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Characterization of surfactant liquid crystal phases suitable for molecular alignment and measurement of dipolar couplings

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Abstract

Media employed for imparting partial alignment onto solute molecules have recently attracted considerable attention, since they permit the measurement of NMR parameters for solute biomolecules commonly associated with solid state NMR. Here we characterize a medium which is based on a quasi-ternary surfactant system comprising cetylpyridinium bromide/hexanol/sodium bromide. We demonstrate that dilute solutions of this system can exist in liquid crystalline phases which orient in the magnetic field and allow the measurement of residual dipolar couplings under a variety of conditions. The present system is extremely versatile and robust, tolerating different buffer conditions, temperature ranges and concentrations.

Introduction

Traditionally, the determination of three-dimensional structures of biological macromolecules employs NOE-derived distance restraints and torsion angle restraints extracted from J-couplings (Wüthrich, 1986; Clore and Gronenborn, 1989). A key limitation inherent to this approach concerns the strictly local nature of these parameters, since they solely define distances and angles between atoms close in space within the structure. Despite this limitation, protein structure determination by NMR has been extremely successful, primarily because the large number of short interproton distances between amino acids far apart in sequence render these distances conformationally highly restrictive.

Nevertheless, the use of only short distance information may limit the accuracy of NMR-derived structures, especially for elongated structures where the cumulative errors may be significant or in cases where only few contacts are available between structural elements. Examples of such systems include modular and multi-domain proteins and linear nucleic acids. Recently, solution NMR methods have been developed which allow the extraction of structural restraints characterizing long-range order, in particular residual dipolar couplings (Kung et al., 1995; Tolman et al., 1995; Tjandra et al., 1996, 1997; Tolman and Prestegard, 1996a,b; Tjandra and Bax, 1997a,b; Hansen et al., 1998a; Wang et al., 1998b). They are most conveniently measured by placing the molecule under investigation into a dilute, aqueous liquid crystalline phase (Bax and Tjandra, 1997; Tjandra and Bax, 1997a; reviewed in Prestegard, 1998; Sanders and Prosser, 1998; Tjandra, 1999). Realization of widespread applications of residual dipolar couplings with respect to NMR structure determination resulted in a flurry of activity, studying alignment media with the aim of improving the initial bicelle system (Ottiger and Bax, 1998, 1999; Losonczi and Prestegard, 1998; Wang et al., 1998a; Cavagnero et al., 1999), as well as the discovery of novel ones (Clore et al., 1998; Hansen et al., 1998b; Prosser et al., 1998; Koenig et al., 1999; Sass et al., 1999).

In the search for alternative and robust liquid crystalline media suitable for partially aligning biomolecules for structural studies, dilute quasi-ternary

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systems of surfactant/salt/alcohol forming Helfrich lamellar phases were investigated (Prosser et al., 1998; Gronenborn, unpublished data). In the 1970s Helfrich predicted that lamellar phases could exist in a form which is stabilized principally by repulsive entropic forces. These phases consist of bilayers, which can be swelled by solvent such that the spacing between the bilayers is much larger than the thickness of the bilayer itself, which undergoes large amplitude fluctuations (Helfrich, 1978). The morphology of a lamellar liquid crystalline (LLC) phase formed from cetylpyridinium chloride (CPCl)/hexanol/1% NaCl consists of ~3 nm thick bilayers with large interlamellar spacings (McGrath, 1997). This Helfrich lamellar phase will readily take up water soluble polymers of considerable size ($r_g \sim 20$ nm), whereas classical lamellar phases are unable to do so or will take up only small amounts. Thus Helfrich lamellar phases may constitute another ideal medium for studying biological macromolecules under partial alignment. Previously, Prosser et al. used a 2% aqueous solution of CPCl/hexanol (1/1) in 200 mM NaCl and demonstrated that residual dipolar couplings up to 15 Hz could be measured on ubiquitin.

Here, we report on a liquid crystalline medium based on cetylpyridinium bromide (CPBr)/hexanol/NaBr which is stable in a variety of buffers and over a wide pH and temperature range. In contrast to the chloride system, only small amounts of salt are necessary to stabilize the lyotropic liquid crystalline phase. We discuss practical considerations with respect to sample preparation and characterize the phase for commonly used conditions in biomolecular NMR. In addition, we demonstrate the utility of this phase for measuring ^{1}H-^{15}N dipolar couplings for the GB1 domain of protein G and compare these to previously obtained data in liquid crystalline phases of lipid bicelles and rod-shaped viruses (Clore et al., 1998).

Experimental

Chemicals

Cetylpyridinium bromide (monohydrate, 98%) and NaBr (99.99+%) were purchased from Aldrich Chemical Co. and used without further purification. Hexanol and all other reagents used for buffer preparations were of the highest purity commercially available.

Uniformly ^{15}N labeled GB1 was prepared as described previously (Gronenborn et al., 1991).

Liquid crystal preparation

The most efficient and consistent procedure for sample preparation consisted of weighing the appropriate amounts of CPBr and hexanol in varying proportions with subsequent additions of a stock solution of salt and H_2O. In total, approximately 400 samples were prepared for probing selected regions of the phase diagram and exploring particular buffer and concentration windows more thoroughly. The final samples used for NMR measurements were prepared from a 6.5% (w/v) stock solution of liquid crystalline phase (CPBr/hexanol; 1/1.33; w/w), followed by the addition of aliquots of stock solutions of 1 M NaBr, appropriate buffer, D_2O and H_2O. The following buffers were used in the present study: Tris-HBr (pH 8.1), phosphate (pH 5.7), acetate (pH 4.2), formate (pH 4.2) and glycine-HBr (pH 3.2 and 2.2). HBr and NaOH were used to make minor pH adjustments. The tubes containing the stock solution were tightly sealed, vortexed, heated to 70 °C until clearance occurred and left to cool to room temperature. After centrifugation at 4000 × g for 2 min the stocks were kept at room temperature and remained stable (no phase separation) for weeks. Dilutions of these liquid crystal stock solutions were performed with a solution containing NaBr, buffer and D_2O at the appropriate concentrations. Diluted samples were handled in 1.5 ml screw cap micro centrifuge tubes, gently mixed and centrifuged at 4000 × g. Any excess of hexanol, observed as a thin upper layer, was carefully removed with a pipette tip. The above procedure ensured absolute reproducibility of all samples. If single samples of liquid crystal solutions are desired, it is possible to simply weigh the appropriate amounts and go through a single mixing, heating and centrifugation cycle.

For the protein-containing sample, a 6.5% stock liquid crystal solution in the desired buffer was mixed with a solution of GB1 in 10 mM glycine-HBr buffer, pH 3.0, 25 mM NaBr. The final concentration was 0.5 mM for the protein and 5% for the liquid crystalline phase.

Samples were pre-aligned for a period of 6 h or overnight underneath the magnet on the probe connector plate to take advantage of the relatively strong magnetic field in this position. Samples prepared in this manner remained aligned on the bench for 1–2 days.

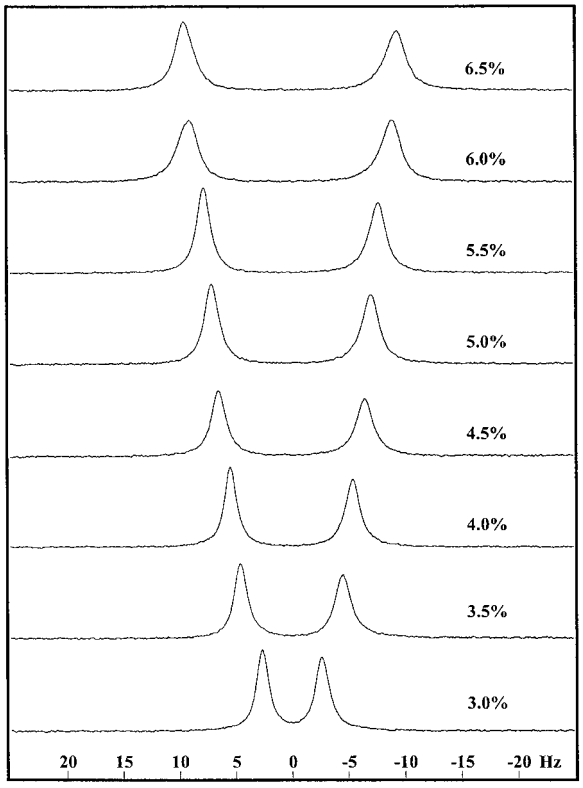

Figure 1. 1D ^{2}H spectra measuring the solvent D_2O quadrupole splitting of CPBr/hexanol (1/1.33; w/w) samples containing increasing amounts of surfactant/co-surfactant in 30 mM NaBr, 90% H_2O/10% D_2O, pH 5.5. All spectra were recorded with 1 scan and a sweep width of 153.8 Hz at 25 °C.

Optical microscopy

A Nikon AFX-IIA optical microscope with a camera attachment was used for observing optical birefringence between crossed polarizers.

NMR

All NMR spectra were recorded on a Bruker DMX500 spectrometer equipped with an x,y,z-shielded gradient triple resonance probe. ^{2}H spectra were acquired with the deuterium field frequency lock turned off. Prealigned samples were equilibrated in the spectrometer for ca. 15 min before measuring the quadrupolar splitting. For the temperature dependence, a 15 min equilibration period was interleaved between successive temperatures. The ^{1}H-^{15}N couplings ($^1J_{NH}$ + $^1D_{NH}$) were measured from ^{15}N,^{1}H-HSQC spectra with no decoupling in the ^{15}N dimension. Dipolar couplings for GB1 were obtained by calculating the difference in the $^1J_{NH}$ splittings measured in isotropic and liquid crystalline media. The error in the measured $^1D_{NH}$ values ranges from 0.2 to 0.4 Hz.

Results and discussion

In order to assess the suitability of amphiphilic aggregates comprising two or more components as media for partially aligning macromolecules, we evaluated the available phase diagrams for the quasi-ternary systems surfactant/alcohol/brine (Porte and Appell, 1981; Benton and Miller, 1983; Ben-Shaul et al., 1986; Porte et al., 1986; Gomati et al., 1987; McGrath, 1997). Those phase diagrams are extremely complex and the morphology of aggregates formed by the assembly of surfactants as well as the changes in morphology and their evolution with additives such as co-surfactants exhibit dramatic diversity. For most systems, complete phase diagrams are not available, but some general patterns of phase behavior have been established. Under dilute aqueous saline conditions it was found that CPCl micelles remain small and globular, while CPBr micelles grow into large flexible cylinders with increasing salt concentration. (Porte et al., 1980, 1986; Porte and Appell, 1981). Addition of alcohol promotes the formation of lyotropic nematic phases of small, anisotropic rod- or disk-like micelles or induces a transition to a lamellar phase. Increasing concentrations of surfactants yield a sponge phase. In agreement with the report by Prosser et al. (1998), we also observed a lyotropic liquid crystalline (LLC) phase consisting of 2–6% CPCl/hexanol/200 mM NaCl, which exhibited alignment in the magnetic field. We focussed on the CPBr system, since long, rod-like, flexible micelles had been reported (Porte et al., 1980) and we wanted to exploit the larger aggregates. In addition, we found that the CPBr/hexanol/NaBr system permitted significantly lower salt concentrations, namely 10–40 mM instead of 200–500 mM, which seemed more desirable for NMR purposes.

As for other alignment media, the alignment of the CPBr/hexanol/NaBr phases can be established from the residual orientation of water deuterons in a H_2O/D_2O mixture and is evidenced by a detectable quadrupolar splitting of the deuterium resonance which arises from exchange between bound water molecules on the surface of the bilayer and bulk water. Figure 1 shows the ^{2}H NMR spectra of a 90% H_2O/10% D_2O solution containing increasing amounts of CPBr/hexanol (1/1.33; w/w) in 30 mM NaBr. The size of the splitting is indepen-

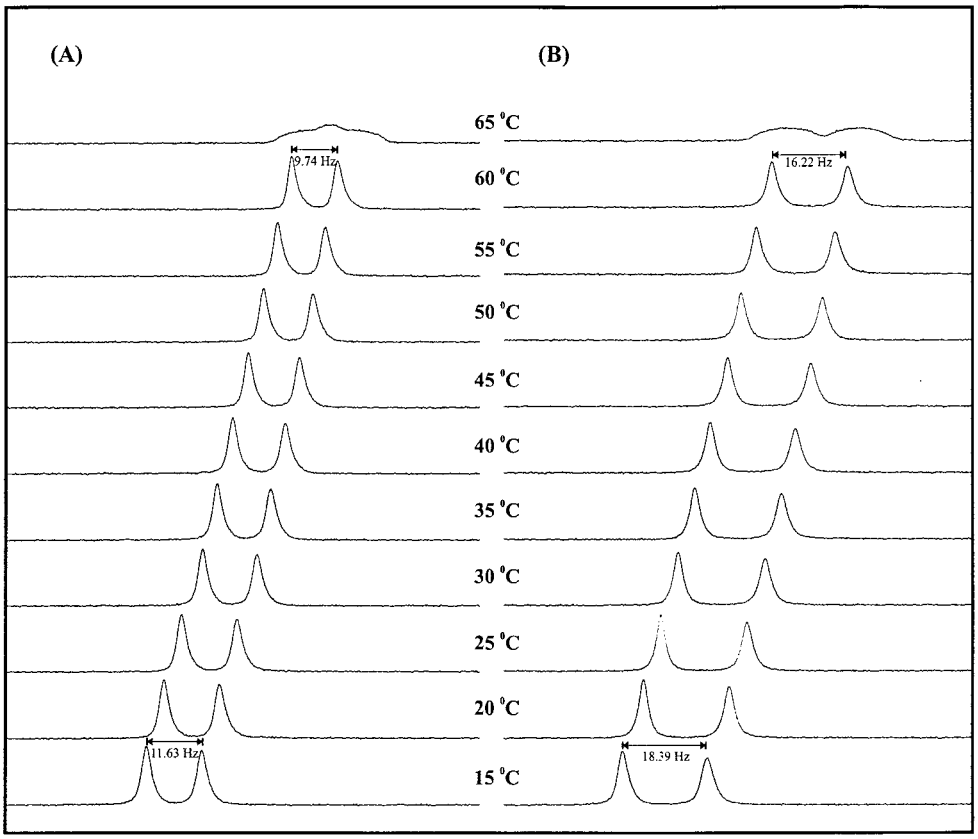

Figure 2. Temperature dependence of the ^2H spectra of a 4% (A) and 6% (B) solution of CPBr/hexanol (1/1.33; w/w) in 10 mM sodium phosphate buffer, pH 5.7, 25 mM NaBr in 90% H_2O/10% D_2O.

Table 1. Conditions probed for liquid crystalline surfactant phases formed by the quasi-ternary system cetylpyridinium bromide, hexanol, NaBr in water

Concentration (%) (w/v)	CPBr/hexanol (w/w)	Salt (NaBr) (mM)	^2H quadrupolar splitting (Hz)	Birefringent	Phases observed
1–10	1:0.5–1:10	50–200	–	No	Biphasic/triphasic/turbid/viscous/salted-out crystals
1–10	1:0.5–1:1	5–30	–	No	Homogenous, clear and opalescent/turbid/viscous/ emulsions/gels
1–10	1:1.67–1:10	5–30	–	No	Homogenous, opalescent/biphasic/turbid/emulsions
1–10	1:1.33	5–20	0–5	No	Homogenous, clear and opalescent/biphasic/turbid/ emulsions
1–2.5	1:1.33	25–30	0–3	No	Homogenous, milky and opalescent
3–3.5	1:1.33	25–30	4–8	No	Homogenous, opalescent
4–6.5	1:1.33	25–30	9–21	Yes	Homogenous, opalescent[a]
7–10	1:1.33	25–30	n.d.	Yes	Biphasic

[a]Putative lamellar phase. This phase is stable over the temperature range 15–60 °C (see Figure 2) and in the presence of commonly used buffers at various pH values (see Figure 3). If phosphate buffer (10 mM) is used, this putative lamellar phase is observed at salt concentrations of 20–25 mM.

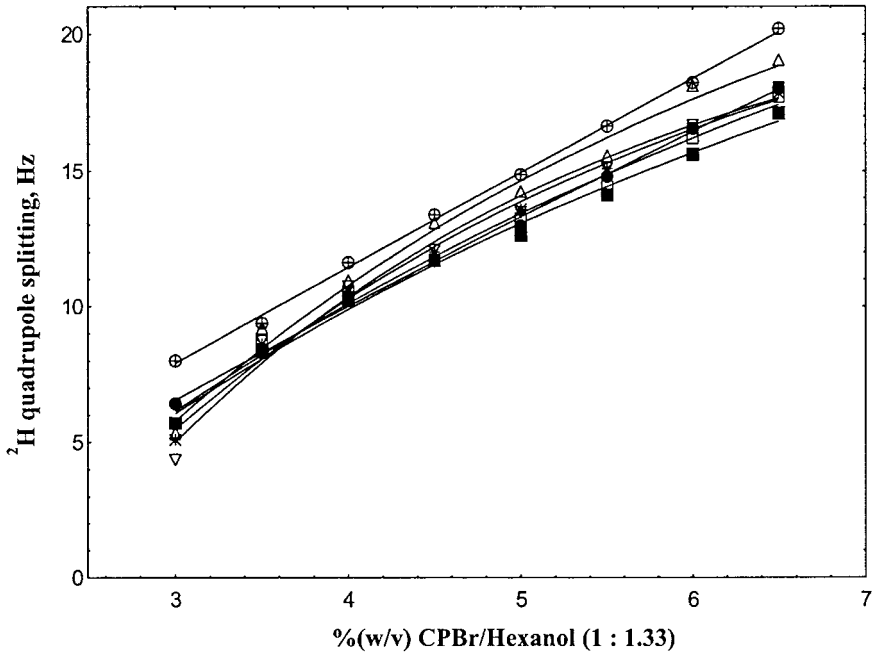

Figure 3. Plot of the ^{2}H quadrupole splittings as a function of surfactant/co-surfactant concentration for different buffers and pH values. △ 30 mM NaBr, pH 5.5, no buffer; ■ 10 mM glycine-HBr buffer, pH 2.2, 30 mM NaBr; □ 10 mM glycine-HBr buffer, pH 3.2, 30 mM NaBr; ▽ 10 mM acetate buffer, pH 4.2, 30 mM NaBr; ✳ 10 mM formate buffer, pH 4.2, 30 mM NaBr; ⊕ 10 mM phosphate buffer, pH 5.7, 25 mM NaBr; ● 10 mM Tris-HBr buffer, pH 8.1, 30 mM NaBr.

dent of the magnetic field strength (tested for 11.7, 17.6 and 18.8 T), thus full order is achieved already at 500 MHz (^{1}H frequency). The observed quadrupole splitting varies approximately linearly with surfactant/co-surfactant concentration in a similar manner as has been observed for phospholipid bicelles and rod-shaped viruses. Thus, the degree of ordering can be adjusted by changing the concentration of medium. In contrast to the liquid crystalline phase formed by phospholipid bicelles, which is thermotropic, i.e. exists over a fairly narrow temperature range, the lyotropic liquid crystalline phase consisting of CPBr/hexanol/NaBr is stable over a wide temperature interval. This is illustrated in Figure 2. In this regard, the LLC phase behaves similar to the liquid crystalline phases which form spontaneously upon concentration of biological rod-like particles such as tobacco mosaic virus (TMV) or filamentous bacteriophages. As is evident from the data in Figure 2, only above 60 °C does the quadrupolar splitting coalesce, indicating the breakdown of this unique liquid crystalline phase. The transition is completely reversible and upon cooling down, identical splittings are observed. Visual inspection of the sample at temperatures above 65 °C revealed isotropic clearance of the mixtures.

One important aspect to consider in evaluating new liquid crystalline phases for their use as media to magnetically align biological molecules with the purpose of determining dipolar couplings is their ability to persist over a wide range of different experimental conditions. We therefore tested not only different percentages of CPBr/hexanol, but also varied the ratio of surfactant to co-surfactant. Table 1 lists a large number of conditions that were examined. For CPBr/hexanol ratios from 1/0.5 to 1/10 (w/w) under high salt conditions (50–1200 mM NaBr), a variety of multiphasic behavior was observed and no birefringence was apparent. A window of CPBr/hexanol ratios from 1/1.2 to 1/1.4 and using lower amounts of salt appeared to be more favorable for liquid crystal formation, yielding measurable ^{2}H quadrupolar splittings. Optimization of the CPBr/hexanol ratio and the salt concentration led to a final ratio of 1/1.33 (w/w) and 25–30 mM NaBr for consistent observation of birefringence and quadrupolar splittings. The most suitable concentration of the liquid crystalline material was found to lie between 2.5% and 6.5%, with lower concentrations yielding too little splitting and higher

Table 2. Alignment tensor for GB1 and rms between observed and calculated residual dipolar couplings, $^1D_{NH}$, in different liquid crystalline media[a]

Liquid crystal	α (degree)	β (degree)	γ (degree)	D_A (Hz)	R[b]	Rms (Hz)[c]	Number of D_{NH}
Lamellar pH 3.0 (5% w/v)	75.9	−27.8	51.8	8.3	0.31	1.41	51
Fd pH 5.4 (∼28 mg/ml)	70.8	−20.4	54.7	−7.7	0.64	1.49	48
Bicelles pH 7.0 (5%)	67.0	89.5	17.5	−9.6	0.24	1.38	49

[a]The Euler angles α, β and γ define the alignment tensor relative to the coordinate frame of the 1.1 Å X-ray structure.
[b]R, rhombicity defined by D_R/D_A, with D_A and D_R representing the axial and rhombic components of the tensor **D**.
[c]Rms difference between the observed and calculated values of $^1D_{NH}$ based on the known orientations of the NH vectors in the X-ray structure.

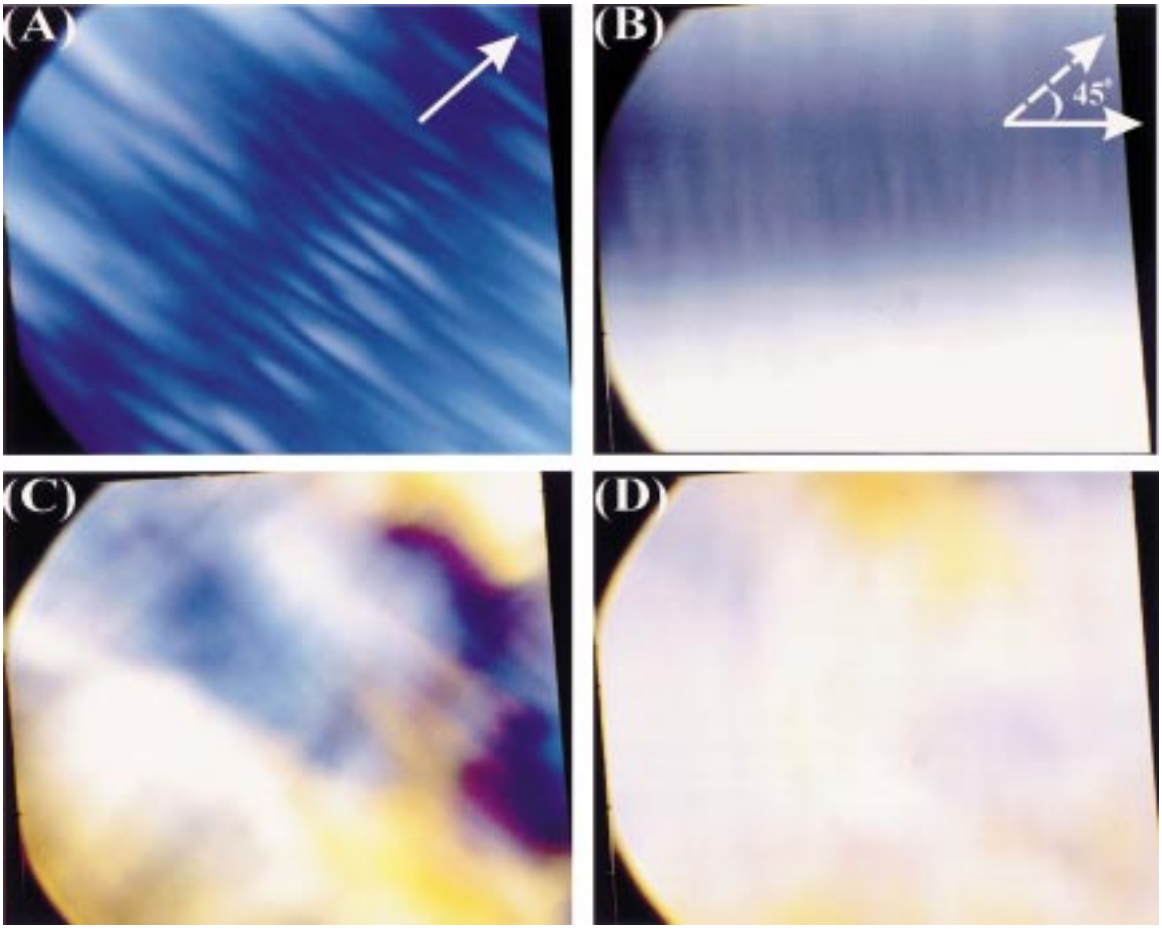

Figure 4. Composite photograph of the LLC phase formed by 6.5% CPBr/hexanol (1/1.33; w/w) in 10 mM phosphate buffer, pH 5.7, 25 mM NaBr, 90% H_2O/10% D_2O in a 5 mm NMR tube. (A) and (B) are two views of the same sample with the polarizing lenses oriented at 90° and 45°, respectively. This sample was aligned in the magnetic field (11.7 T) for 30 min and the solid white arrow marks the long axis of the NMR tube. (C) and (D) are views of a sample of identical composition to (A, B) which has never been subjected to a high magnetic field.

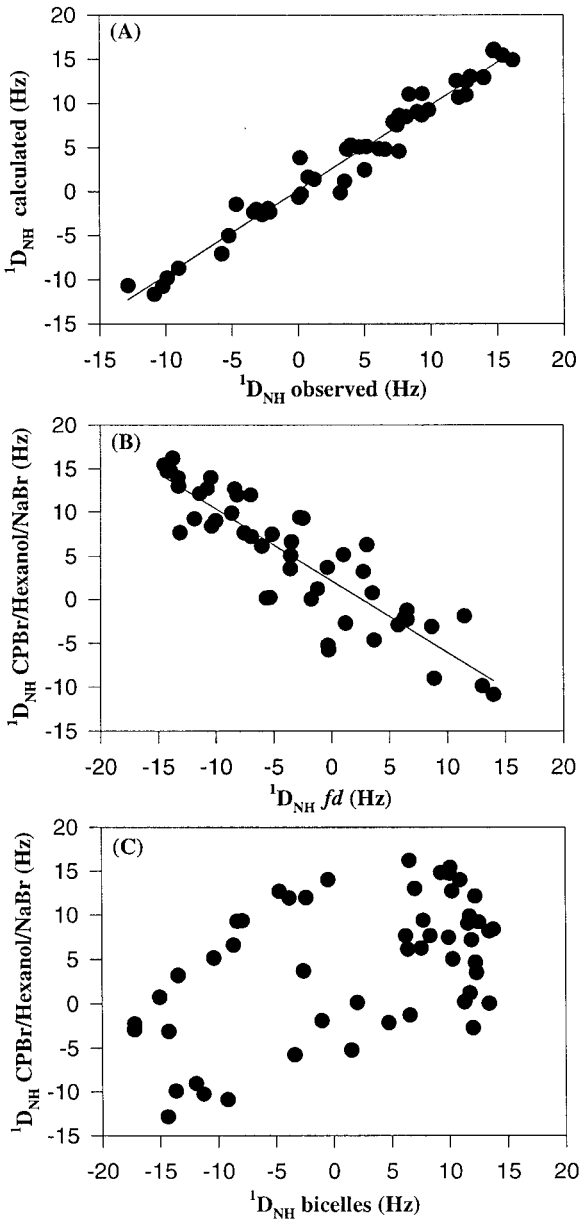

Figure 5. (A) Correlation between the measured residual ^1H-^{15}N dipolar couplings for GB1 versus values predicted from the 1.1 Å crystal structure using the magnitude and orientation of the alignment tensor given in Table 2. The correlation coefficient is 0.97. (B) Correlation between experimental values of $^1D_{NH}$ measured in the LLC phase formed by 5% CPBr/hexanol (1/1.33; w/w) in 10 mM glycine-HBr buffer, pH 3.0, 10 mM NaBr, 90% H_2O/10% D_2O versus those obtained in the nematic phase of fd (~28 mg/ml; in 50 mM sodium phosphate buffer, pH 5.4, 90% H_2O/10% D_2O). The correlation coefficient is 0.78. (C) Correlation between experimental values of $^1D_{NH}$ measured in the LLC phase formed by 5% CPBr/hexanol (1.33/1; w/w) in 10 mM glycine-HBr buffer, pH 3.0, 10 mM NaBr, 90% H_2O/10% D_2O versus those obtained in a 5% liquid crystalline bicelle solution (DMPC/DHPC; 3/1 in 90% H_2O/10% D_2O).

concentrations (> 7%) resulting in turbid, emulsion-like samples. In addition to the basic variations in surfactant/co-surfactant ratio and salt concentration, different buffer solutions, varying in composition as well as pH values were evaluated. Figure 3 illustrates the effects on the ^2H quadrupolar splitting for a variety of buffers. It is obvious from the data shown that only very small effects are observed for widely differing pH values (2.2–8.1) and chemical composition (glycine-HBr, acetate, formate, phosphate and Tris-HBr buffers). The marginally larger values observed for the phosphate buffer sample most likely arises from the slightly reduced ionic strength in this particular sample (25 mM NaBr versus 30 mM NaBr). Similar to the colloidal suspensions of rod-like viruses, which have been shown to exhibit transient magnetic birefringence below the concentration at which the liquid crystalline phase appears (Nakamura and Okano, 1983), the liquid crystalline phase formed by dilute aqueous CPBr/hexanol/NaBr mixtures seems to require magnetic fields for optimal ordering at the concentrations reported here. Two lines of evidence support this notion. First, the observed quadrupole splitting reaches its final value only after equilibration for about 90 min after initial placement of the mixtures into the magnetic field. This time requirement is independent of concentration, at least for the range studied here (3–6.5%). Second, the magnetically induced birefringence, which results in bright color effects when viewed through crossed polarizers, changes upon mixing when the sample is removed from the magnet. Likewise, solutions which have never been placed into the magnetic field show significantly less ordered birefringence when viewed through the polarizing microscope. Figure 4 demonstrates the difference in birefringence for a 'non aligned' and a 'magnetically aligned' sample of a CPBr/hexanol/NaBr LLC phase in phosphate buffer. The composite photograph shows views of NMR tubes containing the phase with polarizing lenses at 90° (A, C) and 45° (B, D). The sample shown in (A) and (B) was placed in the magnetic field prior to being photographed, whereas the sample shown in (C) and (D) is identical in composition but was never subjected to a magnetic field. As can be appreciated, the uniform blue color in (A) disappears when the polarizers are at 45° (B), whereas for the sample which had not been subjected to the magnetic field, patches of blue color in (C) appear a different color in (D). We interpret these effects to indicate partial ordering in the 'non aligned' sample in different areas of the NMR tube, whereas continuous ordering

is present in the aligned sample. It is interesting to note the macroscopically visible layers perpendicular to the long axis (white arrow) of the NMR tube in (A). At the present time, however, we have no explanation as to their origin or which morphological structures are causing this effect.

The alignment of a molecule in liquid crystalline media can be derived from the residual dipolar couplings between pairs of nuclei and, for directly bonded nuclei, is approximately distance independent. If the structure of the protein is known, the direction of the alignment is calculated using a global fitting procedure (Tjandra et al., 1996). The experimental values were measured for a 0.5 mM protein solution in 5% LLC media of CPBr/hexanol in 10 mM glycine-HBr buffer, pH 3.0, 25 mM NaBr at 25 °C. The measured values range from −14 to +18 Hz and Figure 5A shows the correlation between experimental values of the residual dipolar couplings, $^1D_{NH}$, and those calculated from the 1.1 Å crystal structure of GB1 (Derrick and Wrigley, 1994) using the equation of Tjandra and Bax (1997). Good agreement between the experimental and calculated values is observed (correlation coefficient = 0.98). Also shown are the correlations between measured $^1D_{NH}$ values in the present medium and previously characterized alignment media, namely fd (Figure 5B) and bicelles (Figure 5C). It is interesting that a surprisingly good anticorrelation between $^1D_{NH}$ values in the present surfactant LLC phase and the rod-like phage nematic phase exists (correlation coefficient = 0.88), although the rhombicity is quite different. Figure 5C compares the ^{15}N-^1H residual dipolar couplings measured in the present medium and regular DMPC/DHPC bicelles. In this case there appears to be a genuine difference in the rhombicity of the alignment tensor as well as its orientation. A summary of the magnitude and the orientation of the tensor for the three different alignment media is provided in Table 2. It appears that phage or virus suspensions are similar, yet complementary to the present quasi-ternary surfactant system. In particular, phage suspensions are best suited to work at or above pH values of their isoelectric points (pH > 5 for fd), whereas the present LLC phase is ideally suited to work at low pH values. Both systems thus would provide independent information to that provided by the different bicelle systems.

A large variety of different submicroscopic structures and supramolecular aggregates formed by surfactant molecules have been described and discussed extensively in the colloid literature (Hoffmann, 1994). With increasing amounts of co-surfactant the initially formed micelles, which exhibit strongly curved interfaces, develop into systems with planar interfaces towards those with interfaces curved away from the micellar core. As a consequence, these systems undergo several macroscopic phase transitions in dilute solutions, and up to 11 different phases have been reported (Hoffmann, 1994). In addition, the dramatic consequences of ionic charges are well known. The simple picture that bilayers in aqueous solution are principally stabilized by the competition between hydration, van der Waals forces and electrostatic interactions may not always be sufficient to describe the observed experimental phases and structures. Indeed, Helfrich lamellar phases constitute examples of this 'non classical' type. Whether the present phase is such a Helfrich phase or a liquid crystal phase formed by cylindrical, rod-like elongated micelles will require further experimental characterization. From a practical standpoint, however, the present system adds another avenue to partially align biomolecules and exploit residual dipolar couplings for structure refinement.

Acknowledgements

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