Development of magnetically aligned phospholipid bilayers in mixtures of palmitoylstearoylphosphatidylcholine and dihexanoylphosphatidylcholine by solid-state NMR spectroscopy

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Abstract

This study reports the solid-state NMR spectroscopic characterization of a long chain phospholipid bilayer system which spontaneously aligns in a static magnetic field. Magnetically aligned phospholipid bilayers or bicelles are model systems which mimic biological membranes for magnetic resonance studies. The oriented membrane system is composed of a mixture of the bilayer forming phospholipid palmitoylstearoylphosphatidylcholine (PSPC) and the short chain phospholipid dihexanoylphosphatidylcholine (DHPC) that breaks up the extended bilayers into bilayered micelles or bicelles that are highly hydrated (approx. 75% aqueous). Traditionally, the shorter 14 carbon chain phospholipid dimyristoylphosphatidylcholine (DMPC) has been utilized as the bilayer forming phospholipid in bicelle studies. Alignment (perpendicular) was observed with a PSPC/DHPC q ratio between 1.6 and 2.0 slightly above T_m at 50°C with ^{2}H and ^{31}P NMR spectroscopy. Paramagnetic lanthanide ions (Yb^{3+}) were added to flip the bilayer discs such that the bilayer normal was parallel with the static magnetic field. The approx. 1.8 (PSPC/DHPC) molar ratio yields a thicker membrane due to the differences in the chain lengths of the DMPC and PSPC phospholipids. The phosphate-to-phosphate thickness of magnetically aligned PSPC/DHPC phospholipid bilayers in the L_α phase may enhance the activity and/or incorporation of different types of integral membrane proteins for solid-state NMR spectroscopic studies. © 2001 Published by Elsevier Science B.V.

Keywords: Solid-state nuclear magnetic resonance spectroscopy; Magnetically oriented membrane; Lipid bilayer; Membrane protein

1. Introduction

Magnetically aligned phospholipid bilayers (bi-

celles) have been demonstrated to be useful models for studying the structural and dynamic properties of membrane systems and integral membrane proteins using solid-state nuclear magnetic resonance (NMR) spectroscopic techniques [1–8]. The magnetic alignment of bicelles is due to the anisotropy of the overall magnetic susceptibility of the system. The negative sign of the diamagnetic susceptibility anisotropy tensor ($\Delta\chi < 0$) for phospholipid bilayers dictates that the bicelles align with their bilayer normal oriented perpendicular to the direction of the static magnetic field. The addition of paramagnetic lantha-

nide ions with a large positive $\Delta\chi$ (Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}) can cause the bicelles to flip 90° such that the average bilayer normal is colinear with the direction of the static magnetic field. One advantage of flipping the phospholipid bicelles is that the spectral resolution is dramatically increased, because the spectral width is spread over a broader frequency range. Additionally, in a uniaxially aligned system, the highly anisotropic spectral data can yield the orientation and structure of different segments of the protein with respect to the magnetic field and the lipid bilayer [9–11].

The development of dilute magnetically aligned aqueous liquid-crystalline media has dramatically increased the refinement of structural studies of globular proteins with high-resolution NMR spectroscopy [12–15]. Generally, in an isotropic solution, internuclear dipolar couplings average to zero as a result of rotational molecular motion. By dissolving proteins in a dilute magnetically oriented aqueous liquid-crystalline medium (bicelle), a tunable degree of solute alignment with respect to the magnetic field can be created while retaining both the resolution and sensitivity of the regular isotropic NMR spectrum. In this system, the dipolar couplings no longer average to zero and can be accurately measured. This approach has been shown to significantly improve the accuracy of structures determined by solution NMR spectroscopy, and extend the size limit.

The typical bicelle consists of long chain phospholipids such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and a detergent such as 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC). The diameter of the bicelle increases as the molar ratio between the long chain (n_1) and short chain phospholipid (n_2), $q = n_1/n_2$, increases [1,6,16,17]. Phospholipids that are present in a bilayer undergo various temperature dependent phase transitions [18–20]. Above the phase transition temperature (T_m), the phospholipids are in a relatively fluid liquid-crystalline state. This transition can be of great significance for enzyme activity [21]. The lamellar liquid-crystalline phase is also termed the L_α phase [22–24]. The transition from the gel phase to the L_α phase above T_m is a requirement for magnetically aligned phospholipid bilayers and can be explained in terms of the strong forces in the polar head sheets and the weak forces between the acyl chains. When a phos-

pholipid bilayer system is heated above its T_m, the van der Waals forces between the hydrocarbons become weak compared to the thermal motions [6,22]. Thus, the chains are transformed into a state of disorder with a high degree of *gauche* conformation [6,22]. Since a bilayer arrangement of phospholipids is characterized by a distinct central hydrophobic region bounded by two polar interfacial regions, we expect that the thickness of the hydrophobic region will have influence on the structure and function of transmembrane proteins [25–27]. Despite the success of using DMPC/DHPC bicelle systems to study the structural and dynamic properties of membrane proteins with solid-state NMR spectroscopy there are size limitations on the length and size of proteins to be studied [28]. Natural biological lipids are dominated by chains with 16–18 carbons in length. Also, α-helical transmembrane sections of proteins generally contain 25 residues which may be too long to assemble into the standard DMPC/DHPC bicelle matrix. The objective of the current study is to develop a method to magnetically align longer chain phospholipids in a static magnetic field. Due to the longer acyl chains the lipid bicelles of such a system will extend the thickness of the lipid bilayer and has the potential to provide a better model for a biological membrane and possibly enhance protein activity for solid-state NMR investigations.

In the present study, we have investigated the magnetic alignment of a mixture of 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine (PSPC) and DHPC at various phospholipid concentration (q) ratios and temperatures. The results obtained from this new magnetically aligned phospholipid bilayer system are presented.

2. Materials and methods

2.1. Materials

DMPC, PSPC, DHPC, DMPCd_{54} and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC)d_{62} were purchased from Avanti Polar Lipids (Alabaster, AL). All phospholipids were dissolved in chloroform and stored at −20°C prior to use. Ytterbium(III) chloride hexahydrate and HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-2-ethanesulfonic acid) were ob-

tained from Sigma/Aldrich. Deuterium-depleted water was obtained from Isotec (Miamisburg, OH).

2.2. Sample preparation

The standard DMPC/DHPC bicelle sample, consisting of 25% (w/w) phospholipid to solution with a $q = 3.54$, was made in two separate 15 ml or 25 ml pear-shaped flasks. In one flask DMPC and DMPCd_{54} were mixed together in chloroform at millimolar ratios of 0.1/0.005 respectively while in the second flask DHPC was added. The mixtures in both flasks were rotovaped down to remove the chloroform solvent from the phospholipid mixture and both flasks were placed under high vacuum overnight.

The following day a 100 mM HEPES buffer of pH 7.0 was prepared using deuterium-depleted water and the appropriate amount was added to the flask containing the DHPC. The flask was then vortexed briefly, sonicated for 5 min and vortexed again. The sample was sonicated with a FS30 (Fisher Scientific) bath sonicator with the heater turned off. Several freeze (77 K)/thaw cycles (room temperature) made the dispersion more homogenous and also removed all of the air bubbles. The sample was then transferred to the flask containing the DMPC and DMPCd_{54}, and vortexed and sonicated again. Similar procedures were also used to prepare the PSPC/DHPC/DPPCd_{62} bicelle samples except that all the phospholipids were mixed together in one 25 ml pear-shaped flask. Typically, the total mass of the prepared bicelle sample was 300 mg. An aqueous solution of ytterbium(III) chloride hexahydrate was prepared fresh with deuterium-depleted water. The bicelle sample was placed in a 21 mm NMR flat-bottom tube with a 5 mm o.d. on ice via a Pasteur pipette. To monitor bicelle alignment with ^{2}H NMR spectroscopy a 20:1 mole ratio of regular and chain-perdeuterated phospholipids was used in this study.

2.3. NMR spectroscopy

All solid-state NMR experiments were carried out on a modified Bruker AVANCE 7.05 T narrow bore 300/54 magnet configured to conduct high-power solid-state NMR studies. The resonance frequencies were 300.01 MHz for ^{1}H, 46.07 MHz for ^{2}H, and 121.5 MHz for ^{31}P. The solid-state NMR spectra were gathered with a static double-tuned 5 mm round-coil solid-state NMR probe purchased from Doty Scientific. ^{2}H NMR spectra were recorded at 46.07 MHz using a standard quadrupole-echo pulse sequence (3.0 μs 90° pulses, 45 μs innerpulse delay, 5.12 ms acquisition time, 0.4 s recycle delay, and a 150 kHz sweep width). For the ^{2}H NMR spectra, 2048 transients were acquired and the free induction decay was processed with 200 Hz of line broadening. ^{31}P NMR were recorded at 121.51 MHz with proton decoupling under static conditions using a 11.7 μs excitation pulse and a 4 s recycle delay. For the ^{31}P NMR spectra, 512 transients were acquired and the free induction decay was processed with 25 Hz of line broadening. The sweep width was set equal to 250 ppm. ^{31}P chemical shifts were referenced to 85% H_3PO_4. All samples were allowed to equilibrate in the magnet for 30 min prior to signal acquisition.

The NMR data was processed on a 300 MHz Power Macintosh G3 computer running Igor Pro 3.12 (Wavemetrics, OR) and MacNuts (Acorn NMR, CA).

3. Results

3.1. Magnetic alignment of phospholipid bilayers with ^{2}H NMR spectroscopy

^{2}H solid-state NMR spectra of a 25% w/w phospholipid bicelle of DMPC/DHPC to solution with a $q = 3.54$ containing chain-perdeuterated DMPCd_{54} were acquired over a temperature range from 25°C to 60°C. The results of the temperature study are displayed in Fig. 1. At 25°C, the DMPC/DHPC bicelle discs are not magnetically aligned. However, partial orientation is observed at 30°C. At 35°C and 40°C, the spectra are characteristic of well-aligned liquid-crystalline bicelle discs. In this case, the bilayer normal is oriented perpendicular to the direction of the static magnetic field. At 45°C, the DMPC/DHPC bicelle sample loses some of its orientational characteristics. Finally, between 50°C and 60°C powder characteristics are observed along with a strong isotropic component centered at 0 kHz. The standard DMPC/DHPC ^{2}H bicelle NMR spectra
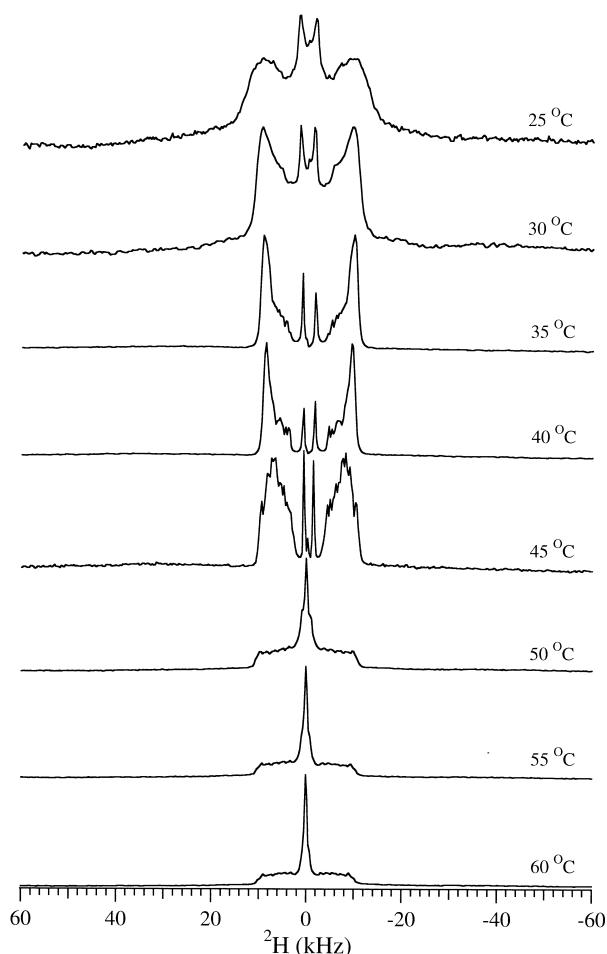
Fig. 1. ^{2}H NMR spectra of a 25% (w/w) $q = 3.54$ DMPC/ DHPC bicelle sample investigated as a function of temperature. The sample was doped with chain-perdeuterated DMPC to monitor bicelle alignment in a deuterium-depleted aqueous buffer.

serve as an excellent basis for comparing the magnetic alignment of different acyl chain length phospholipid bilayer discs.

Similar ^{2}H NMR studies are shown in Fig. 2 for a 25% w/w PSPC/DHPC phospholipid bicelle sample with $q = 1.8$ over a temperature range from 40°C to 60°C. At 40°C and 45°C, the spectra reveal only an isotropic component. The same isotropic component was observed at 25°C, 30°C, and 35°C (data not shown). At 50°C, the ^{2}H NMR spectrum indicates that the PSPC/DHPC phospholipid bicelle discs are magnetically aligned such that the normal of the lipid bilayer is perpendicular to the static magnetic field. Above 50°C, the spectra reveal a powder-like spec-

trum with a large isotropic component centered at 0 kHz.

In order to investigate the orientational properties of PSPC/DHPC bicelle samples at various q ratios and at various temperatures, ^{2}H NMR spectra of samples with PSPC/DHPC q ratios between $q = 1.4$ and 10 were obtained and the corresponding spectra are displayed in Fig. 3 at 50°C. At $q = 1.4$, a large isotropic peak centered at 0 kHz is observed. At $q = 1.5$, a broader unoriented and isotropic component is observed. Between $q = 1.6$ and 2.0, the powder and isotropic components disappear and orientational L_{α}-like phase characteristics are observed. In this case, the bilayer normal is aligned perpendicular to the static magnetic field. Although the individual deuterons along the acyl chain cannot be fully resolved, the spectra resemble the shape and breadth of previously published ^{2}H NMR spectra of DMPC/ DHPC bicelle samples [3,16]. As the q ratios increase from 2.25 to 10.0, orientational characteristics disappear and the spectra broaden out to reveal powder characteristics along with some isotropic components. The lower q ratios for the aligned PSPC/ DHPC bicelle samples indicate that additional detergent is needed to stabilize and cap off the edges of
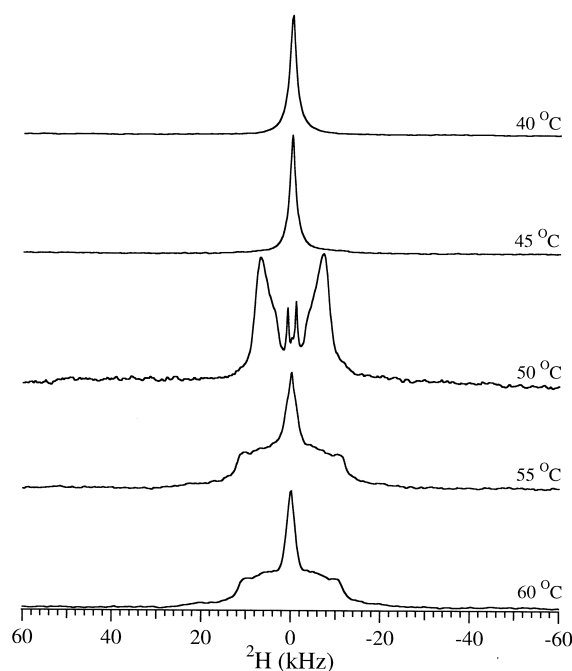

Fig. 2. ^{2}H NMR spectra of a 25% (w/w) $q = 1.8$ PSPC/DHPC bicelle sample investigated as a function of temperature.
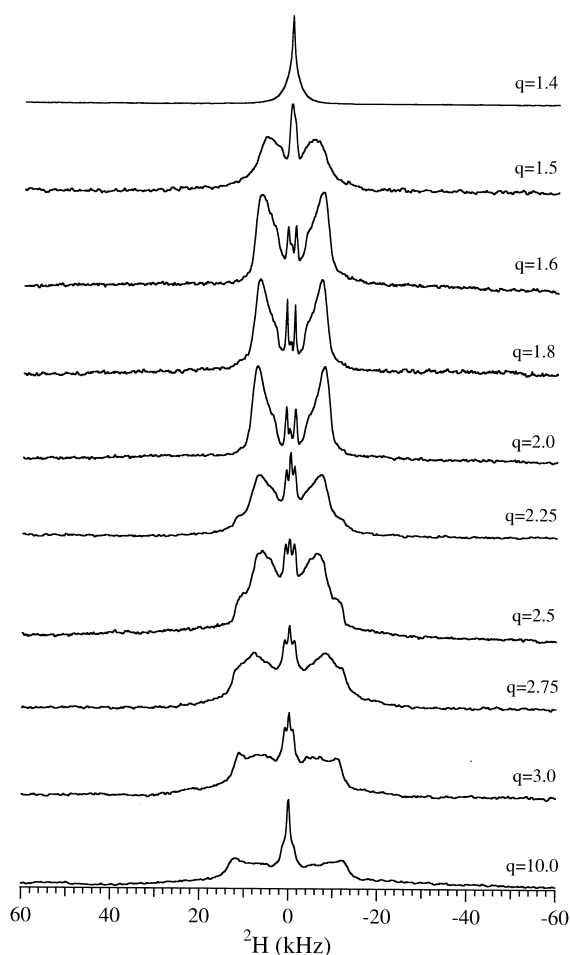
Fig. 3. ^{2}H NMR spectra of 25% (w/w) PSPC/DHPC bicelle samples investigated at 50°C as a function of the PSPC/DHPC molar q ratio ($q = 1.4$–10).

the bicelle discs due to the longer chain PSPC phospholipids. Temperature dependent studies were carried out on the PSPC/DHPC $q = 1.4$–10 bicelle samples (data not shown). Alignment characteristics were only noticed between $q = 1.6$ and $q = 2.0$ at 50°C.

As discussed previously, the addition of lanthanide ions such as Yb^{3+} or Tm^{3+} with a large $\Delta\chi$ to DMPC/DHPC bicelle samples causes the phospholipid discs to flip 90° such that the bilayer normal is aligned parallel with the direction of the static magnetic field. Fig. 4A shows a ^{2}H NMR spectrum of a $q = 2.0$ PSPC/DHPC bicelle sample at 50°C prepared in the absence of lanthanide ions. In order to change the sign of the net magnetic susceptibility anisotropy tensor of the bicelles so that their normal is parallel to the applied magnetic field, 5% molar

Yb^{3+} with respect to PSPC was added to the PSPC/DHPC bicelle sample and the results are shown in Fig. 4B. The quadrupolar splittings observed in the ^{2}H NMR spectrum are spread out and much better resolved. The spectrum indicates that the PSPC/DHPC bicelle discs have flipped such that their bilayer normal is now parallel with the magnetic field. The ^{2}H line shape and breadth of the magnetically aligned lanthanide-doped PSPC/DHPC bicelle sample observed in Fig. 4B are similar to previous DMPC/DHPC/lanthanide spectra in the literature [3,29]. The breadth is slightly larger for the PSPC bicelle spectra in both the parallel and perpendicular orientations when compared to the ^{2}H spectra of standard DMPC/DHPC bicelle samples. The PSPC/DHPC bicelle spectra have a wider quadrupolar splitting than the corresponding DMPC/DHPC bicelle spectra because the L_α phase PSPC phospholipids are closer to T_m when the aligned phase occurs.
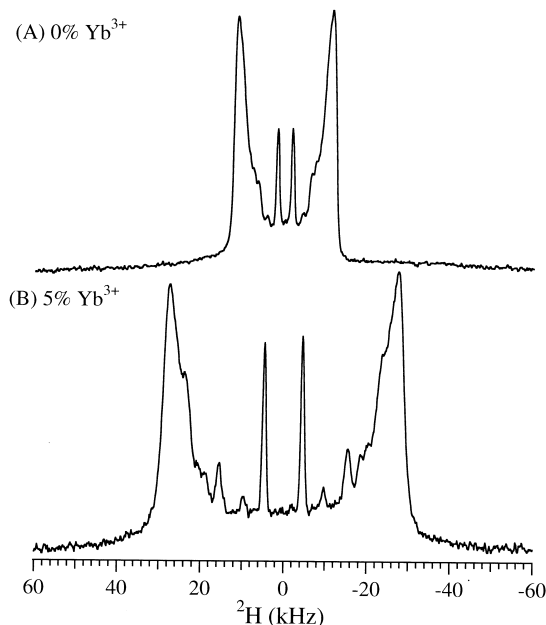

Fig. 4. ^{2}H NMR spectra of a 25% (w/w) $q = 2.0$ PSPC/DHPC bicelle sample investigated with and without the paramagnetic lanthanide ion Yb^{3+}. (A) Bicelle spectrum contains 0% Yb^{3+} and is consistent with the bilayer normal being perpendicular to the magnetic field. (B) Bicelle spectrum contains 5% molar Yb^{3+} (when compared to PSPC) and is consistent with the bilayer normal being parallel with the direction of the static magnetic field.
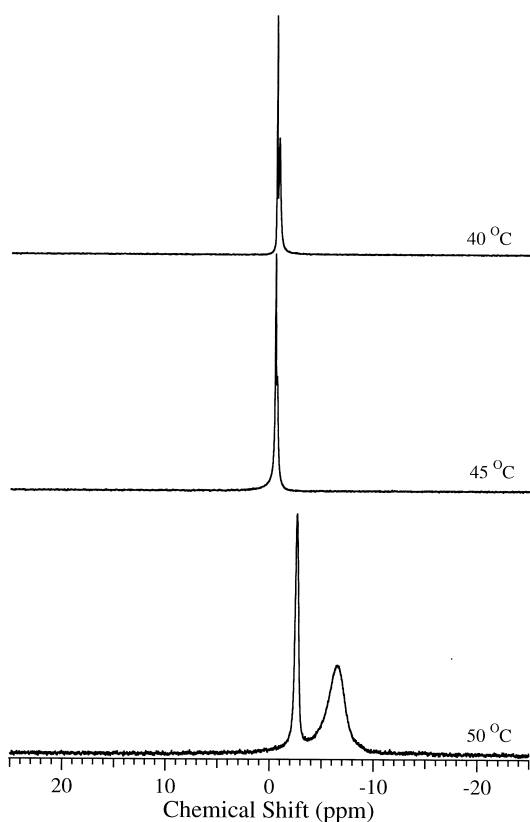
Fig. 5. ^{31}P NMR spectra of a 25% (w/w) $q = 1.8$ PSPC/DHPC bicelle sample investigated as a function of temperature.

3.2. Magnetic alignment of phospholipid bilayers with ^{31}P NMR spectroscopy

^{31}P NMR spectroscopy is an excellent technique for studying magnetically aligned phospholipid bilayers in a magnetic field due to the presence of one phosphorus atom in the phospholipid head group [16,30,31]. ^{31}P NMR resonance line shapes and shifts contain information concerning head group conformation, membrane morphology, and the orientation of the lipid bilayer with respect to the magnetic field [32]. The ^{1}H-decoupled ^{31}P NMR spectra of PSPC/DHPC bicelles with $q = 1.8$ are shown in Fig. 5 from 40°C through 50°C. Below T_m, the two spectra at 40°C and 45°C reveal two closely spaced isotropic peaks at −0.55 ppm and −0.75 ppm. These two spectra agree with the isotropic ^{2}H NMR spectra at 40°C and 45°C shown in Fig. 2. Above T_m at 50°C, two new peaks are clearly observed in the ^{31}P spectrum. These two peaks are shifted upfield from the isotropic peaks.

Based upon previous DMPC/DHPC bicelle ^{31}P NMR studies, the broad peak centered at −7.0 ppm corresponds to the phosphorus atoms of the long chain PSPC phospholipids [16]. The sharp peak observed at −3.0 ppm is due to the short chain DHPC phospholipids located at the edges of the bicelle. Signal intensity integration of the two peaks indicates that the broad peak is twice as large as the narrow resonance transition. This agrees with the peak assignment due to the molar q ratio of 1.8/1 for the PSPC/DHPC bicelle sample.

4. Discussion

Phospholipids with longer hydrocarbon chains were used in place of DMPC for several different reasons in this study. First of all, the application of DMPC/DHPC bicelles may not be optimal for solid-state NMR studies for all integral membrane proteins. The Sanders group has shown that activities vary for the integral membrane protein diacylglycerol kinase (DAGK) at different detergent:phospholipid ratios [28]. They concluded that the phosphate-to-phosphate membrane thickness of lipid bicelles in the L_α phase affects the activity of proteins within the membrane [28]. Thus, by investigating the magnetic alignment characteristics of longer chain phospholipid discs which extend the thickness of the bicelle membrane, we hope to expand the bicelle technique to include additional integral membrane proteins for solid-state NMR studies. Also, larger membrane proteins that contain large hydrophobic regions may be more active or magnetically align better in a longer chain phospholipid disc such as PSPC/DHPC when compared to a standard 14 carbon acyl chain in the DMPC/DHPC bicelle matrix.

Characteristics of PSPC/DHPC bicelle alignment are observed only at 50°C, whereas the DMPC/DHPC phospholipid bilayer arrays align between 34°C and 43°C. DMPC/DHPC bicelle discs are known to magnetically align above the gel to liquid crystalline phase transition temperature of pure DMPC ($T_m = 23$°C) [6,16,26]. Similarly, our results indicate that the PSPC/DHPC bicelles align above the T_m of pure PSPC (approx. 48°C) [26]. Additionally, the range of temperatures at which the PSPC/DHPC bicelle discs align is very narrow when com-

pared to the DMPC/DHPC system which could be due to instability of the system above or below the T_m value of 48°C [26].

For a DMPC/DHPC bicelle system the dimensions of the discs were calculated based on X-ray diffraction studies of the thickness of the DMPC hydrophobic chain and on a simple theoretical model of the mixed phospholipid bicelle [2]. In this model, an expression

$$R = \frac{1}{2}rq[\pi + (\pi^2 + 8/q)^{1/2}] \qquad (1)$$

for the dimensions of the bicelle is calculated by analyzing a round bilayered center with radius R containing the length of the long chain phospholipids and a smaller rim portion containing the length of the short chain lipids with radius r [2]. For a DMPC/DHPC bicelle system with a q ratio of 3.0, the diameter of the planar region has been estimated to be approx. 200 Å [2]. This corresponds to a total bilayer thickness of approx. 40 Å for the DMPC/DHPC bicelle system [2,33]. For our study, the length of the hydrophobic bilayer of pure 16:0 and 18:0 phospholipids has been estimated by X-ray scattering techniques to be about 26 Å and 29.5 Å respectively in the L_α phase [27]. In a similar fashion, we have used the same theoretical mixed phospholipid bicelle model (Eq. 1) and adjusted for the longer average length of the PSPC hydrophobic chain to estimate the dimensions of a PSPC/DHPC bicelle disc. Based upon this model, we estimate that the thickness for the lipid bilayer region of a PSPC/DHPC bicelle disc with a q ratio of 1.8 in the L_α phase should be approx. 46 Å and that the diameter of the planar section is approx. 160 Å [2].

The ^{2}H NMR spectrum in Fig. 4B clearly demonstrates that the PSPC/DHPC bicelles are flipped such that the bilayer normal is parallel with the magnetic field when ytterbium ions are added to the bicelle matrix. Spectral resolution is increased because the ordering of the bilayer director has changed from $S_{zz} = -1/2$ to $S_{zz} = 1$ causing the quadrupolar splittings to approximately double and spread out over a wider frequency range. For the well-resolved terminal methyl groups (CD$_3$) the quadrupolar splitting is 3.8 kHz (perpendicular alignment) and 8.9 kHz (parallel alignment) for the PSPC/DHPC bicelle spectra at 50°C. The quadrupolar splittings of the plateau

methylene (-CD$_2$) deuterons located near the top of the phospholipid head groups are 23 kHz (perpendicular alignment) and 54 kHz (parallel alignment). The quadrupolar splittings do not exactly double as expected from the change in the order parameter [2]. For both the terminal methyl deuterons and the plateau methylene deuterons Δ_Q increases by a factor of 2.3. This is expected because the addition of trivalent cations can increase the quadrupolar splittings up to 10% [34]. For comparison, Δ_Q increases by a factor of between 2.1 and 2.2 for a DMPC/DHPC bicelle system (data not shown) [2]. The individual terminal methyl deuterons are clearly resolved in Fig. 4A,B. The individual methylene deuterons are not fully resolved at the perpendicular orientation, but the resolution is significantly improved at the parallel orientation.

^{31}P NMR spectroscopy can be used to study the different phases formed by phospholipid membranes because molecular arrangements can lead to dramatic changes in the chemical shifts and/or line shape. Below T_m (40°C and 45°C), the ^{31}P spectra of the PSPC/DHPC bicelle sample reveal two closely spaced isotropic peaks suggesting an isotropic phase with no orientational characteristics. Above T_m (50°C), the ^{31}P NMR spectrum shows signs of magnetic alignment based upon comparisons with similar DMPC/DHPC bicelle spectra [16,35]. When DMPC/DHPC bicelle discs are magnetically aligned, the DMPC phospholipid bilayer is in a liquid-crystalline L_α-like phase [6,16]. Our ^{2}H and ^{31}P NMR spectra suggest that the PSPC/DHPC bilayered discs are in a comparable L_α-like phase at 50°C. Thus, the PSPC/DHPC bilayers go directly from an isotropic phase into a liquid-crystalline phase around 50°C. Similarly, temperature dependent ^{2}H and ^{31}P NMR studies of DMPC/DHPC phospholipid bilayers have indicated a highly ordered L_α-like phase above T_m, a mixed morphology consisting of an isotropic phase and a gel-like randomly dispersed phase slightly below T_m, and a completely isotropic phase is observed below T_m [16,36].

The ^{2}H and ^{31}P NMR spectra shown in Figs. 4 and 5 reveal qualitative information on the overall degree of alignment of the phospholipid bilayers with respect to the direction of the static magnetic field. The broad peak at -7.0 ppm arises from ^{31}P PSPC atoms and indicates that the phospholipid bilayer

discs are not perfectly aligned with the magnetic field when compared to DMPC/DHPC ^{31}P bicelle spectra (data not shown) [6,16,35]. Furthermore, the lack of complete resolution of the methylene deuterons in the ^{2}H NMR PSPC/DHPC bicelle spectra shown in Fig. 4 indicates that the bicelle disks are not perfectly aligned when compared to the ^{2}H NMR spectra of magnetically aligned DMPC/DMPCd_{54}/DHPC phospholipid bilayers. Although PSPC/DHPC phospholipid bilayers are not perfectly aligned, they do provide an alternative membrane system for conducting solid-state NMR structural studies of integral membrane proteins. Depending upon the size, hydrophobicity, and/or activity of a particular membrane protein a PSPC/DHPC bicelle system may be a good alternative to the standard DMPC/DHPC matrix.

Our results indicate that longer chain phospholipids such as PSPC when mixed with detergents such as DHPC can be magnetically aligned and they can serve as potential model systems for studying integral membrane proteins. Now that we have optimized magnetic alignment conditions (temperature and q ratio) for solid-state NMR studies, future investigations will be carried out on spin-labeled PSPC/DHPC bicelle systems containing 1-palmitoyl-2-stearoyl(n-DOXYL)-sn-glycero-3-phosphatidylcholine with electron paramagnetic resonance (EPR) spectroscopy [37,38]. The corresponding PSPC spin label is commercially available at positions 5, 7, 10, 12, and 16 (Avanti Polar Lipids). The results of these studies will provide important dynamic information on the bicelle system and will be compared with parallel solid-state NMR results. Finally, the research presented in this manuscript will enable us to carry out independent studies on the structural and dynamic properties of reconstituted integral membrane peptides inserted into magnetically aligned phospholipid bilayers utilizing both solid-state NMR spectroscopy and spin-label EPR spectroscopy [37,38].

Acknowledgements

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