Alignment of Biological Macromolecules in Novel Nonionic Liquid Crystalline Media for NMR Experiments

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Abstract: *n*-Alkyl-poly(ethylene glycol)/*n*-alkyl alcohol and glucopone/*n*-hexanol mixtures are shown to form dilute liquid crystalline phases in aqueous solution which are suitable for partial alignment of biological macromolecules in a magnetic field. The constituent compounds are commercially available and inexpensive. The poly(ethylene glycol)-based systems are uncharged and thus insensitive with respect to pH, little sensitive with respect to salt, and tolerant against high protein concentrations. They are demonstrated to be suitable for measurements of residual dipolar couplings in proteins, DNA, and a protein/DNA complex. The stability range of the lamellar phase with respect to temperature was explored for different mixtures and guidelines are provided for the use of these lyotropic systems in NMR spectroscopy.

Introduction

Residual dipolar couplings measured in dilute liquid crystalline phase provide important restraints for molecular structure determinations by NMR[1] spectroscopy which cannot be obtained otherwise.[2] Several different liquid crystal media have been proposed to achieve the necessary partial alignment of the molecules with respect to the magnetic field, including phospholipid bicelles composed of mixtures of DMPC/DHPC[2] or DIODPC/CHAPSO,[3] and lamellar or nematic phases made of cetylpyridinium chloride/hexanol mixtures,[4] filamentous phages,[5,6] or purple membrane fragments[7,8] in aqueous solution. None of these media, however, is universally applicable. DMPC/DHPC mixtures suffer from irreversible degradation at low pH due to hydrolysis,[9] DIODPC/CHAPSO mixtures are more stable at low pH but do not form a stable liquid crystalline phase at neutral pH,[3] cetylpyridinium chloride/hexanol requires high salt concentrations for formation of a stable phase[4] which is destabilized by high protein concentrations, cetylpyridinium bromide/hexanol seems to work best under low ionic strength conditions,[10] phages work well at neutral pH but tend to aggregate below pH 6,[5] and purple membrane fragments bind proteins rather tightly, resulting in line broadening and orientation tensors which do not merely reflect the shape of the solute molecule.[7,8] Here we propose the application of an alternative set of liquid crystalline media composed of *n*-alkyl-poly(ethylene glycol)/*n*-alkyl alcohol mixtures.[11−13] These media are uncharged, insensitive to pH, fairly insensitive to salt, feature little or no binding capacity for macromolecules, and can be used at temperatures below 0 °C up to almost 40 °C, covering the temperature range of interest for most NMR studies of biological macromolecules. In addition, the technical product "glucopone", which contains different *n*-alkylated carbohydrates, is shown to form a lamellar phase in mixture with *n*-hexanol which is suitable for the measurement of residual dipolar couplings.[14]

Different alkyl-poly(ethylene glycol) molecules are denoted as C*m*E*n*, where *m* is the number of carbons in the *n*-alkyl group and *n* is the number of glycol units in the poly(ethylene glycol) moiety.[15] Phase diagrams have been reported for ternary mixtures of C12E5, *n*-hexanol, and H_2O[11,13,16] and mixtures of C8E5, *n*-octanol, and H_2O.[12] In the present work, we also explored mixtures of C12E6, *n*-hexanol, and H_2O. Under certain conditions, all these systems form a lyotropic liquid crystalline phase referred to as L_α, where the hydrophobic *n*-alkyl chains aggregate into planar bilayers with the hydrophilic poly(ethylene glycol) headgroups directed into the water phase. The L_α phase is optically clear with slight opalescence, forming a lamellar-like superstructure where the spacing of the stacked bilayers

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(1) Abbreviations: ArgR-N, N-terminal domain (residues 1−78) of the *E. coli* arginine repressor; BPTI, bovine pancreatic trypsin inhibitor; C8E5, *n*-octyl-penta(ethylene glycol); C12E5, *n*-dodecyl-penta(ethylene glycol); C12E6, *n*-dodecyl-hexa(ethylene glycol); CHAPSO, 3-(chloramidopropyl)-dimethylammonio-2-hydroxyl-1-propane sulfonate; DHPC, 1,2-di-hexanoyl-*sn*-glycero-3-phosphocholine; DIODPC, 1,2-di-O-dodecyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-di-tetradecanoyl-*sn*-glycero-3-phosphocholine; PEG, poly(ethylene glycol), PEG2000-PE, 1,2-di-myristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000].

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and thus the degree of alignment of guest molecules in the water-rich layers can be tuned by the surfactant/water ratio. The spacing can range from several hundred nanometers (1 wt %) to a few nanometers in the more concentrated regime (>5 wt %). In a magnet, the bilayer surfaces are oriented parallel with respect to the direction of the magnetic field,[17] presumably with a superstructure in which the bilayers bend into a set of concentric tubes of different radii with the axis aligned along the magnetic field.[18] At high surfactant concentration (>50%) and high temperatures (>50 °C), liquid crystalline phases are already formed by binary mixtures without alcohol. The addition of *n*-alkyl alcohols lowers the temperature range of stability for the liquid crystalline phase and, most important, makes the L_α phase accessible even at low surfactant concentrations.[11]

The bilayers formed by C12E5/*n*-hexanol, C8E5/*n*-octanol, and C12E6/*n*-hexanol carry an effective poly(ethylene glycol) (PEG) coating that provides a means of minimizing protein adsorption, in the same way as PEG-derivatized surfaces are suitable for protein chromatography[19] and enhanced biocompatibility of implants,[20,21] and PEG is used as a cosolvent in protein crystallization.[22,23] Interestingly, PEG groups can also physically stabilize bilayers, as the addition of 1% PEG2000-PE increases the stability of DMPC/DHPC bicelles at surfactant/H_2O ratios above 10 wt %.[24] In the present work, we have explored the stability range of the L_α phase formed by different C*m*E*n*/alcohol systems in the concentration range of 3−8 wt %, where residual dipolar couplings are easily measured for one-bond couplings in proteins, without resulting in excessive line broadening due to $^1H-^1H$ couplings.

Experimental Methods

Materials. C12E5 and C12E6 (≥98% purity, Fluka), C8E5 (Sigma), and *n*-hexanol and *n*-octanol (puriss., Fluka) were used without further purification. Glucopone was purchased from Fluka as a 65% aqueous solution of glucopone 215 CS UP.

L_α phases were prepared by dissolving C*m*E*n* in 90% H_2O/10% D_2O, adjusting the pH, and adding *n*-alkyl alcohol in microliter steps to the desired final molar ratio under vigorous shaking. The solutions were biphasic at low alcohol concentrations and became instantaneously transparent and opalescent upon crossing the L_α phase boundary. The L_α phase is relatively viscous, immobilizing any small air bubbles present in solution. They were readily removed by centrifugation. Despite the increased viscosity, conventional pipets worked well to transfer the liquid crystals to an NMR tube.

The composition of the final solution is reported in weight percent for the ratio C*m*E*n* to water, taking into account the presence of deuterium but not the presence of alcohol. The molar ratio of C*m*E*n* to *n*-alkyl alcohol is indicated by the factor *r*. In the glucopone/*n*-hexanol system, the surfactant concentration is indicated as wt % on a hexanol free basis, while the *n*-hexanol concentration is given on a total solution weight basis for the pure liquid crystal in pure H_2O.[14]

NMR Spectroscopy. All spectra were recorded on a Bruker DRX-500 NMR spectrometer operating at 500 MHz for ^1H and at 76.7 MHz for ^2H. One-bond $^1H-^{15}N$ and $^1H-^{13}C$ coupling constants were measured from ^{15}N- and ^{13}C-HSQC spectra recorded without decoupling during the acquisition time.

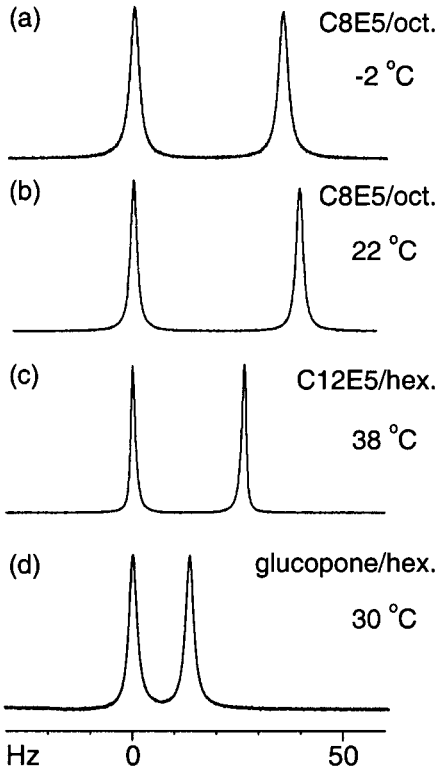

Figure 1. Quadrupolar splitting observed in the ^2H NMR spectra of the solvent in different dilute liquid crystalline systems. All samples were in 90% H_2O/10% D_2O, pH 7. The molar ratio *r* of C8E5 to *n*-octanol was 0.87 and the C8E5/water ratio was 5 wt %. For the C12E5/*n*-hexanol system, *r* was 0.96 and the C12E5/water ratio was 5 wt %. The glucopone solution was 4 wt % in water with 0.57 wt % *n*-hexanol added.

Results

The presence of an ordered lamellar phase was monitored by the observation of quadrupolar splitting of the ^2H NMR signal of the solvent (Figure 1). After placing the sample in the magnet, the quadrupolar splitting appeared within minutes. While the final splitting was typically reached in less than 15 min, the deuterium lock signal observed with liquid crystalline protein solutions continued to change over a period of hours without noticeable changes in the ^1H NMR spectrum of the solute. Establishment of stable sample conditions was slower near the lower boundary of the temperature range in which the L_α phase is stable. Quite generally, narrower ^2H signals were observed at higher temperatures.

As expected, the quadrupolar splitting observed in the ^2H NMR spectrum increased with increasing surfactant/water ratio (Figure 2a). Remarkably, the ordering effect was entirely determined by the surfactant concentration, with no significant difference between C12E5 and C12E6 or different surfactant/alcohol ratios. The splitting was temperature independent over a wide range and decreased only when the temperature was lowered toward the boundary of the stability range of the L_α phase (Figure 2b). This suggests that the lamellar phases become somewhat less ordered and less stable near the low-temperature end of their stability range, in agreement with the observation that stable ^2H NMR spectra took longer to establish under those conditions.

Figure 3 shows the temperature ranges in which lamellar phases made of different surfactant/alcohol systems were stable as judged by the appearance of a well-resolved deuterium quadrupolar splitting within less than 2 h after placing the

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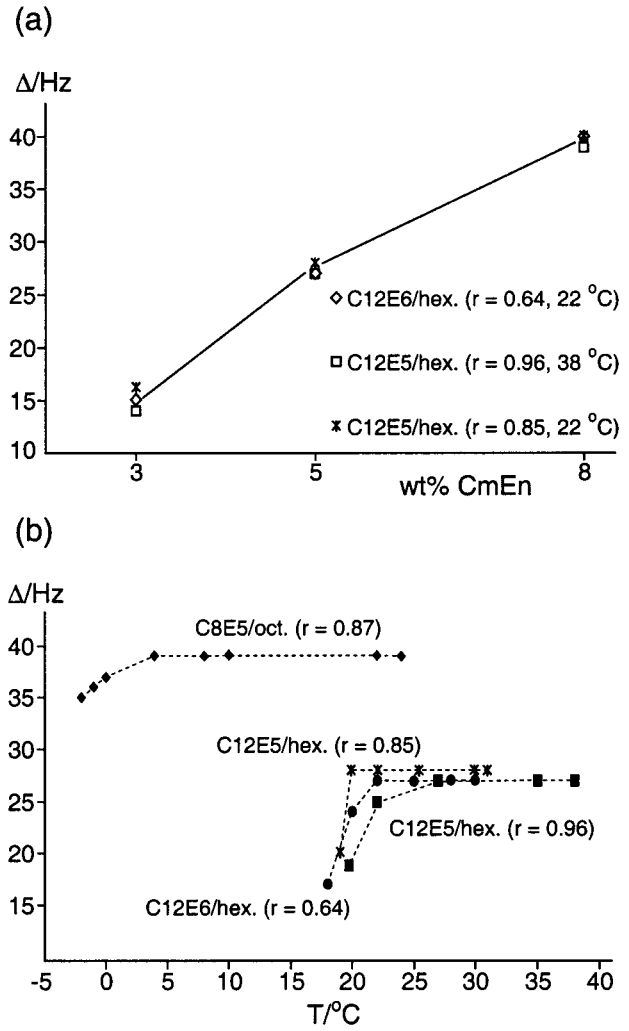

Figure 2. Quadrupolar splitting of the ^{2}H NMR signal of the solvent observed with different C*m*E*n*/alcohol systems as a function of surfactant/water ratio (a) and temperature (b). C*m*E*n*/alcohol ratios are indicated in brackets as molar ratios, *r*. The data in (a) were measured under conditions where the splitting of the ^{2}H NMR signal was insensitive with respect to temperature. The surfactant/water ratio was 5 wt % for all points shown in (b). The solutions were equilibrated in the magnetic field for 2 h before measurement.

sample in the magnet. The bars in Figure 3 correspond to conditions where the ^{2}H NMR signal was characterized by narrow line widths (Figure 1) and splittings close to the high-temperature value (Figure 2b). These criteria were found to correlate strongly with conditions suitable for biomolecular NMR. The bars do not extend to temperatures, where quadrupolar splittings were still observed, but only with broad lines of non-Lorentzian shape; under those conditions, the ^{1}H NMR spectra of dissolved proteins appeared inhomogeneously broadened too. For a given surfactant/alcohol system, the upper end of the stability range was virtually invariant at different liquid crystal concentrations, but the stability range extended to lower temperatures at higher concentrations, as evidenced by comparison of the 5% and 3% C12E5/*n*-hexanol ($r = 0.96$) systems as well as of the 8%, 5%, and 3% C12E6/*n*-hexanol ($r = 0.64$) systems. Increased amounts of alcohol decreased both the upper and lower end of the temperature range, as demonstrated by comparison between 5% C12E5/*n*-hexanol at $r = 0.96$ and 0.85. NaCl had a similar effect, except that it mostly affected the low-temperature end. Thus, 100 mM NaCl was found to extend the stability range of 5% C8E5/*n*-octanol ($r = 0.87$) to −5 °C.

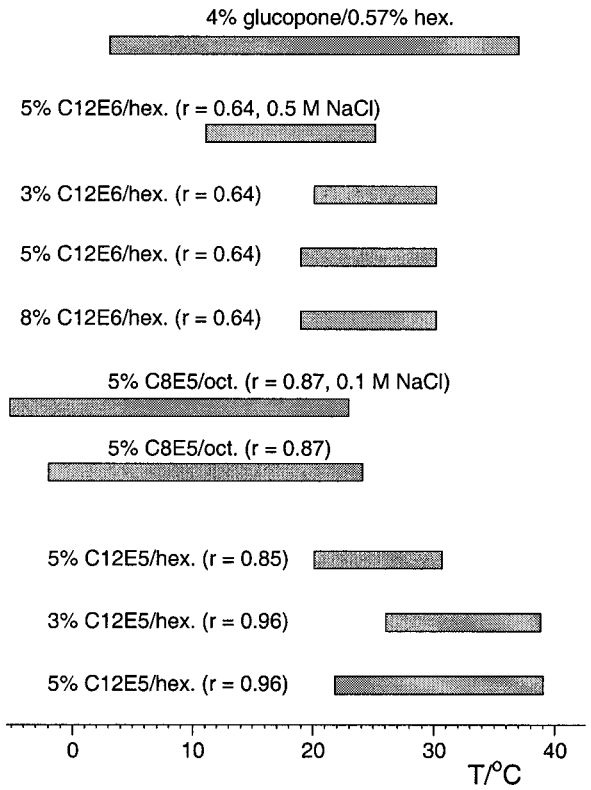

Figure 3. Temperature ranges of stable lamellar phases composed of C*m*E*n*/alcohol/H_2O and glucopone/alcohol/H_2O, respectively. The bars identify conditions suitable for biomolecular NMR spectroscopy, i.e., their borders are well within the phase boundaries of L_α. The systems are identified with the surfactant/H_2O ratio in wt %. The value *r* denotes the molar ratio of surfactant to alcohol. In the glucopone/*n*-hexanol system, the surfactant concentration is indicated as wt % on a hexanol free basis while the *n*-hexanol concentration is given on a total solution weight basis for the pure liquid crystal.[14]

500 mM NaCl did not shift that limit any further. In the case of the 5% C12E6/*n*-hexanol ($r = 0.64$) system, 500 mM NaCl significantly lowered both the upper and lower end of the stability range. Within the stability ranges indicated in Figure 3, quadrupolar splitting and line width of the ^{2}H NMR signal were insensitive to the presence of salt. Similarly, the appearance of the ^{2}H NMR spectrum did not change in the presence of 50 mM phosphate buffer and 1 mM EDTA or 1 mM BPTI. In the presence of 15 mM BPTI, however, the quadrupolar splitting observed in the 5% C12E6/*n*-hexanol ($r = 0.64$) system decreased from 26 to 21 Hz, although the ^{2}H line width remained unchanged. Most interestingly, all lamellar phases made of C*m*E*n*/alcohol mixtures were insensitive to pH, as identical ^{2}H NMR spectra were observed at pH 2, 7, and 11. The widest stability range was measured for 4% glucopone with 0.57% *n*-hexanol. This liquid crystalline medium was not stable at lower surfactant concentration, required longer to equilibrate, yielded very large residual dipolar couplings in BPTI (see below), and therefore was not explored more extensively.

The lamellar phases produced by the C*m*E*n*/alcohol systems proved to be very suitable for aligning proteins, DNA, and a protein/DNA complex. For example, the highly polar N-terminal domain of the *E. coli* arginine repressor (ArgR-N)[25] was found to aggregate with Pf1 phages and liquid crystalline phases made from DHPC/DMPC or cetylpyridinium chloride/hexanol mixtures, while residual dipolar ^{1}H−^{15}N couplings ranging from

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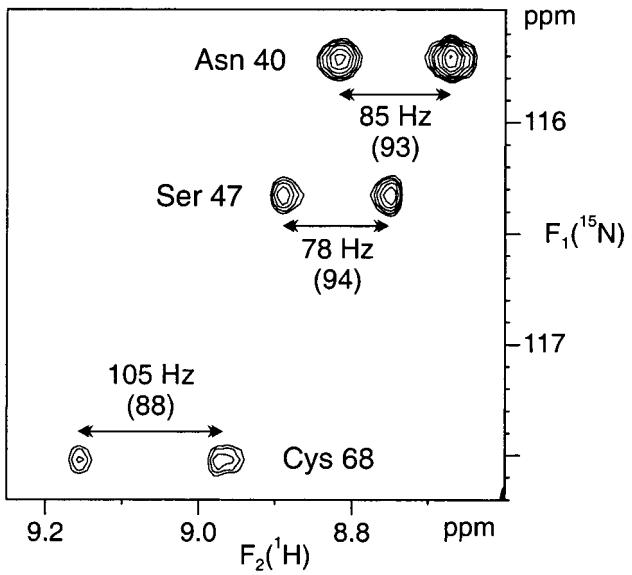

Figure 4. One-bond $^1H-^{15}N$ coupling constants measured in a ^{15}N-HSQC spectrum of a 2 mM solution of $^{15}N/^{13}C$-labeled ArgR-N in a dilute liquid crystalline phase of 5% C8E5/*n*-octanol ($r = 0.87$) at pH 5.8 and 22 °C. The spectrum was recorded without ^{15}N-decoupling in the F_2 dimension. Coupling constants measured in an isotropic solution are indicated in brackets.

−17 to 20 Hz were readily measured in a system of 5% C8E5/*n*-octanol ($r = 0.87$) at 22 °C (Figure 4). Similarly, a 2:1 complex between ArgR-N and operator DNA yielded residual dipolar $^1H-^{15}N$ couplings ranging from −10 to 18 Hz in the lamellar phase made of 5% C12E6/*n*-hexanol ($r = 0.64$) at 25 °C (P. Andersson and G. Otting, unpublished results).

The general viability of the liquid crystalline systems of Figure 3 for protein alignment was tested by measurements of residual dipolar one-bond $^1H-^{13}C$ couplings in 4 mM solutions of BPTI at pH 4.8 and 25 °C or, in the case of C8E5/*n*-octanol, 22 °C. Identical residual dipolar couplings were measured in 5% C12E5/*n*-hexanol ($r = 0.96$) and 5% C12E6/*n*-hexanol ($r = 0.64$), with maximum couplings of about 40 Hz. These two systems also displayed the same quadrupolar splitting (~27 Hz) in the ^2H NMR spectrum. Quite generally, the magnitude of the residual dipolar couplings observed in BPTI was found to scale approximately with the splitting observed in the ^2H NMR spectrum. Thus, 5% C8E5/*n*-octanol ($r = 0.87$) yielded about 1.4 times larger residual dipolar couplings in BPTI than 5% C12E6/*n*-hexanol ($r = 0.64$) (compare Figure 2b), which in turn yielded about 1.4-fold larger residual dipolar couplings than 3% C12E6/*n*-hexanol ($r = 0.64$) (compare Figure 2a). The relationship between quadrupolar splitting and residual dipolar coupling was different for the 4% glucopone/0.57% hexanol system, where residual dipolar $^1H-^{13}C$ couplings larger than 60 Hz were measured in BPTI, although the quadrupolar splitting observed in the ^2H NMR spectrum was only 13 Hz.

All lamellar phases of Figure 3 gave rise to signals in the ^1H NMR spectrum between about 1 and 4 ppm. Most of these signals were, however, very broad so that $^1H-^{13}C$ couplings could be measured at natural isotopic abundance with little interference from the liquid crystalline phases (see Supporting Information).

Discussion

C*m*E*n*/alcohol/water systems form dilute liquid crystalline phases with very attractive properties for the partial alignment of biological macromolecules. As the constituent components are uncharged, the stability of these phases is virtually unaffected by pH and little sensitive with respect to salt. Perhaps most importantly, these bilayers do not associate with charged molecules through electrostatic interactions, providing a system suitable for the alignment of almost any water-soluble macromolecule. Even high protein concentrations, e.g. 15 mM BPTI, are tolerated. Furthermore, the absence of charge means that alignment is based on molecular shape rather than charge distribution. Residual dipolar couplings measured with BPTI for 5% C12E5/*n*-hexanol ($r = 0.96$) and 5% C12E6/*n*-hexanol ($r = 0.64$) were closely comparable to those reported by Ottiger and Bax for 5% DMPC/DHPC mixtures.[9]

The systems also offer several practical advantages. The liquid crystals are easy to prepare outside the magnet, their viscosity is sufficiently low that even 10% solutions are readily transferred with a pipet, they align rapidly in a magnetic field, and they are chemically stable. Composed of simple chemicals, they are free of traces of proteases or RNAses which may occur as impurities in oriented systems made of biological material.

Protein solutions in lamellar phases of C*m*E*n*/alcohol and glucopone/alcohol were stable for weeks without noticeable changes. Whenever breakdown of the lamellar phases was observed after long storage periods at room temperature, they could be fully restored by the addition of a few microliters of *n*-alkyl alcohol. Clearly, it is critical to prevent evaporation of the alcohol because most of the systems of Figure 3 were established with the minimum amount of alcohol needed to generate a lamellar phase. As shown by the phase diagrams of C12E5/*n*-hexanol and C8E5/*n*-octanol,[12,13] L_α phases are very sensitive to the total amount of alcohol present. This sensitivity is also illustrated by the additional *n*-hexanol required for a lamellar phase with C12E6 as compared to C12E5 (Figure 3). Perhaps not unexpected, attempts to generate L_α phases using *n*-octanol instead of *n*-hexanol with C12E5 and other cross combinations between the reagents of Figure 3 failed. Quite generally, the phase diagrams show that the L_α phase is stable over a larger range of temperature and chemical composition at higher surfactant concentration.[12,13] About 3 wt % surfactant may thus be the minimum concentration that is practical for biomolecular applications.

Previously, the existence of a lamellar phase made of glucopone/hexanol/water had been reported only for a single temperature.[14] Glucopone is a large scale technical product, containing a heterogeneous mixture of *n*-alkyl mono- and disaccharides with a distribution of alkyl chain lengths and different types of hexoses, linked by α- and β-glycosidic bonds.[14] The lamellar phase prepared with *n*-hexanol displayed a few narrow resonances in the ^1H NMR spectrum between 1 and 6 ppm and one at about 8.55 ppm, which could not be removed by ultrafiltration of the sample. Heterogeneity of the sample seemed to be required for formation of the L_α phase, as we did not succeed in reconstituting a lamellar phase from 1:1 or 7:3 mixtures of α- and β-octylglucose (5% solution in water) with *n*-hexanol. The ^1H NMR spectrum of BPTI dissolved in 4% glucopone/0.57% hexanol displayed rather broad peaks, presumably due to residual dipolar $^1H-^1H$ couplings induced by strong alignment. Alignment may have been increased by electrostatic attraction between positively charged BPTI and glucopone, as glucopone micelles have been reported to be overall negatively charged between pH 3 and 9.[26]

Proteins are more difficult to recover from lamellar phases of C*m*E*n*/alcohol or glucopone/alcohol mixtures than from filamentous phages. When a solution of ArgR-N in 5% C8E5/

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n-octanol ($r = 0.87$) was cooled to 4 °C, the sample precipitated irreversibly. The protein could, however, be recovered by extensive dialysis followed by cationic exchange chromatography.

Conclusion

Dilute lamellar phases made of C*m*E*n*/alcohol and water present widely applicable systems for the partial alignment of biological macromolecules in a magnetic field. The constituent chemicals are commercially available and inexpensive compared to most other dilute liquid crystalline media currently used in biomolecular NMR spectroscopy. The new liquid crystalline phases thus open the way for residual dipolar couplings as a routine tool not only in biomolecular NMR but also in the conformational analysis of small, water-soluble, organic compounds.

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Supporting Information Available: ^{1}H NMR spectra of the dilute liquid crystals, ^{13}C-HSQC spectra of BPTI at natural abundance identifying the $^{13}C-^{1}H$ cross-peaks of the liquid crystals, and a TOCSY spectrum of a DNA fragment in 5% C12E6/*n*-hexanol ($r = 0.64$) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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