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Investigating fatty acids inserted into magnetically aligned phospholipid bilayers using EPR and solid-state NMR spectroscopy

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Abstract

This is the first time ^{2}H solid-state NMR spectroscopy and spin-labeled EPR spectroscopy have been utilized to probe the structural orientation and dynamics of a stearic acid incorporated into magnetically aligned phospholipid bilayers or bicelles. The data gleaned from the two different techniques provide a more complete description of the bilayer membrane system. Both methods provided similar qualitative information on the phospholipid bilayer, high order, and low motion for the hydrocarbon segment close to the carboxyl groups of the stearic acid and less order and more rapid motion at the end towards the terminal methyl groups. However, the segmental order parameters differed markedly due to the different orientations that the nitroxide and C–D bond axes transform with the various stearic acid acyl chain conformations, and because of the difference in dynamic sensitivity between NMR and EPR over the timescales examined. 5-, 7-, 12-, and 16-doxylstearic acids spin-labels were used in the EPR experiments and stearic acid-d_{35} was used in the solid-state NMR experiments. The influence of the addition of cholesterol and the variation of temperature on the fatty acid hydrocarbon chain ordering in the DMPC/DHPC phospholipid bilayers was also studied. Cholesterol increased the degree of ordering of the hydrocarbon chains. Conversely, as the temperature of the magnetically aligned phospholipid bilayers increased, the order parameters decreased due to the higher random motion of the acyl chain of the stearic acid. The results indicate that magnetically aligned phospholipid bilayers are an excellent model membrane system and can be used for both NMR and EPR studies.

Keywords: Solid-state NMR; Spin-label EPR; Molecular order parameter; Phospholipid bilayer; Cholesterol; Stearic acid-d_{35}

1. Introduction

Magnetically aligned phospholipid bilayers or bicelles have been successfully used to study the structural and dynamic properties of membrane systems and integral membrane proteins by using solid-state NMR [1–14] and spin-label EPR [15–20] spectroscopic techniques. Bilayer structures play an important role in biological membranes and detailed information about the lipid bilayer organization is crucial for a better understanding of membrane properties [21–30].

NMR spectroscopy investigates the dynamics of the stearic acid hydrocarbon chains incorporated into phospholipid bilayers without the local perturbation effects induced by the nitroxide spin-label [29,31–33]. Conversely, EPR spectroscopy shows a higher degree of sensitivity for motion and the timescale of the nitroxide spin-label matches the rate of molecular rotation within the membrane organization [29,34]. The purpose of this study is to employ two different spectroscopic techniques (EPR and solid-state NMR) that provide a more complete description of the degree of motional ordering of the fatty acid hydrocarbon chains in the membrane bicelle system, and to reveal that the degree of segmental ordering is highly dependent upon the position of the probe (whether deuterium-label or spin-label) along the stearic acid chains [22,26,29].

Cholesterol is an essential component in membrane systems and the structural and dynamic properties of

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membrane systems containing cholesterol is important not only in elucidating phospholipid–cholesterol interactions, but also in understanding the functional role of membranes [26,35–42]. Therefore, the present work investigates the influence of cholesterol and temperature on the ordering profile of a bicellar system by calculating the order parameters derived via both ^{2}H-labeled NMR and spin-labeled EPR spectroscopic techniques. Thus, different amounts of cholesterol are added to our bicelle samples to limit wobbling of the long molecular axis (*m*) of the stearic acid spin probe about the bilayer normal (*n*). Fig. 1 illustrates the orientation of the fatty acid with respect to the phospholipids and the membrane. 5-doxylstearic acid (5-DSA), 7-doxylstearic acid (7-DSA), 12-doxylstearic acid (12-DSA), and 16-doxylstearic acid (16-DSA) are the spin-labels used for the X-band EPR studies and perdeuterated stearic acid (d_{35}) is utilized for the solid-state NMR spectroscopic experiments used in this study. Previous studies have determined that the amphiphilic fatty acids make excellent, non-perturbing EPR and NMR spin probes, and also evenly distributed throughout the polar and non-polar regions of the bilayers [32,43]. This study suggests that the properties of non-biological liquid crystalline materials can be more easily studied by using spin-label EPR spectroscopy and solid-state ^{2}H NMR spectroscopy through the direct measurement of hyperfine splittings and quadrupolar splittings of oriented systems, respectively.

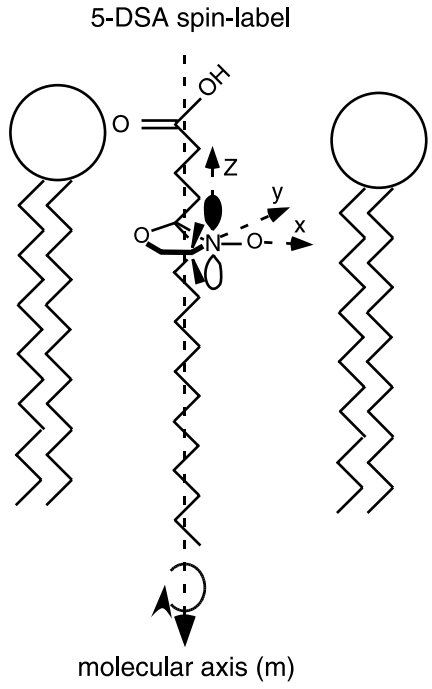

Fig. 1. Drawing that represents 5-doxylstearic acid spin label rotating around its long molecular axis (*m*). The nitroxide group is attached to the stearic acid, the nitrogen $2p\pi$ orbital (*z* axis) is co-linear with the long molecular axis ($\theta = 0°$), the *x* axis is extended in the direction of the NO bond, and the *y* axis is perpendicular to the *xz* plane (plane of the paper).

To accomplish the above objectives, we sought a reliable alignment technique to prepare magnetically aligned phospholipid bilayers (bicelles) [3–6,15–17,20]. The bicelle system consists of a mixture of a long chain phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and a short chain phospholipid 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) [5,15–17,20]. The magnetic alignment depends on several factors including the sign and magnitude of the magnetic susceptibility anisotropy tensor $\Delta\chi$ of the bicelle [44,45]. The negative sign of the susceptibility anisotropy tensor $\Delta\chi$ ($\Delta\chi < 0$) for phospholipid bilayers dictates that the bilayer normal (*n*) is aligned perpendicular to the static magnetic field (Fig. 2). The addition of paramagnetic lanthanide ions (Tm^{+3}, Yb^{+3}) with a large positive magnetic susceptibility anisotropy tensor ($\Delta\chi > 0$) causes the bicelles to flip 90° and align with their bilayer normal (*n*) parallel along the direction of the static magnetic field (B_o) (Fig. 2) [3–6,15,17–19,46].

The fatty acid work presented in this paper utilizing magnetically aligned phospholipid bilayers will be compared with previous EPR and NMR studies conducted on phospholipid bilayers. In one of the studies by Moser and coworkers, they utilized both spin-labeled EPR spectroscopy and deuterium-labeled solid-state NMR spectroscopy to compare the chain configuration and flexibility gradient in phospholipid bilayer membranes [29]. The orientational molecular order parameters and correlation times for the overall motion between the spin-labeled EPR and deuterium-labeled NMR spectra agreed well with each other. However, their study did not apply to the segmental molecular order parameters at the various chain positions, whereas the chain conformation and *trans-gauche* isomerization rate displayed a flexibility gradient with increasing motion towards the terminal methyl end of the chain.

1.1. Spin-label X-band EPR spectroscopy

A Cartesian coordinate system *x*, *y*, and *z* is defined to describe the orientation characteristics of the N–O group of the nitroxide label such that the *x* axis is extended in the direction of the NO bond, the *z* axis is extended in the direction of the nitrogen $2p\pi$ orbital, and the *y* axis is perpendicular to the *xz* plane [17,30]. For a fatty acid nitroxide spin-label, the nitrogen $2p\pi$ orbital is oriented parallel to the long molecular axis (*m*) as shown in Fig. 1 for 5-DSA, when the chain has a *trans* configuration. If the molecular motion increases, the flexibility of the hydrocarbon chain will gradually diminish this orientational effect. The order parameter (*S*) describes the average motional amplitude and the average orientation of the bilayer matrix [23,30,47–49]. Therefore, the variation of (*S*) is related to the orientation and

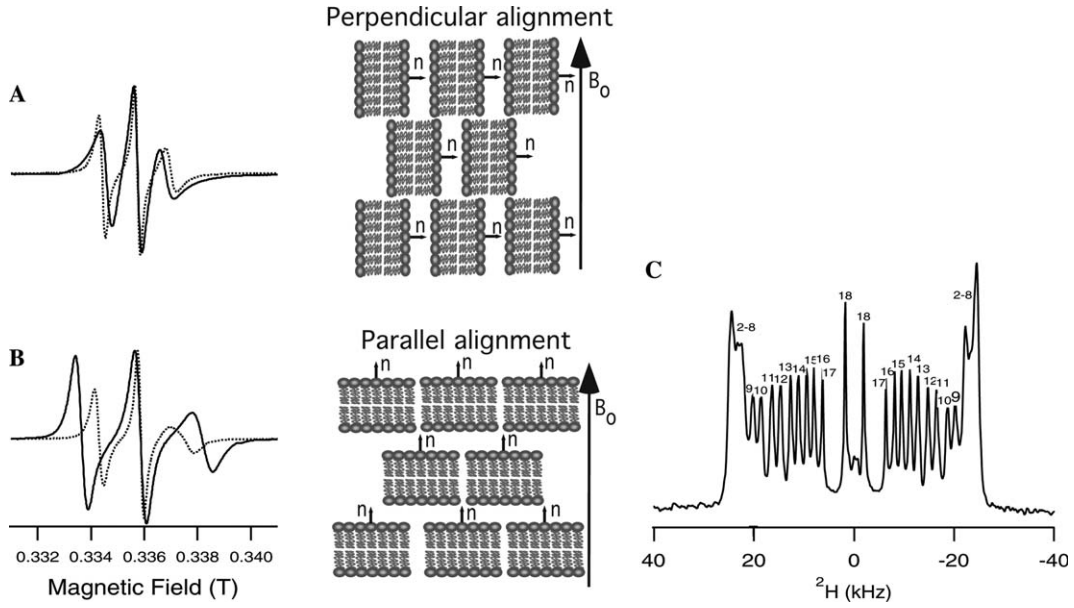

Fig. 2. EPR spectra of bicelles containing DMPC, DHPC, 10 mol% cholesterol with respect to DMPC, and either 5-DSA (___) or 12-DSA (. . ..) at 318 K. (A) The bilayer normal is perpendicular with respect to the static magnetic field (the sample is doped with Dy^{3+}). (B) The bilayer normal is parallel with respect to the static magnetic field (the sample is doped with Tm^{3+}). (C) ^{2}H NMR spectrum of bicelles containing DMPC, DHPC, perdeuterated stearic acid (d_{35}), and 10 mol% cholesterol with respect to DMPC at 318 K. The bilayer normal is parallel with respect to the static magnetic field (the sample is doped with Yb^{3+}). The acyl chain assignments are labeled on the top of the peaks.

motion within the system. In this coordinate system, S_{33} corresponds to the fluctuations of the z axis about the long molecular axis (m) [23,30].

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

where the hyperfine splitting is described by an axial symmetric tensor (A). Therefore, $A_{\perp}$ and $A_{\parallel}$ are the hyperfine splittings measured experimentally between the low field and the center field peaks, when the long molecular axis (m) of the stearic acid spin label is perpendicular and parallel to the static magnetic field, respectively. Typical principle values for a nitroxide hyperfine tensor are $A_{XX} = 6\,\mathrm{G}$, $A_{YY} = 6\,\mathrm{G}$, and $A_{ZZ} = 32\,\mathrm{G}$ [17,30]. a_N is the isotropic hyperfine splitting constant and a'_N represents the measured isotropic hyperfine splitting constant to correct for the polarity difference [50]. In general, we are interested in evaluating the segmental molecular order parameter S_{mol}. S_{mol} represents a measure of the randomness of the motion of the long molecular axis (m) of the labeled fatty acid. S_{mol} can be determined through the following equation [30]:

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

where θ is the angle between the long molecular axis and the corresponding z axis. In the case of the fatty acid spin-label, we consider the hypothetical situation where the z axis remains co-linear with the long molecular axis and the nitrogen $2p\pi$ orbital (z axis) is rotating about the long molecular axis (m) (Fig. 1). This indicates that the segmental molecular order parameter S_{mol} is approximately equal to S_{33} ($S_{mol} \cong S_{33}$). Values for (S_{mol}) range from 0 for unrestricted motion of every individual molecule to 1.0 for a perfectly oriented immobile sample when all the molecules are oriented in one direction [51].

1.2. Deuterium-labeled solid-state NMR spectroscopy

For a static carbon–deuterium sp^3 bond, the quadrupolar coupling constant (e^2qQ/h) has a value of approximately 168 kHz [3,32]. $\Delta^i_p = 3/2(e^2qQ/h)$ is the splitting that would be observed for a deuteron in a CD bond pointing along the direction of the external magnetic field, $\Delta^i_p = 3/2\ (168) = 252\,\mathrm{kHz}$ [3,12,38]. The order parameter (S_{CD}) represents the local orientational or dynamic perturbations of the CD bond vector from its standard state and can be determined by [3,12]:

$$S_{CD} = \Delta^i_B/\Delta^i_p = \Delta^i_B/252, \quad (3)$$

where Δ^i_B is the experimentally measured quadrupolar splitting. Therefore, the corresponding ordering parameters for the individual C–D methylene groups and the terminal methyl groups of the acyl chains were directly evaluated from the quadrupolar splittings of the ^{2}H NMR spectra. The molecular order parameter S_{mol} is defined as the degree of ordering of the long molecular axis at a particular segment with respect to the static magnetic field. Therefore, S_{mol} is represented by the following equations [12,43]:

$$S_{mol} = S_{CD2}/[(3\cos^2 90^\circ - 1)/2] = -2S_{CD2}, \quad (4)$$

$$\begin{aligned} S_{mol} &= S_{CD3}/[((3\cos^2 109.5^\circ - 1)/2) \\ &\quad \times ((3\cos^2 35.25^\circ - 1)/2)] \\ &= -6S_{CD3}. \end{aligned} \quad (5)$$

All (C–D) bonds of the methylene groups would make equal angles ($\theta = 90°$ in Eq. (4)) with respect to the long molecular axis of the deuterated stearic acid acyl chain (m). However, the terminal methyl groups would make equal angles ($\theta = 109.5°$ in Eq. (5)) with respect to the last C–C bond of the deuterated stearic acid acyl chain, and ($\theta = 35.25°$ in Eq. (5)) accounts for the segment orientation of the terminal methyl group along the C–C bond with respect to the long molecular axis (m).

2. Results

The EPR spectra in Figs. 2A and B represent a magnetically aligned DMPC/DHPC bicelle sample that contains 10 mol% cholesterol with respect to DMPC with either 5-DSA (solid line) or 12-DSA (dashed line) at 318 K. In Fig. 2A, Dy^{3+} has been added to the DMPC/DHPC bicelle matrix to induce a large negative $\Delta\chi$ on the phospholipid bilayers [17]. Under these conditions, the lipid bilayer normal (n) is aligned perpendicular to the direction of the static magnetic field. Conversely, in Fig. 2B, a Tm^{3+}-DMPC/DHPC bicelle matrix is aligned such that membrane normal is parallel with respect to the static magnetic field. In this sample, the membrane bicelle system has a positive $\Delta\chi$ value. The spectra in Figs. 2A and B provide an illustration of the degree of ordering for magnetically aligned phospholipid bilayers at two different orientations with respect to the static magnetic field.

The EPR spectra were recorded in the liquid crystalline phase in order to determine the dynamics and degree of motion along the hydrophobic region of the stearic acid incorporated into the phospholipid bilayers. In an aligned spectrum (parallel or perpendicular), the orientation that the spin label makes with respect to the magnetic field and the motion about its molecular axis will determine the observed hyperfine splitting [17].

The theoretical value of the hyperfine splitting for a randomly dispersed isotropic system containing n-DSA is equal to $(A_{XX} + A_{YY} + A_{ZZ})/3 = 14.1$ G [17,30]. The literature hyperfine splitting values for DMPC/DHPC bilayers in the perpendicular Dy^{3+}-bicelle alignment and in the parallel Tm^{3+}-bicelle alignment are equal to 11.8 and 22.1 G, respectively [17,18]. There are apparent differences in the hyperfine splitting values and line shapes of the spectra describing the degree of motional order of (C-5) for the 5-DSA at the portion of the stearic acid hydrocarbon chain nearest to the polar head group, and the (C-12) of the 12-DSA closer to the end of the hydrocarbon chain. The measured hyperfine splittings for the perpendicular and parallel aligned systems containing 5-DSA (solid line in Figs. 2A and B) are 11.6 and 22.0 G, respectively. However, the measured hyperfine splittings for perpendicular and parallel aligned system containing 12-DSA (dashed line in Figs. 2A and B) are 13.5 and 16.0 G, respectively. In the perpendicular aligned spectra (Fig. 2A), the resultant hyperfine splittings increase as the doxyl group is transferred from the C-5 position to the C-12 position of the stearic acid hydrocarbon chain. In the corresponding parallel aligned spectra (Fig. 2B), the hyperfine splittings decrease as the doxyl group is moved toward the end of the hydrocarbon chain. In the aligned spectra in Figs. 2A and B, the hyperfine splittings of the parallel and perpendicular spectra gradually approach the hyperfine splittings of the random spectra as the doxyl group is transferred from the upper part of the stearic acid acyl chain (C-5) towards the end of the stearic acid acyl chain (C-12).

In Fig. 2C, the stearic acid acyl chains are ^{2}H-labeled and Yb^{3+} has been added to a magnetically aligned DMPC/DHPC sample so that the lipid bilayer normal is aligned parallel to the static magnetic field. The sharp lines indicate that the stearic acid is well aligned in the DMPC/DHPC bicelle sample. The corresponding ordering parameters for the individual C–D methylene groups and the terminal methyl groups of the acyl chains were directly evaluated from the quadrupolar splittings of the ^{2}H NMR spectra. The ^{2}H peaks in the NMR spectra were assigned based upon the dynamic properties of the individual CD_2 and CD_3 groups. The quadrupolar splittings for the CD_3 methyl groups at the end of the phospholipid acyl chains are the smallest and closest to 0 kHz. The next smallest splitting was assigned to the deuterons attached to C-17 and so forth along the acyl chain of the stearic acid. The splittings for the deuterons in the plateau region were estimated by integration of the last broad peak according to the literature [52].

2.1. Segmental molecular order parameters of stearic acid incorporated DMPC/DHPC bilayers

Fig. 3 shows the dependence of the molecular order parameter S_{mol} on the position of the label on the acyl chain of both the deuterium-labeled stearic acid (open diamonds) and spin-labeled stearic acid (filled circles) at positions 5, 7, 12, and 16. The molecular order parameters obtained from the deuterium-labeled stearic acid in the aligned DMPC/DHPC phospholipid bicelles decrease gradually in the plateau region until carbon 8 where it shows a steeper decrease after that point towards the end of the hydrocarbon chain. In addition, the molecular order parameters of the spin-labeled stearic acid (5, 7, 12, and 16-DSA) decrease as the spin-label is positioned towards the end of the acyl chain. Moreover, the values of the order parameters derived from the deuterium NMR probes are larger than those determined by the EPR spin probes at any point after carbon 5 due to the bulkiness of the nitroxide moiety [53].

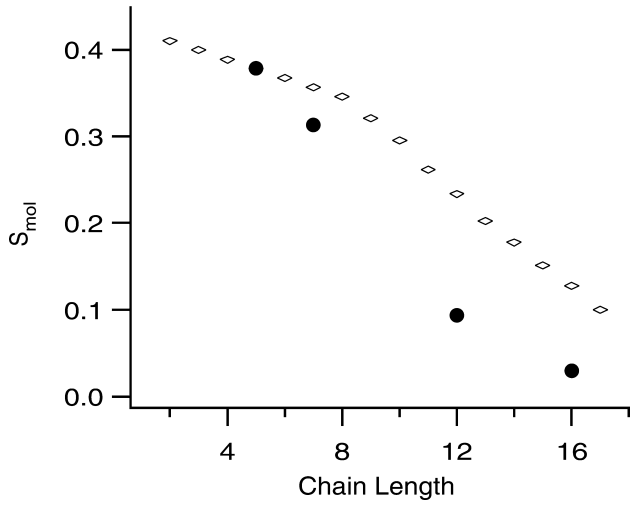

Fig. 3. Molecular order parameters (S_{mol}) with respect to the acyl chain length of the ^{2}H-labeled stearic acid (open diamonds) and the spin-labeled stearic acids (5-, 7-, 12-, and 16-DSA) with (filled circles). The S_{mol} data was collected at a temperature of 318 K and 10 mol% cholesterol with respect to DMPC.

DMPC/DHPC bicelle samples were prepared and various amounts of cholesterol were added in order to study the effect of cholesterol on the dynamics of the stearic acid chains. Fig. 4 shows the molecular order parameters measured in DMPC/DHPC phospholipid bicelles as a function of cholesterol concentration. To establish an appropriate comparison between the results of the spin-labeled EPR and the deuterium-labeled NMR spectroscopic techniques, molecular order parameters calculated from the spectra representing the matrix with either 5-DSA (filled circles) or 12-DSA (filled triangles) are compared with the deuterium-labeled stearic acid at the C^{5th}–D (open circles) and C^{12th}–D (open triangles) positions, respectively. Thus, both the doxyl group and the deuterium labels will be at the same stearic acid position in the bicelle lipid matrix.

As observed in Fig. 4, cholesterol enhances the ordering of the 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. In addition, the molecular order parameters of the 5-DSA or C^{5th}–D are higher than those of the 12-DSA or the C^{12th}–D. The EPR spin-label and the ^{2}H NMR label indicate that the degree of ordering decreases as the label is moved toward the hydrocarbon end from the C-5 to the C-12 position. There is a distinct difference between the two techniques, the C^{12th}–D point reveals larger values for S_{mol} than the 12-DSA label at various cholesterol concentrations (Fig. 4). This increase is due to the local perturbing influence of the nitroxide group in the spin-labeled EPR experiment because the hydrophobic acyl chain end is able to move more freely. Nevertheless, the 5-DSA molecular order parameter is approximately equal to the C^{5th}–D molecular order

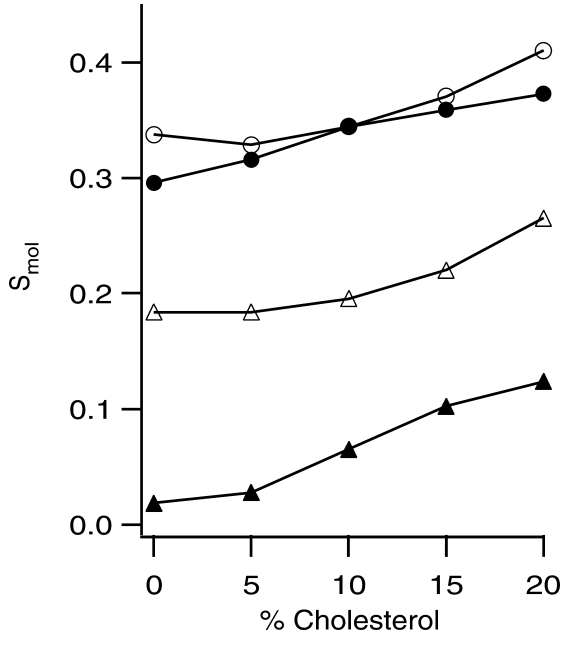

Fig. 4. Molecular order parameters (S_{mol}) plotted against the mol% of cholesterol (with respect to DMPC) incorporated into the phospholipid bilayer. The results compare the spin-labeled stearic acid (EPR, filled symbols) with the ^{2}H-labeled stearic acid (NMR, open symbols) at 323 K. The following symbols are represented by —○—, C^{5th}-D, NMR; —●—, 5-DSA, EPR; —△—, C^{12th}-D, NMR; and —▲—, 12-DSA, EPR.

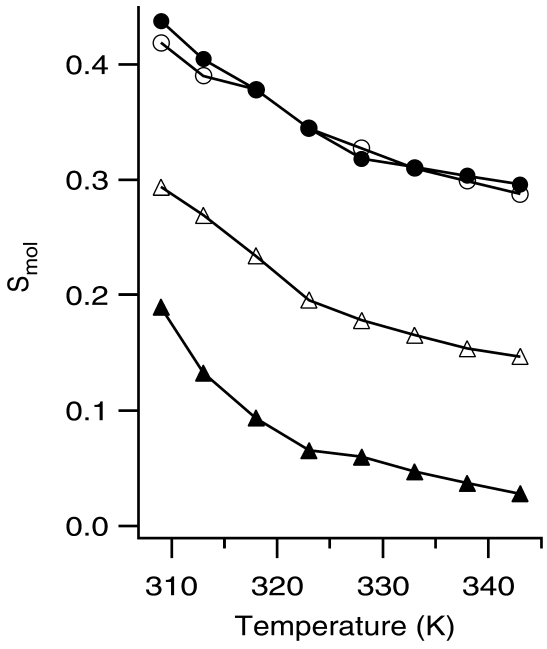

Fig. 5. Temperature dependence of the degree of molecular order S_{mol} at 10 mol% cholesterol with respect to DMPC for both spin-labeled EPR (filled markers) and the deuterium-labeled NMR (open markers) in the phospholipid bilayer. The following symbols are represented by —○—, C^{5th}-D, NMR; —●—, 5-DSA, EPR; —△—, C^{12th}-D, NMR; and —▲—, 12-DSA, EPR.

parameter at carbon number five near the polar region of the phospholipid. Therefore, the nitroxide moiety does not have a remarkable effect on the flexibility profile at position 5, because the hydrophilic end of the chains is anchored near the lipid–water interface and is restricted in motion. In addition, the introduction of cholesterol into the bicelle sample has a pronounced influence on increasing the ordering profile of the system, specifically near the top of the hydrocarbon chain due to the packing effect of the rigid planar steroid backbone [39]. Therefore, both the sterol ring and the polar region together create a rigid-like environment that limits the perturbing influence caused by the nitroxide group on position 5 of the acyl chain.

In Fig. 5, the corresponding molecular order parameters were calculated for magnetically aligned DMPC/DHPC samples with 5-DSA (filled circles), 12-DSA (filled triangles), C^{5th}–D (open circles) or C^{12th}–D (open triangles) and compared as a function of temperature. 10 mol% cholesterol was added to these samples. The data indicates that increasing the temperature from 308 to 348 K decreases the S_{mol} parameter of the stearic acids incorporated into the magnetically aligned phospholipid bilayers. This result is due to the effect of temperature on increasing the degree of motion and flexibility along the hydrocarbon chains of the fatty acids.

3. Discussion

The hyperfine splittings and the quadrupolar splittings observed in Fig. 2 determine the degree of ordering along the fatty acid hydrocarbon chain in magnetically aligned phospholipid bilayers. The discrepancy between the hyperfine splitting values observed for the 5-DSA and 12-DSA labels incorporated into the parallel and perpendicular aligned phospholipid bilayer systems in Fig. 2 indicates that near the polar surface of the phospholipid bilayer, the motional freedom is rather restricted, while the motion increases randomly towards the end of the bilayer due to rotational isomerization between *trans* and *gauche* conformations about each C–C bond in the hydrocarbon chain [3,22,29,31,32,47,52].

Both the spin-label and the deuterium-label methods are complementary to each other and provide an illustration of the motional degree of freedom of the phospholipid bilayers. The lipid bilayer has been described as ordered near the hydrophilic headgroups at the plateau region (carbons 2 up to 7) of the stearic acid and disordered in the interior part starting at carbon 8 towards the end of the hydrocarbon chain at the terminal methyl group, as has been illustrated in Fig. 3. In a study of the bilayer systems in the liquid crystalline phase that consists of sodium decanoate, decanol, and water utilizing ^{2}H NMR and spin-label EPR [54], it has been found that the ^{2}H NMR results show an almost constant slope order parameter over most of the chain until carbon 7 and a decrease over the last three carbon atoms 8, 9, and 10. In contrast, their spin-label EPR experiments revealed a continuous decrease in the resultant order parameter (S_{mol}). Both the deuterium and nitroxide spin probe (S_{mol}) order parameters overlap at position 5. In a similar study, the order parameters obtained from deuterated and spin-labeled stearic acids in oriented multibilayers of egg lecithin [32] indicated that the order parameters in the ^{2}H NMR experiments has a constant slope up to carbon atom 8, and then decrease to a small value at carbon 18. In contrast, the order parameters in the spin-label EPR experiments decline continuously toward the methyl end of the stearic acid. The S_{mol} data presented in this paper using magnetically aligned bilayers is consistent with previous studies using alternative phospholipid bilayer systems.

^{2}H solid-state NMR spectroscopy shows higher ordering magnitudes than the EPR spectroscopic results after carbon 5 of the acyl chains as shown in Fig. 3. This discrepancy is a result of the difference in sensitivity of the two techniques over the various molecular motion time scales. EPR spectra are sensitive to motions with correlation times between 10^{-10} and 10^{-8} s [55], whereas ^{2}H NMR spectra are particularly sensitive to motions with correlation times between 10^{-3} and 10^{-9} s [56]. The orientational ordering that characterizes the rotation and wobbling motions of the stearic acid molecule will be in the fast-motion regime on the NMR time scale [29,57]. In addition, the deuterium-labels reveal the structure of the unperturbed bilayer, while the spin-labels detect the response of that segment of the bilayer attached to the doxyl group under a small physical stress due to the presence of the nitroxide moiety. The nitroxide moiety increases the probability of the intramolecular motion in the magnetically aligned bilayers. Intramolecular motions on the chain configuration play a pivotal role in the segmental ordering of the phospholipid bilayers and consist of *trans–gauche* isomerizations.

Meanwhile, both of these spectroscopic techniques have the same degree of ordering at carbon 5 as shown in Fig. 3, closer to the polar headgroups, due to the decrease in the average number of *gauche* isomerizations at that region as a result of strong interactions within the polar head groups. This study on the fatty acid spin-label system by EPR spectroscopy is valuable because the effect of the nitroxide group on the bicelle system is similar to the effect of the incorporation of the protein into the membrane, which may locally perturb the neighboring lipid chain in a similar fashion. Therefore, the nitroxide spin label mimics a situation similar to a biological significant integral membrane system. Furthermore, when the amount of cholesterol in the bilayers decrease or the temperature increases, the system will be more fluid and experience faster motion. Under these

conditions, the correlation time fits better with the EPR time scale. Therefore, spin-label EPR spectroscopy has a higher degree of sensitivity than ^{2}H NMR spectroscopy for probing faster motions. This demonstrates why the molecular order parameter values obtained from EPR and NMR data are more consistent under conditions of higher ordering and diverge as the bilayers become more flexible and the degree of ordering decreases [58].

Cholesterol is known to be an important constituent of many natural membranes and has been suggested to control the hydrocarbon chain fluidity of the lipid components of membranes and stabilizing them at various temperatures [39]. In Fig. 4, both the ^{2}H label and the spin label at carbon position 5 of the stearic acid most likely have a similar degree of ordering at various cholesterol concentrations. The addition of cholesterol reduces the probability of intrinsic flexibility in the acyl chains next to the rigid sterol ring system, which decreases the population of kinks in the upper part of the hydrocarbon chains. The acyl chains adjacent to cholesterol could possibly not tilt as far as other acyl chains with respect to the bilayer normal. At higher cholesterol concentrations, the acyl chains are more ordered due to a decrease in fluidity of the membrane [26,32,33,35,38,41,42,52,59–61]. The segmental order parameters decrease continuously as the temperature increases; this effect is clearly the result of increased motional freedom along the hydrocarbon chains [3,21,22,24,29,32,37,38,41]. Therefore, our results on magnetically aligned phospholipid bilayers agree with similar studies performed on other membrane systems. Thus, our data indicates that magnetically aligned phospholipid bilayers represent an excellent model system for studying the structural and dynamic properties of membrane systems utilizing both solid-state NMR spectroscopy and spin-label EPR spectroscopy. Furthermore, previous studies have demonstrated that bicelles proved to be a successful model membrane systems to study integral membrane proteins [62].

4. Conclusion

In this work, we have presented spin-labeled EPR and ^{2}H-labeled solid-state NMR studies that document the segmental molecular order parameter of a fatty acid incorporated into DMPC/DHPC bicelles. The utilization of aligned phospholipid bilayers in both solid-state NMR and EPR spectroscopy provides pertinent structural and dynamic information about membrane-associated molecules at two different time scales. We have shown that hydrocarbon chains of the stearic acid and the phospholipids are restricted in motion in the segments close to the polar headgroup, while further down the acyl chain they are able to move more freely towards the terminal methyl group. Also, increasing the temperatures enhance the extent of this motion. Furthermore, the addition of cholesterol increases the degree of ordering of the fatty acid chains and decreases the amplitude of motion along their long axes.

5. Experimental

5.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), perdeuterated stearic acid (d_{35}) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoetholamine-*N*-[poly (ethylene glycol) 2000] (PEG2000) were purchased from Avanti Polar Lipids. Thulium (III) chloride hexahydrate, Ytterbium (III) chloride hexahydrate, Dysprosium (III) chloride hexahydrate, and HEPES (*N*-[2-hydroxy-ethyl] piperazine-*N*-2-ethanesulfonic acid) were obtained from Sigma/Aldrich. 5-, 7-, 12-, and 16-Doxylstearic acids were purchased from ICN. The cholesterol was obtained from Avocado Research Chemicals and has dissolved in chloroform before each experiment. All lipids were dissolved in chloroform and stored at −20 °C prior to use. An aqueous solution of $TmCl_3$, $YbCl_3$, and $DyCl_3$ was prepared fresh each day. All aqueous solutions were prepared with deuterium-depleted water purchased from Isotec (Miamisburg, OH).

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 the EPR experiments, one flask containing DMPC, DHPC, PEG2000-PE, cholesterol, and the spin label (5-, 7-, 12-, or 16-DSA) were mixed together at molar ratios of 3.5/1/0.035/0.35/0.0196, respectively. For the NMR experiments, DMPC, DHPC, and perdeuterated stearic acid (d_{35}) were mixed in ratios of 3.54/1/0.47, respectively. After that, the same sample preparation procedure was performed for both EPR and NMR methods. The chloroform in the flask was removed by rotoevaporation and then the flask was placed under high vacuum overnight.

The following day, 100 mM HEPES buffer at pH 6.8 was added to the flask so the amount of lipids in the sample was 25% (wt%). The flask was then vortexed and put into an ice bath periodically until the sample became homogeneous and clear. The sample was sonicated for about 30 min with a FS30 (Fisher Scientific) ice bath sonicator with the heater turned off. The flask was left in an ice bath for approximately 20 min. Next, the sample was subjected to three to four freeze (77 K by liquid nitrogen)/thaw cycles (room temperature) to homogenize the sample and to remove any air bubbles. At 0 °C

(ice bucket), 20 mol% (molar ratio to DMPC) of either $TmCl_3$ or $DyCl_3$ was added and mixed into the EPR samples. The total mass of the prepared samples was 200 mg. For NMR samples, in 0°C (ice bucket), 5 mol% (molar ratio to DMPC) of $YbCl_3$ was added and mixed into the sample. Typically, the total mass of the prepared samples was 300 mg.

5.3. EPR spectroscopy

The bicelle samples were placed into 1 mm ID capillary tubes (Kimax) via a syringe. Both ends of the capillary tubes were sealed with Critoseal (Fisher Scientific) and placed inside standard quartz EPR tubes (Wilmad, 707-SQ-250M) filled with light mineral oil. All EPR experiments were carried out on a Bruker EMX X-band CW-EPR spectrometer consisting of an ER041XG microwave bridge and a TE_{102} cavity coupled with a BVT 3000 nitrogen gas temperature controller (temperature stability of ± 0.2 °C). All EPR spectra were gathered with a center field of 0.3350 T, sweep width of 140 G, a microwave frequency of 9.39 GHz, modulation frequency of 100 kHz, modulation amplitude of 1.0 G, and a power of 6.3 mW. All magnetically oriented phospholipid bilayer samples were aligned at a maximum magnetic field strength of 6300 G.

5.4. NMR spectroscopy

The NMR samples were loaded via a Pasteur pipet into 5 mm O.D. NMR tubes and placed into a round coil solid-state NMR probe. All solid-state NMR experiments were carried out on a modified 300 MHz Bruker Avance narrow bore (7.05 T) magnet configured to conduct high-power solid-state NMR studies. The resonance frequencies were 300.01 MHz for ^1H and 46.07 MHz for ^2H. The solid-state NMR spectra were gathered with a static double-tuned 5 mm round-coil solid-state NMR probe purchased from Doty Scientific. ^2H NMR spectra were recorded at 46.07 MHz using a standard quadrupole-echo pulse sequence (3.0 μs 90° pulses, 45 μs inter pulse delay, 5.12 ms acquisition time, 0.4 s recycle delay) [63]. 2048 transients were acquired and the free induction decay processed with 200 Hz of line broadening.

All of the EPR and NMR spectra and resulting graphs were processed on a G4 Mac computer utilizing the Igor Carbon software package (Wavemetrics, Lake Oswego, OR).

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