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Phospholipid Bicelles That Align with Their Normals Parallel to the Magnetic Field

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Abstract: We have recently reported phospholipid bicelles (bilayered micelles) that have positive anisotropy of the magnetic susceptibility and align with their normals parallel to an external magnetic field [*J. Am. Chem. Soc.* **2001**, *123*, 1537]. Improvements have been made via the synthesis of a new phospholipid, 1-dodecanoyl-2-(4-(4-biphenyl)butanoyl)-*sn*-glycero-3-phosphocholine (DBBPC). Bicelles can be formed by mixing DBBPC with a short-chain phospholipid, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) in a ratio between 5.1:1 and 6.5:1 in an aqueous medium. The ^{31}P NMR spectra clearly show that these bicelles align with their principal axes parallel to the magnetic field within a wide temperature range. The ^{31}P chemical shifts indicate that the conformation of the polar headgroup in these bicelles may be different from that in common bicelles. The phase behavior of a mixture of DBBPC/DHPC with 6:1 mole ratio was investigated in the temperature range of 10−75 °C using ^{31}P, ^{2}H, and ^{23}Na NMR. At lower temperatures (10−54 °C), the system is dominated by the bicellar phase. At higher temperatures (54−75 °C), isotropic micelles are formed and coexist with the bicelles. The partial alignment of maltotriose in the DBBPC/DHPC system was studied at three temperatures, and the ^{1}H−^{13}C dipolar coupling constants are compared with those obtained for two other bicelle solutions.

Introduction

Phospholipid (PC) bicelles were initially described as disklike bilayered micelles.[1] They consist of an appropriate combination of long-chain phospholipid, such as 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC, where M stands for myristoyl) or 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC, where L stands for lauroyl, **2**), and a short-chain phospholipid, such as 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC, **1**), in an aqueous medium. Bicelles exhibit typical lyotropic liquid crystalline properties with in a limited temperature range.[1a,2] It was suggested that the long-chain PC forms the plane of the disk and the short-chain PC forms the edge.[1a,3] The long-chain PC can be replaced by other phospholipids to increase the chemical stability[4] or impart a charge[5] on the bicelles. Recently, based on the results of small-angle neutron scattering[6] and translational diffusion anisotropy measurements of a probe molecule,[7] it was suggested that the morphology of the bicelles can be better described as a heavily perforated, highly dynamic lamellar bilayer phase. In other words, the bilayers resemble slices of Swiss cheese, a picture first suggested for the bicelles when (and only when) Tm^{3+} is added to the system.[8] In this description, the rims surrounding the holes in the bilayer consist of DHPC,[7] and the holes are probably dynamic rather than static.

The perforated lamellar phase (previously considered to be nematic) of bicelles can be macroscopically aligned in a magnetic field, and this has been used extensively for nuclear magnetic resonance (NMR) studies of biological molecules. The magnetically aligned bicelles are also useful in low-angle X-ray and neutron diffraction studies.[6] Initially, the emphasis was on the use of bicelles as model membranes for NMR studies of membrane-associated peptides and proteins.[1,9] Since the publication of the seminal work by Bax and Tjandra,[10] the use of dilute bicelle solutions as ordering media for water-soluble proteins and DNA has found wide application.

Common PC bicelles have a negative anisotropy of the magnetic susceptibility ($\Delta\chi$), so that their normals align in all directions perpendicular to the external field, spanning a 360° distribution. Consequently, the NMR peaks of species dissolved in the interior or bound to the exterior might show partial powder

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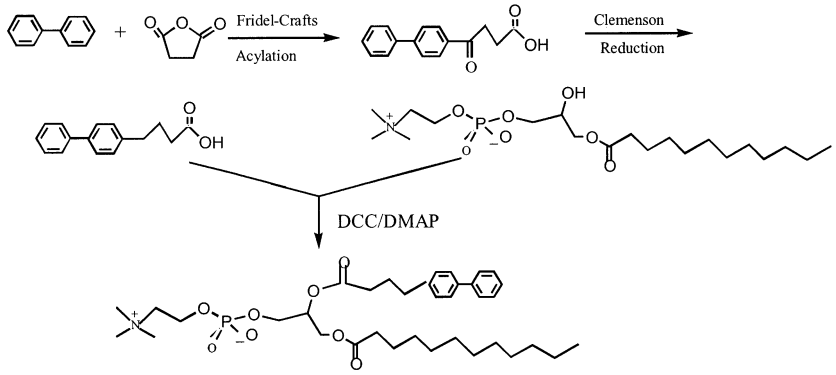
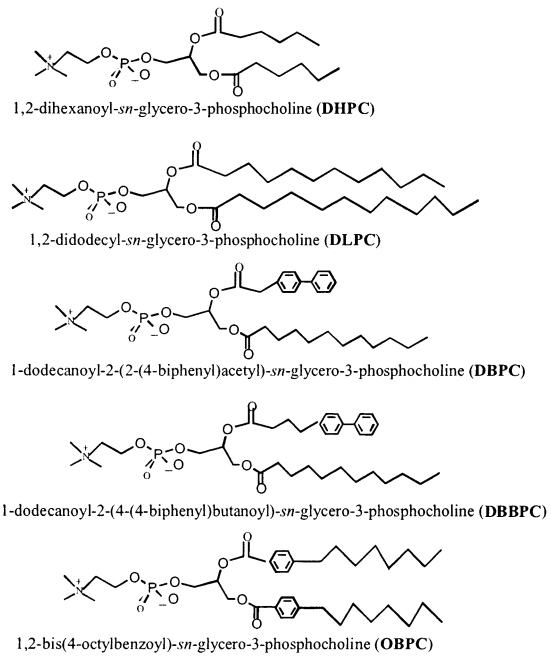
Scheme 1. Synthetic Scheme for DBBPC



patterns, limiting the usefulness of the NMR studies. Furthermore, because the magnetic susceptibility anisotropy of phospholipids and of the α-helical protein segments have opposite signs, the addition of proteins containing membrane-spanning α-helices may cancel the tendency for the bilayers to align well.[8] Although the direction of alignment can be changed with variable-angle rapid sample spinning,[11] a uniform alignment of the bicelle axes along the direction of the magnetic field is most desirable. For these reasons, efforts have been made to construct bicelles that would align with their normals parallel to the external field and have a uniform alignment. For the study of water-soluble species, the direction of alignment of the bicelles is not critical because they function only as an ordering matrix. However, in many cases it is very beneficial to carry out the NMR study in media with two different alignments[12] so that the data can be analyzed to yield more detailed structural information. Therefore, PC bicelles that align with their normals parallel to the magnetic field will also be useful in these cases.

Two approaches had been used to change the alignment of the bicelle normal from perpendicular to parallel. The first method is to use amphiphilic aromatic compounds to mix with phosphatidylcholine[13] because the phenyl ring has a large positive $\Delta\chi$,[14] but the amount of the additive required is large and the results are usually not very satisfactory. The second method is to use paramagnetic lanthanide ions to change the sign of $\Delta\chi$ to make the bicelles turn around,[8,15] but the effect of the paramagnetic ions is a concern. To overcome these problems, we developed a modified PC (DBPC, **3**), in which one of the long aliphatic chains was replaced by a biphenyl moiety, to formulate PC bicelles without using other additives.[16] It was shown that mixtures of DBPC/DHPC with a ratio very close to 6 can form bicellar solutions which are stable from 8 to 40 °C. Because the two phenyl rings at DBPC impart a large positive $\Delta\chi$ on the bicelles, they do "flip over" in a magnetic field to achieve the desired magnetic alignment. Subsequently, we have made an improvement on this system by synthesizing another PC that also contains a biphenyl unit but a longer spacer, 1-dodecanoyl-2-(4-(4-biphenyl)-butanoyl)-*sn*-glycero-3-phosphocholine (DBBPC, **4**). The phase behavior of mixtures formed



by mixing this new PC and DHPC was investigated by using ^{31}P, ^{23}Na, and ^{2}H NMR spectroscopy. It was found that bicelles are formed in a wider range of composition and with better thermal stability. The results are reported here.

Experimental Section

DLPC, DHPC, and 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids Co.; all other compounds were purchased from Aldrich Chemicals. DLPC rather than DMPC was used because the bicellar phase begins to form at a lower temperature. These starting materials were used directly without further purification. The synthetic scheme is shown in Scheme 1, and the procedures are described in the following.

4-(4-Biphenyl)-4-oxobutanoic Acid.[17] To a 100 mL round-bottom flask containing 3.0 g (30 mmol) of succinic anhydride and 5.0 g (32 mmol) of biphenyl 30 mL of nitrobenzene was added. The mixture was heated until a clear solution was obtained and then cooled to room temperature. The solution became opaque at room temperature. Then, 8.0 g (60 mmol) of powdered anhydrous aluminum chloride was added slowly while the mixture was being stirred, taking about 5 min. The solution became clear and finally turned black. After the stirring was

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continued for 24 h at room temperature with an HCl trap, the reaction was stopped by adding 15 mL of water and 5 mL of concentrated hydrochloric acid. The yellow precipitate was filtered and dried, and then washed thoroughly with ether. The crude product was purified using silica column chromatography (chloroform/acetone 2:1 as eluent) to obtain 4-(4-biphenyl)-4-oxobutanoic acid as a white solid, with 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.6 (d, 2H, Ar), 7.5 (d, 2H, Ar), 7.4 (m, 2H, Ar), 7.3 (m, 1H, Ar), 7.24 (d, 2H, Ar), 5.15 (m, 1H, CH), 2.7 (m, 2H, CH_2), 2.4 (m, 2H, CH_2), 2.0 (m, 2H, CH_2).

4-(4-Biphenyl)butanoic Acid.[17] Amalgamated zinc was prepared from zinc powder contained in a 100 mL round flask: a mixture of 1.3 g of zinc powder, 0.1 g of mercury(II) chloride, 0.06 mL of concentrated hydrochloric acid, and 1.5 mL of water was stirred for 5 min. The liquid was decanted as completely as possible. Then 0.8 mL of water, 2 mL of concentrated HCl, 2 mL of pure toluene, and 0.255 g (1 mmol) of 4-(4-biphenyl)-4-oxobutanoic acid were added consecutively. The flask was fitted with a reflux condenser connected to a gas absorption trap, and the reaction mixture was boiled vigorously for 30 h. During the refluxing period, three 0.4 mL portions of concentrated HCl were added at approximately 6 h intervals to maintain the concentration of the acid. The mixture was allowed to cool to room temperature to separate into two layers. The aqueous portion was diluted with 10 mL of H_2O and extracted several times with ether. The combined extracts were dried over $MgSO_4$, and the solvent was removed under reduced pressure to give the crude product, which was purified by silica column chromatography (ethyl acetate/*n*-hexane 1:1 as eluent) to give 4-(4-biphenyl)butanoic acid, a white needle solid, with 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.6 (d, 2H, Ar), 7.55 (d, 2H, Ar), 7.45 (m, 2H, Ar), 7.36 (m, 1H, Ar), 7.28 (d, 2H, Ar), 2.7 (m, 2H, CH_2), 2.1 (m, 2H, CH_2).

1-Dodecanoyl-2-(4-(4-biphenyl)butanoyl)-*sn*-glycero-3-phosphocholine (DBBPC, 4).[16,18] 1-Lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine (0.22 g, 0.501 mmol) and 4-(4-biphenyl)butanoic acid (0.32 g, 1.328 mmol) were dissolved in CH_2Cl_2 (7 mL). 4-(Dimethylamino)-pyridine (DMAP) (0.50 mmol) and a solution of 1,3-dicyclohexylcarbodiimide (DCC) (1.34 mmol) in CH_2Cl_2 (2 mL) were added, and the solution was stirred at room temperature for 2 days. A white precipitate was removed by filtration through a small cotton wool plug in a pipet. The product was obtained by rotary evaporation of the filtrate and purified twice by column chromatography (65:35:4 $CHCl_3/CH_3OH/H_2O$) to give DBBPC (60% yield): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (d, 2H, Ar), 7.5 (d, 2H, Ar), 7.4 (m, 2H, Ar), 7.3 (m, 1H, Ar), 7.2 (d, 2H, Ar), 5.15 (m, 1H, CH), 4.25 (dd, 1H, CH), 4.2 (m, 2H, POCH_2-CH_2N), 4.1 (dd, 1H, CH), 3.9 (m, 2H, CH_2OCO), 3.7 (m, 2H, CH_2N), 3.3 (s, 9H, N(CH_3)$_3$), 2.6 (t, 2H, CH_2), 2.3 (t, 2H, CH_2), 2.2 (t, 2H, CH_2), 1.9 (t, 2H, CH_2), 1.4 (m, 2H, $-$CH_2-), 1.16 (s, 16H, fatty CH_2), 0.8 (t, 3H, $-$CH_3); mass spectrum (FAB): *m*/*z* 662.5 (calculated 661.8).

1,2-(4-Octylbenzoyl)-*sn*-glycero-3-phosphocholine (OBPC, 5).[18,19] 1-α-Glycerophosphocholine (1:1 cadmium chloride adduct) (200 mg, 0.447 mmol) and 4-octylbenzoic acid (425 mg, 1.816 mmol) were dissolved in CH_2Cl_2 (6 mL). 4-(Dimethylamino)pyridine (DMAP) (1.816 mmol) and a solution of 1,3-dicyclohexylcarbodiimide (DCC) (1.816 mmol) in CH_2Cl_2 (2 mL) were added, and the solution was stirred at room temperature for 4 days. Then the reaction mixture was cooled to -5 °C, the precipitate was filtered off, and the filtrate was evaporated. The residue was dissolved in a 4/5/1 chloroform/methanol/water mixture, and the solution was slowly passed through a column packed with a mixture of 15 mL of Amberlite IRC-50 and 15 mL of Amberlite IR-96. An additional 50 mL of 4/5/1 chloroform/methanol/water mixture was passed through the column, and the combined filtrate was evaporated in vacuo. The crude product was purified twice by column chromatography (65:35:4 $CHCl_3/CH_3OH/H_2O$) to give OBPC (48% yield): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.8 (m, 8H, Ar), 7.1 (m, 8H, Ar), 5.54 (m, 1H, CH), 4.52 (dd, 1H, CH), 4.2 (m, 2H, POCH_2CH$_2$N), 4.1 (dd, 1H, CH), 3.9 (m, 2H, CH_2OCO), 3.7 (m, 2H, CH_2N), 3.2 (s, 9H, N(CH_3)$_3$), 2.6 (t, 2H, CH_2), 1.5 (m, 2H, $-$CH_2-), 1.23 (s, 12H, fatty CH_2), 0.85 (t, 3H, $-$CH_3).

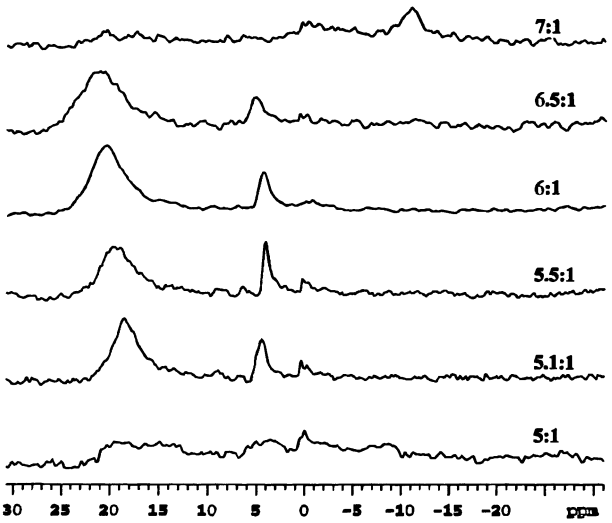

Figure 1. ^{31}P spectra for mixtures of DBBPC/DHPC (10% in D_2O) with different mole ratios at 25 °C.

To prepare samples for NMR studies, 0.035 g of DBBPC was put in an NMR tube, and 0.4 mL of 0.1 M NaCl in D_2O was added. A piece of Teflon tape was placed on top of the tube, which was then capped tightly. Then, the NMR tube was subjected to repeated cycles of heating, vortexing, and back-and-forth centrifugation until the solid dissolved. A calculated amount of DHPC solution (30 wt % in D_2O) was then added into the NMR tube, and several cycles of heating, vortexing, and centrifugation were repeated until the solution became homogeneous. For the comparison of flipped bicelles with the common bicelles, a sample of a 2.5:1 mixture of DLPC/DHPC in a solution of 0.1 M NaCl in D_2O was prepared as well. ^{31}P (162 MHz), ^{23}Na (106 MHz), and ^{2}H (61.4 MHz) spectra were acquired using a Varian UNITY/INOVA 400 spectrometer at 9.4 T, usually with 100 scans.

Results and Discussion

An initial attempt was made to formulate bicelles from mixtures of DHPC and the synthetic aromatic PC OBPC (**5**), which has two identical chains with phenyl rings. The reasoning is that OBPC is more similar to DMPC and DLPC than DBPC or DBBPC is, and the presence of two phenyl rings would also result in $\Delta\chi > 0$ so that the bicellar normal would align parallel to the magnetic field. Unfortunately, samples with a wide range of composition did not form a homogeneous phase over the temperature range of 20−75 °C. Therefore, subsequent effort was concentrated on the study of DBBPC/DHPC systems. For a mole ratio between 5.1:1 and 6.5:1, it was found that the mixtures form stable bicelle solutions from 10 to 75 °C, while the bicelles coexist with isotropic micelles above 54 °C. The details are discussed in the following.

The ^{31}P NMR spectra of several mixtures of DBBPC/DHPC at 25 °C are shown in Figure 1. When the DBBPC/DHPC mole ratio (*q*) was 7:1 and 5:1, the spectra are very broad, showing typical partial powder patterns. This is an indication that the lamellar bilayers of the PCs do not have macroscopic alignment in the magnetic field. When *q* is between 6.5:1 and 5.1:1, the ^{31}P spectra show two peaks at about 19 and 5 ppm, respectively.

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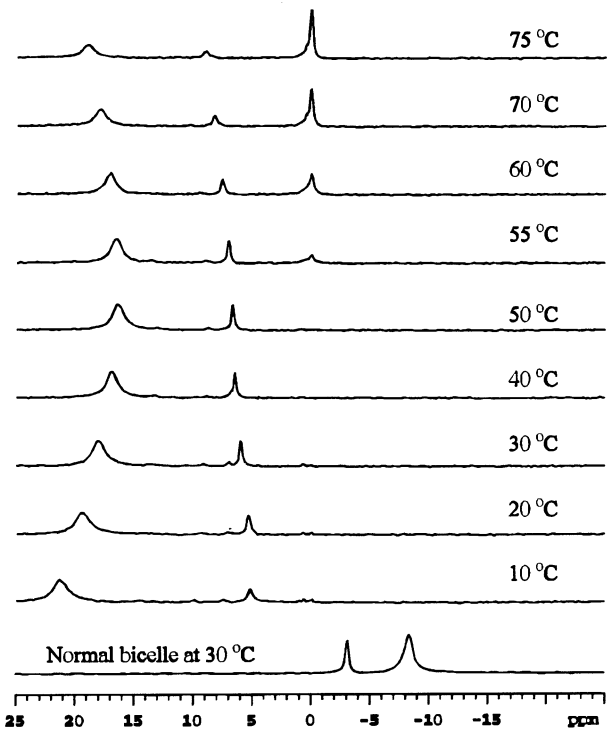

Figure 2. ^{31}P spectra for a 6:1 DBBPC/DHPC mixture (10% in D_2O) at different temperatures. The small peaks at ca. 0, 1, 7, and 10 ppm are due to impurities present in the starting material.

The relative areas of the peaks are consistent with the corresponding mole ratio of the two PCs. By comparing the spectral pattern with that of common bicelles[1a,2] and those calculated from theoretical considerations,[20] it is clear that the normal of the bicelles formed by the DBBPC/DHPC mixtures aligns parallel to the magnetic field. This situation is similar to that for bicelles formulated by mixing DBPC (**3**) and DHPC.[16] However, the DBPC/DHPC bicelles are stable only within a narrow range of composition. The increased stability of the DBBPC/DHPC bicelles over a larger range of q is likely due to the presence of two more CH_2 spacers in the chain containing the biphenyl unit, making the chain more flexible.

The thermal stability of the DBBPC/DHPC bicelles with $q = 6$ was studied by following the ^{31}P spectra (Figure 2). The two ^{31}P peaks characteristic of the "flipped" bicelles indicate that they are thermally stable over the temperature range (10−75 °C). With increasing temperature up to about 54 °C, the chemical shifts of the major and minor peaks change in opposite directions (Figure 3, squares). Above 54 °C, the ^{31}P chemical shift of the major peak begins to increase with temperature. In the meantime, a new peak near 0 ppm, which is characteristic of isotropic PC micelles, starts to appear, and its intensity increases with temperature at the expense of the peaks of the bicelles (Figure 3, diamonds). The change of the spectra with temperature is reversible. This indicates that there is an equilibrium between bicelles and micelles above 54 °C, and a schematic diagram of the temperature dependence of the DBBPC/DHPC system is shown in Figure 4. Since the short-chain DHPC molecules are more favorable to form aggregates with larger curvatures, it is most likely that the isotropic micelles are composed of mainly DHPC, as depicted in the schematic

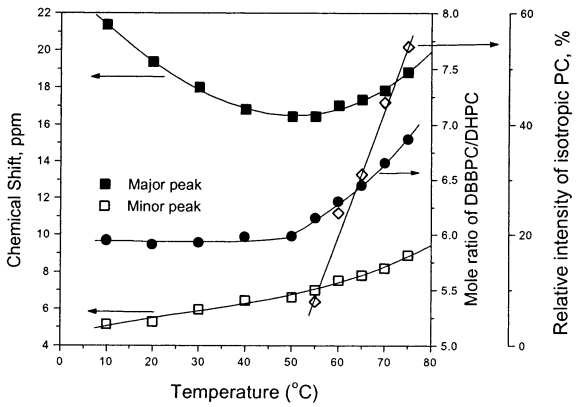

Figure 3. Temperature dependence of the ^{31}P chemical shifts of the major peak (solid squares) and the minor peak (open squares), mole ratio of DBBPC/DHPC in the bicelle fraction (solid circles), and the relative intensity of isotropic PC micelles (diamonds). The lines are drawn to guide the eye only.

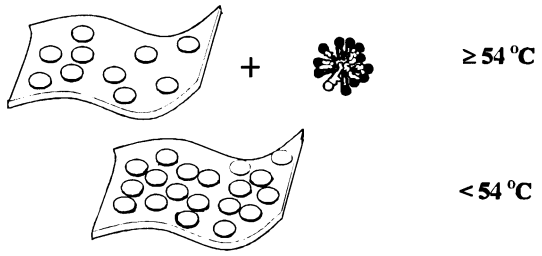

Figure 4. Schematic representation of morphological changes of the DBBPC/DHPC mixtures from low temperature to high temperature. The DHPC molecules are denoted with a solid headgroup; the sizes of the bicelles and micelles are not in proportion. Small amounts of monomers would also be present, but they are not shown in the diagram.

diagram. Because of this, the ratio of DBBPC/DHPC in the bicellar fraction increases with increasing temperature (Figure 3, solid circles), which would reduce the number of holes in the perforated bilayers. This situation is quite different from that of the DBPC/DHPC bicelle, for which there is a more complicated equilibrium from 45 to 50 °C, but the bicelles disappear above 55 °C, leaving only isotropic micelles.[16] The aliphatic DLPC/DHPC bicelles also have lower thermal stability, and phase separate at about 50 °C.

A simulation of the ^{31}P spectra of phospholipid bicelles indicated that the areas under the peaks are dependent on the mole ratio q, while the chemical shifts are not.[20] When the bicelle normal changes its alignment from perpendicular to parallel without adding paramagnetic ions, the ^{31}P peaks would have twice the chemical shifts (with respect to the isotropic peak, which is close to 0 ppm with respect to H_3PO_4) and opposite signs.[20] Although this simulation was made using the disklike model, the argument would hold for the perforated bilayer model as well. However, even though the observed ^{31}P peaks of the DBBPC/DHPC bicelles do have signs opposite those of DLPC/DHPC bicelles, the shifts are not exactly twice (Figure 3). At 30 °C, the chemical shift difference between the major and minor peaks in the DLPC/DHPC bicelles is −4.8 ppm, whereas the corresponding difference for DBBPC/DHPC bicelles is 12.3 ppm, which is much more than a factor of −2 times the first value. A similar result was observed in our previous studies in DBPC/DHPC system,[16] and it was suggested that the reason for this is the different conformations of the polar headgroup of the long-chain PC in the two types of bicelles. Obviously

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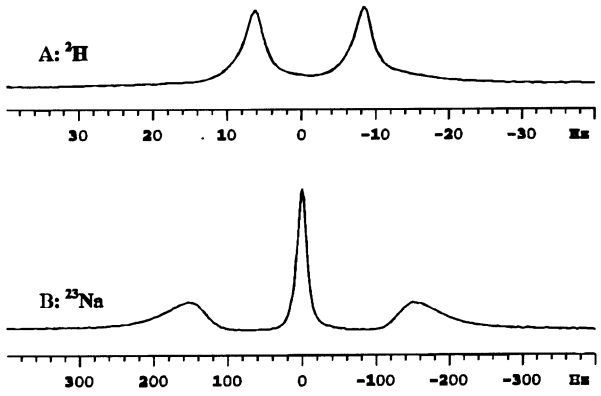

Figure 5. ^{2}H and ^{23}Na spectra of a 6:1 DBBPC/DHPC mixture (10% in D_2O) at 25 °C.

the asymmetry in the two chains contributes to the conformational change of the polar headgroup. When the temperature increases from 10 to 54 °C, the conformation of the headgroup in DBBPC would change gradually. In the meantime, the order parameter of the normals of the bicelles would decrease slightly due to increasing thermal motions of the bilayer.

Both of these factors would contribute to the decrease in the ^{31}P chemical shift of the major peak, which is due to the DBBPC molecules, with increasing temperature below 54 °C (Figure 3, solid squares). The minor peak is due to the DHPC molecules which mostly occupy the rims of the holes. They are expected to be less affected by the decrease in the order parameter of the bicelle normal, and the change in their headgroup conformation with temperature is expected to be much less due to the symmetrical nature of the two chains in DHPC. Consequently, the ^{31}P chemical shift of the minor peak increases with temperature, but much more slowly (Figure 3, open squares). Above 54 °C, the conformation of the DBBPC headgroup is expected to continue its gradual change, but the reduction in the number of holes (Figure 4) would make the bilayers slightly less flexible and raise the order parameter of the normal. Therefore, the ^{31}P chemical shift of the major peak starts to increase; the minor peak is less affected, but the slope of the temperature dependence does show a change at about 54 °C (Figure 3). Because of the complexity of the two contributions, we have not attempted a quantitative analysis of the ^{31}P chemical shift data.

To further investigate the behavior of the DBBPC/DHPC bicelles, we studied the ^{2}H NMR of D_2O and the ^{23}Na NMR of NaCl in the mixtures to obtain complementary information.

In most aqueous lyotropic liquid crystals, ^{2}H quadrupole splitting can be observed for D_2O. If the mesophase is macroscopically aligned in the magnetic field, the ^{2}H spectrum shows a doublet; if there is no macroscopic alignment, the spectrum exhibits a partial powder pattern. The ^{2}H spectrum of D_2O in the DBBPC/DHPC mixture does show two distinct peaks (Figure 5A), consistent with the presence of macroscopic alignment of the bicelles. For most thermotropic liquid crystals and many lytropic liquid crystals, the ^{2}H quadrupole splitting decreases with increasing temperature because of the decrease in the order parameter. However, the opposite trend has been observed for some lytropic liquid crystals[21] because of the

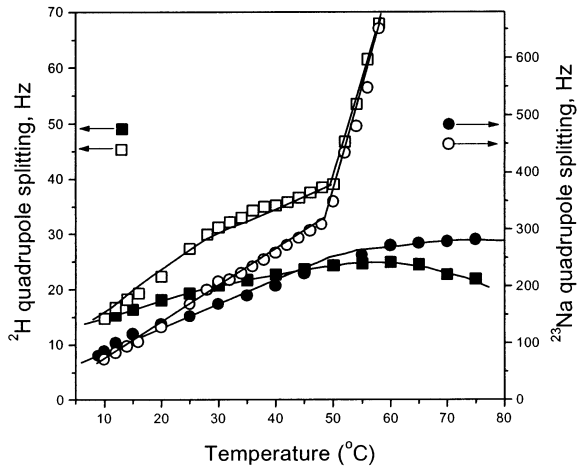

Figure 6. Temperature dependence of ^{2}H (squares) and ^{23}Na (circles) quadrupole splitting. The open symbols are data for DLPC/DHPC bicelles (mole ratio 2.5:1, 21% in D_2O with 0.1 M NaCl), and the closed symbols are data for DBBPC/DHPC bicelles (mole ratio 6:1, 10% in D_2O with 0.1 M NaCl). The lines are drawn to guide the eye only.

temperature dependence of the orientational distribution of the bound water molecules with respect to the direction of the lipid aggregate.[22] For the DLPC/DHPC bicelles above ca. 50 °C, an isotropic ^{2}H peak appears in the middle of the doublet because the system undergoes phase separation. As a consequence, the ^{2}H quadrupole splitting in the liquid crystalline phase shows a sudden change in the slope (Figure 6). In comparison, the ^{2}H doublet splitting for the DBBPC/DHPC bicelles is smaller, but it changes smoothly with temperature, showing no isotropic peak. The temperature dependence of the ^{2}H quadrupole splitting (Figure 6) mirrors that of the major ^{31}P peak. This indicates that the orientational ordering of the D_2O molecules is mainly determined by those bound to the headgroups of DBBPC, which is perfectly reasonable.

For the ^{23}Na NMR of the Na^+ ions, quadrupole splitting were observed for the DLPC/DHPC bicelles as well as for the "flipped" DBPC/DHPC and DBBPC/DHPC bicelles (Figure 5B; in our previous work it was erroneously stated that the DLPC/DHPC bicellar system showed no ^{23}Na quadrupole splitting[16]). The presence of ^{23}Na splitting is due to an induced asymmetry in the hydration sheath of the cations on the bilayer surface, which are in rapid exchange with the Na^+ ions in the bulk.[23] Similar to the ^{2}H splitting, the temperature dependence of the ^{23}Na quadrupole coupling for the DLPC/DHPC bicelles shows a break at ca. 50 °C (Figure 6) due to phase separation. The isotropic ^{23}Na peak coincides with the central peak of the signal in the micellar phase, and the peak intensity increases rapidly with the increase in temperature. On the other hand, the ^{23}Na quadrupole splitting for the DBBPC/DHPC bicellar system increases smoothly with temperature (Figure 6) since there is no phase separation. Because of the presence of the hydration sheath, the Na^+ ions cannot approach the polar headgroups of the phospholipids as closely as the water molecules. Therefore, the morphological change in the system above 54 °C has a smaller effect on the ^{23}Na quadrupole splitting than the ^{2}H quadrupole splitting (Figure 6).

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Table 1. Some $^{1}H-^{13}C$ Dipolar Coupling Constants (D, in Hz) of 30 mM Maltotriose in Three Bicellar Solutions in D_2O[a]

	1β	1α	4″	5α	2β
DLPC/DHPC bicelles[b]	2.5	−4.0	−8.0	2.6	4.6
DBPC/DHPC[c]	−2.7	10.2	18.3	−2.1	−7.9
DBBPC/DHPC (25 °C)[d]	−2.61	7.4	15.7	−1.0	−5.6
DBBPC/DHPC (50 °C)[d]	−2.31	8.3	15.9	−0.1	−6.0
DBBPC/DHPC (75 °C)[c]	−3.14	7.6	15.4	−0.3	−5.0

[a] The values were calculated from the splittings (Δ) using the formular $\Delta = 2D + J$, where J's are scalar coupling constants determined from a solution in pure D_2O.[24] 1β, 1α, 4″, 5α, and 2β are peak assignments. [b] DLPC/DHPC (3/1, 10% w/v, 30 °C). [c] DBPC/DHPC (6/1, 11% w/v, 25 °C).[16] [d] DBBPC/DHPC (6/1, 10% w/v).

It is to be noted that both the ^{2}H and the ^{23}Na quadrupole splittings for the DBBPC/DHPC solution are smaller than those for the DLPC/DHPC solution. The reasons for this would include different lipid concentrations and differences in the alignment of the bicelle normal and the headgroup conformations in the two systems, but estimations of the contribution of each factor are difficult to make.

To test the application of the new DBBPC/DHPC bicellar system as an ordering medium for biological molecules, HMQC (heteronuclear multiple quantum correlation) NMR experiments were performed for a trisaccharide, maltotriose, in a DBBPC/DHPC bicellar solution. The results for the best resolved ^{13}C peaks are listed in Table 1, and those for the DBPC/DHPC and DLPC/DHPC systems are also listed for comparison. The data in the table show that the signs of the $^{1}H-^{13}C$ dipolar coupling constants of maltotriose in the DBBPC/DHPC bicellar solutions are the same as those in DBPC/DHPC solutions and opposite those in DLPC/DHPC bicelles at all three temperatures, confirming the conclusion that the bicelle normal does align parallel to the magnetic field. At 75 °C, there is an equilibrium between bicelles and micelles (Figure 4), which would reduce the amount of bilayers responsible for the alignment. However, this is compensated for by the increase in the order parameter of the bilayer normal, so that the $^{1}H-^{13}C$ dipolar coupling constants are comparable to those at 25 °C.

In conclusion, it has been shown that the DBBPC/DHPC bicelles align with their normals parallel to the magnetic field. Because they are stable in larger composition and temperature ranges compared with the DBPC/DHPC bicelles, they are a better complementary system to common PC bicelles. The better thermal stability of these "flipped" bicelles would also be advantageous for the study of protein folding or thermophilic proteins.

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