-d_6 NMR data yield larger molecular order parameter values with less dependence upon temperature than the

cholestane EPR data. The order parameters given by cholestane show a larger dependence upon temperature and cholesterol concentration. The correspondence between the magnitudes of the molecular order parameters for the two probes is best in situations of high inherent order (high cholesterol concentration and low temperature). The S_{mol} values from EPR and NMR data diverge as the membrane becomes more flexible and the degree of ordering decreases. The degree of ordering in the membrane with cholestane is more sensitive to changes in membrane composition and environment probably due to the existence of the nitroxide radicals. In a comparison study of phospholipid bilayer systems that contain the cholestane spin probe or a cholesterol-3α-*d* deuterium probe, the NMR results show less dependence upon temperature and cholesterol concentration, which agrees with our results [8]. The discrepancy between the EPR and NMR data observed in this paper is also caused by the difference in sensitivity of the two techniques over the various molecular motion time scales. ^{2}H NMR spectra are sensitive to motions with correlation times between 10^{-3} and 10^{-9} s [39], whereas EPR spectra of nitroxide spin probes are particularly sensitive to faster motions with correlation times between 10^{-10} and 10^{-8} s [21]. The phospholipids molecular chain rotation and wobbling will be at the fast-motional limit of NMR time scale. When the temperature increases or the amount of cholesterol in the bilayers decreases, the membrane will be more fluid and the molecules will undergo faster motions. Under these conditions, the correlation time fits better on the EPR time scale. Thus, spin-label EPR spectroscopy is more sensitive for probing faster motions, when compared to solid-state ^{2}H NMR spectroscopy. This explains why the molecular order parameter values obtained by EPR spectroscopy and NMR spectroscopy studies are more consistent under conditions of higher ordering in the membrane.

In both the EPR and NMR spectroscopic studies, the minimum alignment temperature of the bicelles increases as the cholesterol concentration increases. The DMPC/DHPC phospholipid bilayers in the absence of cholesterol are aligned at a minimum temperature of 308 K. The DMPC/DHPC phospholipid bilayers with 20 mol% cholesterol with respect to DMPC are aligned at a minimum temperature of 318 K (10 K higher). The alignment of the bicelles is a cooperative phenomenon. The alignment of bicelles only occurs when the sample temperature is raised above the lipid gel-to-liquid crystalline phase transition temperature. In the gel phase, the ^{2}H NMR spectrum exhibits only an isotropic peak, which suggests the formation of small rapidly tumbling bicelles in an isotropic phase [20,30]. Above the gel-to-liquid crystalline phase transition temperature, the bicelles become aligned in the magnetic field. The phase of the phospholipid bilayers is sensitive to membrane compositions, membrane hydration, temperature, and other factors [31,39]. The incorporation of cholesterol alters the physical and phase properties of the phospholipid bilayers. Cholesterol increases the molecular ordering of the phospholipid molecules at the temperature above the gel-to-liquid crystalline phase transition temperature, most likely by restricting the motions of the neighboring acyl chains, reducing the fraction of *gauche* bonds of the acyl chains, making the acyl chains pack tighter together. As discussed above, the membrane nematic-to-smectic phase transition temperature also increases as the cholesterol concentration is raised in the DMPC/DHPC phospholipid bilayer samples. The data suggests that the lipid gel-to-liquid phase transition temperature is raised by increasing the cholesterol concentration, and the minimum bicelle alignment temperature increases as well.

4. Conclusion

We present here for the first time a comparison of both spin-labeled EPR and ^{2}H-labeled NMR techniques to study the effect cholesterol has on DMPC/DHPC magnetically aligned phospholipid bilayer membranes. Both techniques are ideal ways to study the unique phospholipid membrane system. Our study provides a relatively complete picture of the cholesterol effect on bicelles by both EPR and solid-state NMR techniques with different sensitivity. Cholesterol molecules are rather rigid. Cholesterol incorporated into the phospholipid bilayers increases the molecular ordering of the phospholipid acyl chains, stabilizes the liquid crystalline phase of the phospholipids, increases the minimum alignment temperature of the DMPC/DHPC phospholipid bilayers, and increases the liquid crystalline nematic-to-smectic phase transition temperature. The S_{mol} values obtained in this paper for DMPC/DHPC bilayers are consistent with previous EPR and solid-state NMR studies on other phospholipid bilayer systems [8,9,18,22,40,41]. Thus, magnetically aligned phospholipid bilayers represent an excellent model membrane system for both spin-labeled EPR and solid-state NMR studies.

5. Materials and methods

5.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC), deuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC-d_{54}), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-2000] (PEG 2000-PE) were purchased

from Avanti Polar Lipids (Alabaster, AL). Thulium (III) chloride hexahydrate, Dysprosium (III) chloride hexahydrate, 3β-doxyl-5α cholestane (cholestane), and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes) were obtained from Sigma/Aldrich (St. Louis, MO). The cholesterol was purchased from Avocado Research Chemicals (Heysham, England). β-[2,2,3,4,4,6-$^2H_6$]Cholesterol was purchased from Cambridge Isotope Laboratories (Andover, MA). Deuterium-depleted water was obtained from Isotec (Miamisburg, OH). All phospholipids were dissolved in chloroform and stored at −20 °C prior to use. Aqueous solutions of lanthanide ions were prepared fresh each day. All aqueous solutions were prepared with nanopure water.

5.2. Sample preparation

The standard DMPC/DHPC bicelle samples, consisting of 25% (w/w) phospholipids to solution with a q ratio of 3.5, were made in 25 mL pear-shaped flasks. Cholesterol concentrations were used from 0 to 20 mol% in 5 mol% increments (molar ratio to DMPC). For EPR samples, DMPC, PEG2000-PE, DHPC, and spin-label cholestane were mixed in molar ratio of 3.5:0.035:1:0.0196, respectively. Different amounts of nondeuterated cholesterol were also added. For the ^2H NMR deuterated cholesterol samples, DMPC, PEG2000-PE, and DHPC were mixed in the molar ratio of 3.5:0.035:1. Deuterated cholesterol was added from 0, 5, 10, 15, to 20 mol% with respect to DMPC. For deuterated DMPC-d_{54} samples, DMPC, PEG2000-PE, DHPC, and DMPC-d_{54} were mixed in ratios of 3.22:0.035:1:0.22. Nondeuterated cholesterol was then added to the mixture. The lipids were rotoevaporated down at room temperature and placed in a vacuum desiccator overnight to dry.

The following day 100 mM Hepes buffer of pH 7.0 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 solubilized. Then the samples were sonicated in a Fisher Scientific FS 30 bath sonicator (Florence, KY) for 30 min with the heater turned off and ice added to the bath. The sample was subjected to several freeze/thaw cycles at room temperature. Finally, Tm^{3+} or Dy^{3+} (aq) was added so that the amount of Tm^{3+} (Dy^{3+}) was 20 mol% (molar ratio to DMPC). The sample was gently tilted and slowly rotated for a few minutes until the sample looked homogeneous.

5.3. EPR spectroscopy

The bicelle samples were drawn into 1 mm ID capillary tubes via a syringe. Both ends of the tube were sealed off with Critoseal purchased from Fisher Scientific (Florence, KY). The capillary tube was placed into the standard quartz EPR tube (707-SQ-250M) purchased from Wilmad Glass (Buena, NJ) and filled with light mineral oil.

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 was set to 2.0 mW. All oriented samples were aligned by warming the sample from 298 to 318 K at a maximum magnetic field strength of 0.64 T (6400 G) [30].

5.4. Solid-state ^2H NMR spectroscopy

All solid-state NMR experiments were carried out on a Bruker AVANCE 11.4 T wide bore solid-state NMR spectrometer. The solid-state NMR spectra were gathered with a static double-resonance 5 mm round-coil solid-state NMR probe purchased from Bruker. ^2H NMR spectra were recorded at 76.77 MHz using a standard quad-echo pulse sequence [42]. The following spectrometer conditions were used: 2.6 μs 90° pulses for deuterium-labeled cholesterol, 3.1 μs 90° pulse for deuterium-labeled DMPC, 20 μs innerpulse delay, 10 ms acquisition time, 0.4 s recycle delay, and a 170 kHz spectral width for deuterium-labeled cholesterol, and a 150 kHz spectral width for deuterium-labeled DMPC. Typically 10 K scans were accumulated for the deuterium-labeled DMPC samples, and 16–32 K scans were accumulated for the deuterium-labeled cholesterol in the quadrature detection mode. An exponential line broadening of 300 Hz was applied to the free induction decay before Fourier transformation. The experiments were carried out over the temperature range from 308 to 348 K in 5 K increments. For deuterium-labeled cholesterol bicelle samples, only Tm^{3+}-doped bicelles were studied. Both Tm^{3+}-doped and Dy^{3+}-doped bicelles were studied for deuterium-labeled DMPC-d_{54} bicelles.

5.5. Theory

5.5.1. Spin-label EPR spectroscopy

The EPR spectrum of magnetically aligned phospholipid bilayers with cholestane consists of three peaks (Figs. 1 and 2) as a result of the coupling of the unpaired electron of the nitroxide spin label to a ^{14}N nucleus. A Cartesian coordinate system containing the x, y, and z nitroxide moiety is used with the x-axis extending along the direction of the N–O bond and the z-axis in the direction of the nitrogen 2pπ orbital [34]. For

the co-aligned cholestane in the bicelles, the *y*-axis represents the long molecular axis (*m*). The cholestane is assumed to rotate rapidly about the long axis [25].

From single-crystal studies, it is known that $A_{xx} \approx A_{yy}$ and the following equation can be used to calculate the S_{33} order parameter [43]:

$$S_{33} = [(A_{\parallel} - A_{\perp})a_N]/[(A_{zz} - A_{xx})a'_N], \quad (1)$$

where $A_{\parallel}$ and $A_{\perp}$ are the observed hyperfine splittings measured between the $m_I = +1$ and 0 spectral lines of oriented samples when the long molecular axis of cholestane is, respectively, perpendicular and parallel to the static magnetic field. A_{xx}, A_{yy}, and A_{zz} are the static hyperfine splitting tensor values obtained from the single-crystal data. The values $A_{xx} = 5.8\,\mathrm{G}$, $A_{yy} = 5.8\,\mathrm{G}$, and $A_{zz} = 30.8\,\mathrm{G}$ are used in the calculations [34]. a_N represents the isotropic hyperfine splitting constant ($a_N = (A_{xx} + A_{yy} + A_{zz})/3$). This hyperfine splitting constant a_N is sensitive to changes in the solvent polarity, the polarity of the spin label environment. The measured isotropic hyperfine splitting constant can be determined according to $a'_N = (A_{\parallel} + 2A_{\perp})/3$.

In general, we are most interested in the molecular order parameter S_{mol} of the long molecular axis.

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

where θ denotes the angle between the long molecular axis (*m*) and the corresponding N–O axis (*x*). In this case, $\theta = 90°$, $S_{\mathrm{mol}} = \mid 2S_{33} \mid$.

5.5.2. Deuterium-labeled solid-state NMR spectroscopy

^{2}H NMR spectra of aligned DMPC/DHPC phospholipids bilayers normally consist of a collection of symmetrical doublets (Fig. 5), characteristic of molecules undergoing fast, axially symmetrical motion. We can relate the residual quadrupolar splittings, $\Delta\nu_i$ to S^i_{CD} order parameters, of the C–^{2}H bond vector according to [29]:

$$\Delta\nu_i = \Delta p_i S^i_{\mathrm{CD}}(3\cos^2\theta - 1)/2, \quad (3)$$

where Δp_i is the splitting for a stationary deuteron in a C–^{2}H bond pointing along the external magnetic field and $\Delta p_i = 3/2e^2qQ/h$. The quadrupole coupling constant (e^2qQ/h) of 168 kHz is used here for deuterons bound to an sp^3-hybridized carbon. θ is the angle between the average bicelle normal (n) and the magnetic field in the laboratory frame. When the bicelles are perfectly aligned relative to the magnetic field, θ is equal to 90° for the perpendicular alignment, whereas θ is equal to 0° for the parallel alignment. S^i_{CD} represents the degree of ordering of the *ith* C–^{2}H bond with respect to the average bilayer normal. If *gauche* conformations about C–C bonds are allowed, a wide variety of orientations of the C–^{2}H moieties are possible. The order parameter S^i_{CD} represents the average of all allowed orientations of the C–^{2}H moieties. S^i_{mol}, the molecular order parameter represents the angular fluctuations of the axis of motion of the molecules with respect to the bilayer normal.

$$S^i_{\mathrm{mol}} = \mid S^i_{\mathrm{CD}}/[(3\cos^2\theta_i - 1)/2] \mid, \quad (4)$$

where θ_i is the angle between the C–^{2}H bond and the principal axis of the interaction tensor (*m*) at the *i*th C–^{2}H bond. For deuterium-labeled phospholipid molecules, we choose the "molecular axis (*m*)" the normal to the plane containing the three atoms in a methylene group. Therefore, for all C–^{2}H bonds of the methylene groups, θ_i is equal to 90°.

$$S^i_{\mathrm{mol}} = \mid 2S^i_{\mathrm{CD}} \mid. \quad (5)$$

The terminal methyl groups make equal angles ($\theta = 109.5°$) with respect to the last C–C bond of the deuterated acyl chain. However, the C–C bond is not parallel to the long molecular axis (*m*). $\theta = 35.25°$ accounts for the segment orientation of the terminal methyl group along the C–C bond with respect to the long molecular axis [19,43]:

$$S_{\mathrm{mol}} = \mid S_{\mathrm{CD}}/[(3\cos^2 109.5° - 1)/2 \times (3\cos^2 35.25° - 1)/2] \mid. \quad (6)$$

For deuterium-labeled cholesterol, we assume that the sterol ring is rigid. The angle θ_i given by Dufourc et al. is [9]:

$$\cos\theta_i = (l_i\cos\gamma\sin\beta + m_i\sin\gamma\sin\beta + n_i\cos\beta)/\sqrt{(l_i^2 + m_i^2 + n_i^2)}. \quad (7)$$

The axis system is defined such that the *x*-axis is co-linear with the C_3-axial bond and the *z*-axis corresponds to the OH–C_3–^{2}H plane. l_i, m_i, and n_i are the direction cosines of the C–^{2}H bond vector in the C_3 reference frame taken from the neutron scattering structure [44]. The original coordinates are transformed into the C_3 reference system by one translation and three successive rotations. $\beta = 11°$, $\gamma = 9°$ are the polar coordinates of the symmetry axis of motion (*m*) in the reference frame given by the neutron diffraction structure [22]. β-[2,2,3,4,4,6-^{2}H$_6$]Cholesterol has six different C–^{2}H bonds, which will give six different values of S_{CD}. Individual quadrupolar splittings were obtained by simulation using the DMFIT program [37]. S_{mol} is calculated using Eq. (4).

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