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Dilute Bicellar Solutions for Structural NMR Work

Jochem Struppe and Regitze R. Vold[1]

Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0359

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Deuterium NMR spectroscopy has been employed to characterize the concentration dependence of orientational order in DMPC/DHPC bicellar solutions with molar ratios q = [DMPC]/[DHPC] = 3.3, 2.7, and 2.3. The stability of a discotic nematic phase can, in general, be predicted from a simple Onsager picture involving the size and concentration of the mesogenic unit, but for the bicellar solutions this model is not adequate. Specifically, macroscopic alignment is observed at total lipid concentrations well below that, 1–10% (w/w) predicted by Onsager's model. Thus the discotic nematic phase is stable to ≈3–5% (w/w) for q = 3.3–2.3, and the bicellar order is highest just before phase separation occurs at the minimum total phospholipid concentration. This implies the presence of a $DHPC_{bic} \rightleftarrows DHPC_{sol}$ equilibrium in establishing bicellar size, thereby extending the range of concentrations for which alignment occurs. Bicellar morphology has been verified for a wide range of concentrations, temperatures, and q-values, but as viscosity measurements demonstrate, major morphological changes take place as the temperature is reduced below 30°C.

Key Words: **phospholipid Bicelles; ^{2}H NMR; ^{31}P NMR; viscosities.**

INTRODUCTION

During the past few years magnetically ordered phospholipid bicelles (*1, 2*) have attracted a great deal of attention because of their potential as membrane model systems in structural studies of membrane-associated peptides and proteins (*3*). A bicellar solution contains disk-shaped aggregates composed of a normal long-chain phospholipid, e.g., dimyristoylphosphatidylcholine (DMPC), surrounded by a rim of surfactant, such as dihexanoylphosphatidylcholine (DHPC). Bicellar solutions are typical lyotropic liquid crystalline solutions. They form a large variety of phases as the relative and total lipid concentrations are varied, and at biologically relevant temperatures bicellar solutions form a discotic nematic phase which undergoes macroscopic alignment in magnetic fields normally used in NMR. Bicelles align with their normals perpendicular to the magnetic field because the anisotropy of the phospholipid magnetic susceptibility is negative. For DMPC/DHPC bicelles with $q > 2$ (q is the molar ratio between DMPC and DHPC) the negatively ordered, macroscopically aligned phase is stable between 30 and 45°C when the total lipid concentration, c_L, is between 3 and 40% (w/w) (*4*). Thus far, DMPC/DHPC bicelles have been proposed for use in structural studies of membrane associated molecules in three different ways: (i) Originally (*1–3*), in their negatively ordered, macroscopically aligned nematic phase with $2 < q < 5$ and $c_L \sim 15$–25% for structure determination of membrane associated substances; (ii) in a similar, but positively ordered nematic or smectic phase induced by lanthanide ions with positive magnetic susceptibility anisotropy (*5, 6*); and (iii) in the isotropic phase with $0.5 < q < 1$ and $c_L \sim 10$–15% for high-resolution NMR structural studies (*7*) of membrane binding peptides. Recently (*8*), more dilute bicellar solutions (c_L = 3–5% (w/w)) have been proposed as suitable media for refinement of high-resolution NMR structures of proteins that do not associate specifically with phospholipid bilayers and consequently align only weakly. Given this wide range of possible ways of using bicelles in structural studies, it seemed desirable to make available a partial characterization of the bicellar phase diagram.

With this objective in mind, we have used deuterium NMR to characterize nematic DMPC/DHPC bicellar solutions near 37°C for q = 3.3, 2.7, and 2.3 and c_L = 2–20% (w/w). Our results imply that a small, but significant, fraction of the DHPC is present as monomers and that the equilibrium $DHPC_{bic} \rightleftarrows DHPC_{sol}$ between monomeric (sol) and aggregated (bic) DHPC dictates the bicellar size and order and, consequently, the range and stability of the macroscopically ordered phase. In addition, we have carried out some simple capillary-flow viscosity measurements of the bicellar solutions, and the results demonstrate that gross morphological changes take place at temperatures below the nematic range.

EXPERIMENTAL

Materials

1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dihexanoyl-d_{22}-*sn*-glycero-3-phosphocholine (DHPC-d_{22}), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine (DMPC-d_{54}) were purchased from Avanti Polar Lipids (Alabaster, AL). The phospholipids were used as received, handled only in a "dry-bag" before dissolution in water, and otherwise stored at −20°C. Deuterium-depleted water and deuterium oxide were

[1] To whom correspondence should be addressed.

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obtained from Cambridge Isotope Laboratories (Cambridge, MA).

Sample Preparation

A 20% (w/w) solution of DHPC-d_{22} and a 20% (w/w) slurry of DMPC-d_{54} were prepared with deuterium depleted water containing either 2 mM MES buffer or 10 mM K_2HPO_4, both at pH 6.7. Bicellar samples for the NMR experiments were obtained by mixing the calculated amounts of these mixtures, adding deuterium-depleted water as needed. Thorough mixing was achieved by vortexing the chilled mixtures at 10–15°C, exploiting the low-viscosity, isotropic phase present in this temperature range. The following bicelle solutions were prepared by weight: DMPC-d_{54}/DHPC-d_{22} with $q = 3.3$ and 2.7, DMPC-d_{54}/DHPC with $q = 2.3$, DMPC/DHPC-d_{22} with $q = 3.3$, and, for viscosity measurements, DMPC/DHPC with $q = 3.3$ and 2.7. All stock solutions and NMR samples were stored at −20°C.

We used 5-mm-o.d. sample tubes (Wilmad) for both the ^2H and ^{31}P NMR experiments. Standard high-resolution 5-mm sample tubes were used for ^{31}P NMR, while those for ^2H were 25 mm long and had flat bottoms. Alignment was achieved for most samples within a few minutes after insertion in the magnetic field at 35–40°C, but a 30-min equilibration period was normally used before starting an experiment. We achieved optimal alignment of a few samples with higher viscosity only by ramping the temperature down from slightly above 40°C to 36.6 or 38.9°C, where most of the NMR measurements were performed.

NMR Measurements

Deuterium quadrupole echo spectra were obtained at 38.4 MHz using a GN500 spectrometer controlled by a Tecmag LIBRA unit and interfaced to an ENI LPI-10 rf amplifier and a 5.9 T Oxford Instruments magnet. The sample temperature in our home-built probe was maintained within ±0.1 K using a LakeShore 91C controller, and the temperature gradient was less than 0.1 K across the sample. Between 1024 and 8192 transients with 4 k data points were accumulated using the standard quadrupole echo sequence, $\pi/2\text{-}\tau\text{-}\pi/2\text{-}\tau_1\text{-}acq$ (*9*) with $\tau = 50$ μs and $\tau_1 = 35$ μs, $\pi/2$ pulses of 6.5 μs and 1 s repetition time. The transients were acquired with a sampling rate of 500 kHz in order to facilitate location of the quadrupole echo maximum. The second half of the echo was fractionally left-shifted, zero-filled, and exponentially apodized with a line broadening of 50 or 100 Hz prior to Fourier transformation.

^{31}P NMR experiments were performed at 242.9 MHz on the $q = 3.3$ and 2.7 samples on a Bruker DRX600 spectrometer equipped with a BBI broadband triple-axis gradient probe. The temperature was calibrated using the temperature dependence of the methanol chemical shifts (*10, 11*). Phosphorus spectra were obtained using 30° flip angle pulses, 1.35 s acquisition times and delays of 2 s, the WALTZ16 sequence (*12, 13*) for broadband proton decoupling, and 64 k data points. Integration of the DMPC and DHPC phosphate resonances was used to assess q for the bicellar samples, and the results indicated that the actual value of q was 0.2 smaller than expected from the molar mass of dry phospholipids and careful weighing of the individual constituents in a drybag. This difference exceeds our estimate of the experimental errors associated with sample preparation and may be associated with unknown amounts of water in the DMPC and DHPC powders. Relaxation influence can also be excluded, as 1-, 5-, and 10-s delays were applied for some spectra and did not show significantly different integrals. The values quoted for q in this paper are those determined from the ^{31}P integrals. No isotropic ^{31}P NMR lines representing free DHPC monomer were observed in any of the samples.

Viscosity Measurements

Bulk viscosities were measured with standard capillary-flow viscometers (Cannon, State College, PA), whose calibration was checked periodically. The temperature of the water bath was regulated to within ±0.1°C, calibrated with NBS standard thermometers, and allowed to equilibrate for 30 min before viscosity measurements were done.

RESULTS AND DISCUSSION

Deuterium NMR Spectra

Deuterium quadrupole echo NMR spectra were obtained at 36.6 and 38.9°C for DMPC-d_{54}/DHPC-d_{22} bicelles with $q = 3.3$ and 2.7 and for DMPC-d_{54}/DHPC bicelles at $q = 2.3$ at 5–8 different total phospholipid concentrations between 2 and 20% (w/w). The spectra shown in Fig. 1 were recorded in order of decreasing concentration, terminating at $c_L = 4$–2% depending on q, when powder spectra were observed and the samples were found to be opaque from precipitated DMPC. The deuteron spectra are characteristic of macroscopically aligned phospholipid bilayers oriented with their normals orthogonal to the magnetic field direction, and as usual (*4*), bicelles with higher q are better ordered and yield spectra with larger quadrupolar splittings. For the $q = 3.3$ sample (Fig. 1A), the DMPC and DHPC spectra are well resolved except for partial overlap of the DMPC methyl doublet with the DHPC β-, γ-, and δ-methylene doublets. The two doublets belonging to chemically nonequivalent DHPC methyl groups can be distinguished near the center of the $q = 3.3$ and 2.7 spectra (Figs. 1A and 1B).

Low-intensity quadrupolar doublets arising from the DHPC α-methylene groups can be distinguished in Figs. 1A and 1B, where they are marked with arrows. The α-methylene splittings are important as an indicator of bicellar morphology, but unfortunately, as previously reported (*7, 14*), Avanti provides chain-deuterated DHPC in which the deuterons of the α-methylene groups are almost completely replaced by protons. In

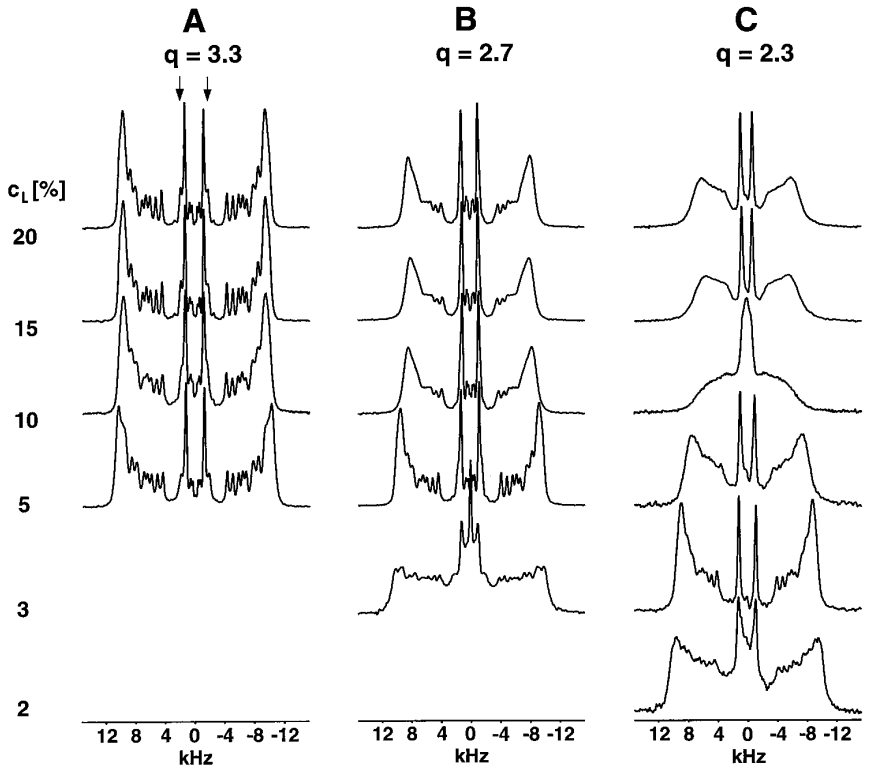

FIG. 1. Deuterium quadrupole echo spectra of DMPC/DHPC bicellar solutions recorded at 38.4 MHz and 36.6°C as a function of total phospholipid concentration c_L for $q = 3.3$ (A), $q = 2.7$ (B), and $q = 2.3$ (C). All spectra are plotted with the same maximum amplitude as dictated by the height of the CD_3-doublets. The spectra shown in (A) and (B) were obtained from bicellar samples containing both chain-deuterated DMPC and DHPC, and the very low intensity doublets arising from residual deuterons in the DHPC α-methylene groups are indicated by small vertical arrows. The $q = 2.3$ samples (C) contained regular DHPC only. Phase separation leading to precipitation of DMPC from solution occurred at $c_L = 4\%$ for the $q = 3.3$ samples and, as indicated by the appearance of powder patterns, at $c_L = 3$ and 2% for $q = 2.7$ and $q = 2.3$, respectively. Otherwise, all samples were stable for at least 5–10 h in the magnet at any given temperature. For $q = 2.7$, an isotropic peak is clearly visible in the spectra of the $c_L = 3$ sample. This peak could stem from the hydrolysis product lysophosphatidylcholine, small DMPC/DHPC aggregates, or monomeric DHPC. ^{31}P NMR measurements on aqueous solutions of DHPC and DMPC/DHPC bicelle solutions suggest that this isotropic peak arises from a hydrolysis product, but this has not been substantiated.

other words, the product should be sold as DHPC-d_{18}. Nevertheless, to test the predictions of the "ideal bicelle model" (*14*), we compared the quadrupolar splittings Δ_s observed for the DHPC α-deuterons with the splittings Δ_l for the DMPC plateau region at two temperatures. In the ideal bicelle, a short-chain phospholipid molecule is assumed to occupy the same surface area on the rim of the bicelle as a long-chain phospholipid molecule does in the planar section. Furthermore, the internal molecular motion, and consequently the internal order parameter, for methylene groups near the glycerol moiety is assumed to be identical in the long- and short-chain phospholipids. Given those assumptions, the ratio $\rho = \Delta_s/\Delta_l$ between the deuterium quadrupolar splittings was predicted to lie in the range 0.23–0.24 for bicelles with $2 \leq q \leq 4$. We determined ρ from the DMPC/DHPC bicelle spectra obtained at 36.6 and 38.9°C, and as shown in Fig. 2, ρ falls in the range 0.22–0.31. Except as noted below, we consider these values to be in reasonable agreement with prediction (*14*), given the simplicity of the model and the large uncertainty associated with measuring the α-deuteron splittings for DHPC. Furthermore, as illustrated by the $q = 3.3$ spectra recorded at $c_L = 5$ and 10%, conformational changes within the DMPC molecules makes it difficult to define the "plateau splitting" consistently. Nevertheless, for the $q = 3.3$ sample (triangles) ρ is observed to be considerably larger at 38.9°C than at 36.6°C. A possible explanation for this behavior may be obtained by considering the values of Δ_s and Δ_l themselves. The data presented in Fig. 3 show that all the resolvable quadrupolar splittings in DHPC increase with increasing temperature, while the DMPC splittings decrease. This result suggests that some DHPC molecules may diffuse into the bilayer section of the bicelle and appear to be more highly ordered. The same trend is observed for the $q = 2.7$ bicelles, as indicated by the values of ρ plotted (squares) in Fig. 2.

The concentration dependence of the quadrupolar splittings observed in Fig. 1 is difficult to rationalize without invoking the presence of a significant, however small, concentration of DHPC monomer in the bicellar solutions. No isotropic DHPC monomer line was observed in either the ^{2}H or ^{31}P NMR spectra, but this may simply mean that the DHPC molecules

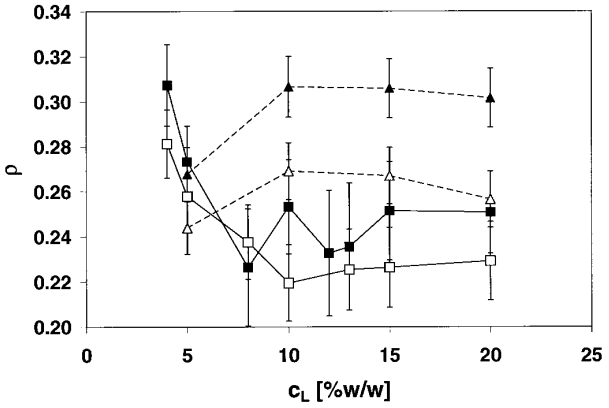

FIG. 2. Concentration dependence of the ratio $\rho = \Delta_s/\Delta_l$ between the plateau splittings for DHPC and DMPC obtained at 36.6°C (open symbols) and 38.9°C (solid symbols) for $q = 2.9$ (squares) and $q = 3.5$ (triangles). The experimental uncertainty (±10%) is largely determined by the low S/N of the DHPC α-deuteron resonances. For other comments, please refer to the text.

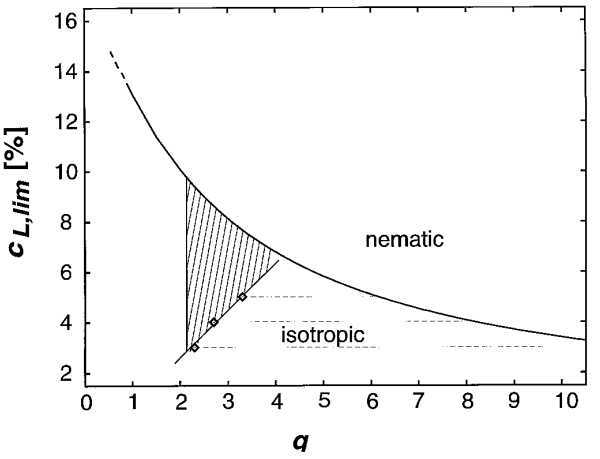

FIG. 4. Minimum phospholipid concentration $c_{L,lim}$ required for establishment of a discotic nematic phase at different q-values according to a Onsager model for flat cylinders. Also indicated (diamonds) are the actual limiting concentrations where nematic bicelles were in fact stable. The region corresponding to the extended nematic phase, induced by loss of DHPC from the bicelles to the bulk solution, is cross hatched.

exchange rapidly between the bicelles and bulk solution according to the $DHPC_{bic} \rightleftarrows DHPC_{sol}$ equilibrium. If no such equilibrium exists, Onsager's calculation of the excluded volume for rotating cylinders may be used to predict the concentration and q dependence of the nematic → isotropic phase transition for the DMPC/DHPC bicellar solutions. According to Onsager (*15*), a transition to an ordered phase takes place when the excluded volume per particle (or mesogenic unit) exceeds the volume available to each particle, i.e., when

$$V_{excl} \geq V_{bic}/c_L, \qquad [1]$$

where (see Eq. 34 of Ref. (*15*))

$$V_{excl} = \pi(R + r)\left[2r^2 + (\pi + 3)r(R + r) + \frac{\pi}{2}(R + r)^2\right] \qquad [2]$$

$$V_{bic} = \pi r\left(2R^2 + \pi Rr + \frac{4}{3}r^2\right) \qquad [3]$$

and the radius R of the bilayered section of the bicelle is given by

$$R = \frac{rq}{2}(\pi + \sqrt{\pi^2 + 8/q}) \qquad [4]$$

in terms of q and r, the radius of the bicelles rim section. The limiting concentration $c_{L,lim}$ is defined when the equal sign is chosen in Eq. [1], and the resulting values are plotted as a function of q in Fig. 4. Also shown in Fig. 4 are the lowest phospholipid concentrations leading to stable bicellar solutions according to the experimental data shown in Fig. 1, and it is clear that stable bicelles exist below the limiting concentration predicted by the Onsager model. Cross hatching indicates the extended concentration range where bicelles appear stable. Following Onsager (*15*), we may now use the argument that for nematic order to exist at $c_{L,lim} = 5$–3%, considerably larger bicelles with $q \cong 6$–10 must be present, rather than bicelles with $q = 2.3$–3.3. This in turn leads to the conclusion that the

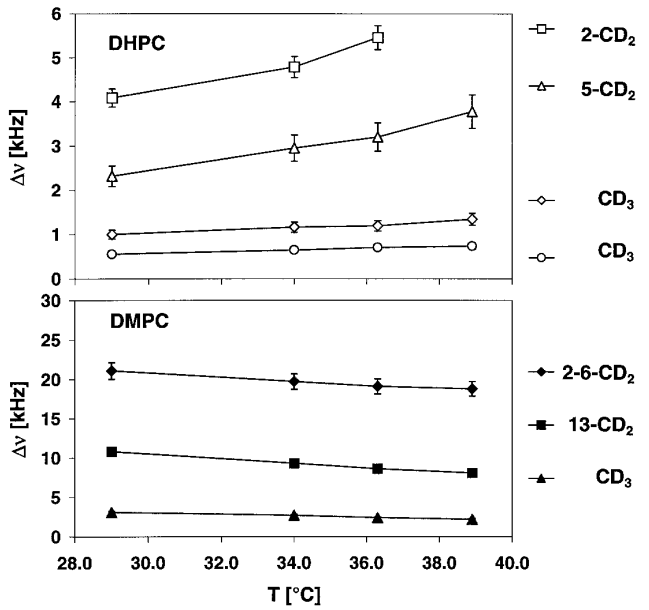

FIG. 3. Temperature dependence of selected deuterium quadrupolar splittings observed for samples with $q = 3.3$ and $c_L = 10\%$ (w/w). The splittings Δ_l for DMPC were measured from the spectra presented in Fig. 1A, while those for DHPC, Δ_s, were obtained from a similar DMPC/DHPC-d_{22} bicellar solution. The numbering of the acyl-chains begins with 1 at the carboxyl carbon. 2–6 CD_2 refers to the so-called plateau region with almost the same order parameter.

three samples contain ~7–10 mM monomeric DHPC. To test this conclusion, we centrifuged the (phase-separated) $q = 2.7$, $c_L = 3\%$ sample and measured the DHPC monomer concentration in the supernatant. The DHPC concentration was found to be 5 ± 1 mM, well below the critical micelle concentration for DHPC, which was determined to be 6.9 mg/mL (15 mM) or 6.5 mg/mL (14 mM) from, respectively, surface tension (*16*) and light scattering (*17*) measurements.

It should be noted that Eq. [4] includes only first order terms for the evaluation of the excluded volume (*15*). Leaving out the second order terms has been shown to be an excellent approximation for long, thin cylinders, but for disks this is less well justified (*18*). In fact, as pointed out by Onsager (*15*), the second order term is positive and results in a larger V_{excl} than those calculated here. The effect of the second order terms would therefore be to lower the theoretical curve in Fig. 4, bringing the estimated DHPC monomer concentration more in line with that observed. Given the many other simplifying assumptions about bicellar shape and size and our neglect of any volume of hydration, we did not think it worthwhile to consider this matter in more detail.

Returning to Fig. 1, we find upon closer inspection of the $q = 3.3$ spectra (A) a slight increase in line width and a ~7% decrease in the quadrupolar splittings when c_L is reduced from 20 to 10%. This slight decrease in apparent order is then followed by a slight increase in both resolution and splittings upon further dilution to $c_L = 5\%$, below which phase separation occurs. The changes are subtle for the $q = 3.3$ samples, but are reproduced at higher temperatures (data not shown). Figures 1B and 1C for $q = 2.7$ and $q = 2.3$ show similar but more dramatic changes in ordering. At c_L between approximately 5 and 10% the deuteron spectra are characteristic of less well-ordered bicelles (*4*, *14*). At lower lipid concentrations, however, a substantial increase in quadrupolar splitting and resolution is observed, followed at even lower concentration by the appearance of powder spectra at 3% for $q = 2.7$ and at 2% for $q = 2.3$. Starting from concentrated bicellar solution and adding water might initially lead to lower ordering of the bicelles, because the extent of bicellar "wobble" would increase, provided that the bicellar size remain constant. However, the existence of the equilibrium $DHPC_{bic} \rightleftarrows DHPC_{sol}$ entails removal of DHPC from the bicellar rim, leading to a higher value of q, larger bicelles, and a compensating effect on the bicellar motion and the observed order.

It remains to be pointed out that the free energy of magnetic alignment of an individual bicelle is far too small to compete with $kT = 4.3 \times 10^{-21}$ J at 310 K. The anisotropy of the volume magnetic susceptibility χ_v for DMPC was determined by Scholz *et al.* (*19*) as 0.145 +/− 0.015 J T^{-2} m^{-3}. That translates into a free energy of alignment of $\sim -7 \times 10^{-23}$ J for a bicelle with $q = 3.5$ and $R = 2.32 \times 10^{-8}$ m in a field of 8.5 T. Like for all nematic liquid crystals the establishment of long-range magnetic order is a consequence of the sterically induced local order just discussed.

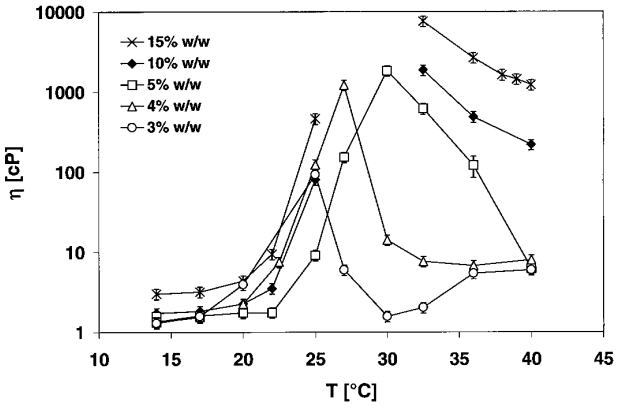

FIG. 5. Temperature dependence of the capillary viscosity for $q = 2.9$ bicelles and five values of the total phospholipid concentration c_L. For the most concentrated solutions, the rapid increase in viscosity with increasing temperature ($\partial\eta/\partial T \sim 0.2$ P K^{-1}) associated with the transition near 30°C from isotropic to nematic solution was difficult or impossible to monitor properly with the equipment available to us.

Viscosity Experiments

The temperature and concentration dependence of the capillary viscosity for the $q = 2.7$ and $q = 3.3$ DMPC/DHPC solutions is shown in Fig. 5. Below 20°C the solutions have a viscosity close to that of pure water, but as the temperature is increased the viscosity increases by up to nearly four orders of magnitude over a very narrow temperature range accompanying the order–disorder phase transition observed in the ^2H NMR spectra. Above ~30°C, where the solutions become macroscopically aligned in the magnetic field for $c_L \geq 5\%$, the viscosity then drops monotonically with increasing temperature. The initial drop near 30°C is presumably caused by flow alignment of the bicelles formed in the fully developed nematic phase.

As might be expected, the height of the viscosity maximum decreases with decreasing lipid concentration, and the position of the maximum shifts to lower temperature. At concentrations below 5%, the temperature dependence of the viscosity suggests that partial phase separation occurs between 25 and 30°C. In this range the samples became translucent, indicative of the formation of large aggregates (but above 35°C the solutions align in the magnet and become clear), and a slight increase in viscosity coincides with the stabilization of the nematic bicellar phase observed at 36.6°C for $q = 2.7$ (see Fig. 1B). This latter observation is particularly relevant for high-resolution biopolymer studies (*8*), the success of which depends on low lipid concentration and viscosity combined with minimal macroscopic orientational ordering of the solutes.

CONCLUDING REMARKS

Stable macroscopically aligned, nematic DMPC/DHPC bicellar solutions can be prepared at total lipid concentrations

between c_L = 20 and 3% and temperatures between 35 and 40°C for molar ratios q = 3.3, 2.7, and 2.3 between long- and short-chain phospholipids. The bicellar nature of these samples was confirmed by comparing the deuterium quadrupolar splittings of DHPC and DMPC, but the nematic phase was observed to be stable well below the range predicted by the Onsager model for hard disks. The nematic range of stability depends, as in all lyotropic liquid crystalline solutions, on the relationship between concentration and size of the mesogenic unit, here the DMPC/DHPC bicelle. According to Onsager's model for the excluded volume of rotating disks, the nematic phase should not be stable below 7–10% (w/w) total phospholipid for the range of q-values investigated here, yet macroscopic bicellar alignment was observed down to c_L = 3–5% (w/w). Our observations can be rationalized by invoking the presence of an equilibrium $DHPC_{bic} \rightleftarrows DHPC_{sol}$ between monomeric DHPC in the bicelles and DHPC in solution. As DHPC is removed from the bicelles upon dilution, q and the bicellar diameter increase, and alignment takes place at lower concentrations than predicted by Onsager's model. In agreement with this modified picture, macroscopic ordering was observed to increase at phospholipid concentrations below $c_L \sim 10\%$ and reach a maximum close to the low concentration limit. This limit is characterized by visible precipitation of large aggregates—phase separation occurs when enough DHPC has been removed from the bicelles. Use of Onsager's model predicts a monomer concentration in the range 7–10 mM, and the determination of a $[DHPC]_{mon} = 5 \pm 1$ mM supports our interpretation within the simplifications made.

We finally note that the *macroscopic* ordering observed in the NMR experiment cannot be predicted from the magnitude of the anisotropy of the magnetic susceptibility of an individual bicelle, whether its size is estimated from the value of q obtained from the ^{31}P NMR spectra or from that calculated by use of the Onsager model. The anisotropy of the magnetic susceptibility of a single bicelle with q = 7–2 is of the order of 10–100 times smaller than that required for magnetic ordering. Liquid crystalline order is established when the correlation length for orientational order exceeds by severalfold the dimension of a single mesogenic unit, and the establishment of magnetic ordering in the bicellar solutions is, as in all liquid crystals, governed by such orientational correlations. But the present estimates of excluded volumes following Onsager's original treatment explains the existence of bicellar order for DMPC/DHPC solutions, and the extension of orientational order to concentrations below that predicted by the Onsager model can be nicely accounted for by a DHPC monomer-bicelle equilibrium.

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