Use of a Novel Aqueous Liquid Crystalline Medium for High-Resolution NMR of Macromolecules in Solution

R. Scott Prosser,*,† J. A. Losonczi,‡ and I. V. Shiyanovskaya†

Department of Chemistry and the Liquid Crystal Institute
Kent State University, Kent, Ohio 44242
Complex Carbohydrate Research Center
University of Georgia, 220 Riverbend Rd.
Athens, Georgia 30602-4712

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This paper describes experiments which demonstrate the potential of a novel lyotropic liquid crystal to weakly align biomolecules for purposes of obtaining dipolar couplings in high-resolution NMR spectra. Until recently, three-dimensional structures of proteins and nucleic acids in solution have been derived from NMR measurements of interproton nuclear Overhauser effects (NOEs) and three-bond scalar couplings.[1] In cases where the three-dimensional structure is characterized by distinct domains (for example nonglobular proteins), long-range interproton distance measurements between such domains may be sparse and, therefore, insufficient to reliably define the entire structure. For proteins or biomolecules with sufficient magnetic susceptibility anisotropies, it is possible to obtain a small degree of alignment of the molecule in a magnetic field,[2] making possible the measurement of $^1H-^{15}N$, $^1H-^{13}C$, $^{13}C-^{13}C$, and $^1H-^1H$ dipolar couplings. These dipole couplings, which complement the above NOEs and scalar couplings, can be used to refine protein structure. The measurement of dipolar couplings in field-oriented proteins was first shown to be possible in paramagnetic systems[3] and later demonstrated in diamagnetic proteins.[4] This approach was dramatically improved when it was shown that a water soluble protein could be aligned simply by confining it to the aqueous domain of a magnetically aligned dilute liquid crystal, consisting of the phospholipids, dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC).[5a,b] The degree of alignment in this so-called dilute bicelle[5c] is typically larger than that observed in field-oriented proteins, and the resulting dipole couplings are, therefore, generally easier to measure. Currently, the main limitation to the use of dilute liquid crystals to measure dipole couplings in biomolecules is the temperature range over which the liquid crystal is observed to align. The DMPC/DHPC mixture retains alignment from roughly 35 to 45 °C although some progress has been made in extending this limit slightly by substitution of DMPC for a shorter chain amphiphile.[6] A second limitation of the existing dilute liquid crystal medium is that certain proteins may bind to the bicelle, resulting in excessive dipole couplings which become impossible to measure by high-resolution NMR techniques. Finally, dilute bicelles often precipitate after long periods of time, depending on the solute and buffer concentration. Although these instabilities can sometimes be avoided by the addition of charged amphiphiles,[7] it would clearly be desirable to employ a dilute liquid crystal which is robust in terms of sample conditions, temperature, and buffer concentration and is inert in terms of binding of aqueous membrane-associated proteins. The lyotropic liquid crystal consisting of a 2−5% (w/w) aqueous solution of an equal weight mixture of cetylpyridinium chloride and n-hexanol in 200 mM NaCl is shown in this paper to exhibit most of these features and should therefore prove to be a useful alternative to the dilute bicelle system.

The combination of cetylpyridinium chloride (CPCl) and hexanol in brine is known to result in a variety of phases including a sponge phase, an isotropic micellar phase, and a lamellar liquid crystalline phase.[8] In particular, if equal weight fractions of CPCl and hexanol are combined in 0.20 M NaCl such that the liquid crystal concentration is 1−5% (w/w), a dilute or swollen lamellar phase is expected in which the thickness of the dry lamellae is estimated to be 27 Å, whereas the corresponding water thickness is should range from 500−2700 Å.[8b] In high-resolution NMR applications, the magnetic anisotropy of the pyridinium ring is expected to dictate magnetic alignment, and the symmetry axis of the lamellae would be expected to be parallel to the applied field. By varying the water concentration and, hence, the degree of confinement of the biomolecule, a scalable degree of order can then be obtained for the biomolecule.

The dipolar coupling, D_{ij}, between two spins, i and j, may be simply expressed as

$$D_{ij} = (\xi_{ij}/r_{ij}^3)\langle A_a(3\cos^2\theta_{ij} - 1) + {}^3/_2 A_r \sin^2\theta_{ij}\cos 2\phi_{ij}\rangle \quad (1)$$

where ξ_{ij} represents an interaction constant that depends on the extent of internal motion of the vector ij and on the product of gyromagnetic ratios of the nuclei. r_{ij} is the distance between the spins, A_a and A_r represent the axial and rhombic components of the molecular alignment tensor, **A**, and θ_{ij} and ϕ_{ij} define the polar coordinates of the ij vector in the principal axis system of the alignment tensor.[9] In the absence of the aligned liquid crystal (and in situations where there is little propensity for the biomolecule to align), the above angular term is averaged to zero. The dipole couplings, obtained directly from measurements in dilute liquid crystals, are fundamentally different from those obtained via NOEs which depend on relaxation effects of the dipole interaction. If the dipole measurement involves a one-bond heteronuclear coupling, then the observed variation in D_{ij} can be used to restrict internal orientations in structure refinements. Assuming the liquid crystal confers a sufficient degree of alignment to the solute molecule, the dipole coupling can be routinely measured by standard high-resolution NMR experiments.[5] For example, a routine $^1H-^{15}N$ heteronuclear HSQC pulse sequence need only be modified by removing a ^1H refocusing pulse in the t_1 dimension, to evolve the scalar plus dipolar

* To whom correspondence should be addressed.
† Kent State University.
‡ University of Georgia.

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(9) (a) Saupe, A. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 97−112. (b) Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114. The brackets ⟨⟩ represent a motionally averaged term in eq 1.

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coupling, $J_{ij} + D_{ij}$. Since the sign and magnitude of J_{ij} is generally very well-known, the sign and magnitude of D_{ij} can be determined, as long as $|D_{ij}|$ is significantly smaller than $|J_{ij}|$.

The degree of order in the dilute CPCl/hexanol system is clearly critical to the success of the measurement of solute dipole couplings by high-resolution NMR. A straightforward means of assessing this order is to monitor the quadrupolar splittings resulting from the ^{2}H signal of ^2H_2O. A nonzero splitting results in the case of an aligned liquid crystal, since the spectrum represents a dynamic average of the bulk (isotropic) water and bound (oriented) water. Since the quadrupolar splittings in dilute bicelles are observed to be on the order of 10 Hz, the composition and concentration of the CPCl/hexanol system was therefore tailored until similar splittings were observed. The ^{2}H spectra and ^{13}C spectra (not shown) of equal weight mixtures of 1−5% CPCl/hexanol both reveal a stable phase from 0−70 °C. The CPCl/hexanol ratio could be changed within a 10% range without seriously altering the order or phase properties of the system, though it was necessary to use 0.20 M NaCl concentrations in order that the sample was transparent and robust. Increasing the water content resulted in corresponding decreases in the ^{2}H splittings. The samples were in general very easy to prepare and were observed to orient within a few minutes to hours in the magnetic field. We also carefully tested stability and robustness of these samples using a variety of solutes (0.2 mM human ubiquitin and 0.5 mM cytochrome *c*). In both cases, the appearance and corresponding ^{1}H NMR spectra of the samples were observed to be perfectly constant over periods of weeks.

The 2D slices from the ^{1}H−^{15}N HSQC spectra, contrast representative couplings of ^{15}N-labeled ubiquitin in isotropic and oriented CPCl/hexanol systems (Figure 1). The spectra clearly demonstrate the utility of the dilute CPCl/hexanol liquid crystal for high-resolution NMR measurements of dipole couplings. In the case of the isotropic solution and the dilute liquid crystal samples (2% and 4.5% w/w CPCl/hexanol), all of the observed resonances corresponded closely to published spectra, suggesting that the liquid crystal environment does not alter the structure of the protein.[5,10] Furthermore, the HSQC peaks are not observed to shift upon successive addition of liquid crystal solute, implying that there is no specific binding of the protein to the lamellar surface. Dipole couplings were determined simply by taking the difference of the observed couplings ($J_{ij} + D_{ij}$) in the dilute liquid crystal system, from the corresponding measured couplings (J_{ij}) in the isotropic sample. The magnitude of the observed dipole couplings,[11] which span 15 Hz in the 2% sample and 45 Hz in the 4.5% sample, are comparable to those reported by Bax and co-workers in the dilute bicelle system (∼30 Hz). No degradation in line widths was observed upon addition of 2% CPCl/hexanol, although only about 50% of the ($J_{ij} + D_{ij}$) couplings could be observed upon addition of 4.5% CPCl/hexanol and line widths in the ^{1}H dimension were degraded by at least a factor of 2. Parts C−E of Figure 1 clearly show the effect of increasing liquid crystal solute concentration on the observed couplings.

The particular advantage of this liquid crystal is its robust temperature range over which well-resolved high-resolution spectra of biomolecules can be obtained. This could have important applications in studies of protein folding or thermophilic proteins. The liquid crystal also boasts a biologically relevant salt concentration (200 mM) and may turn out to be more inert to biomolecules which exhibit binding or strong interactions with membranes (and thus, with bicelles). The $J + D$ dipole couplings shown in the spectra in Figure 1, illustrate the potential of this lyotropic liquid crystal system for dipole coupling measurements. In quantitative situations where it is desirable to obtain dipole couplings to high accuracy, other pulse sequences might be considered.[4b,12]

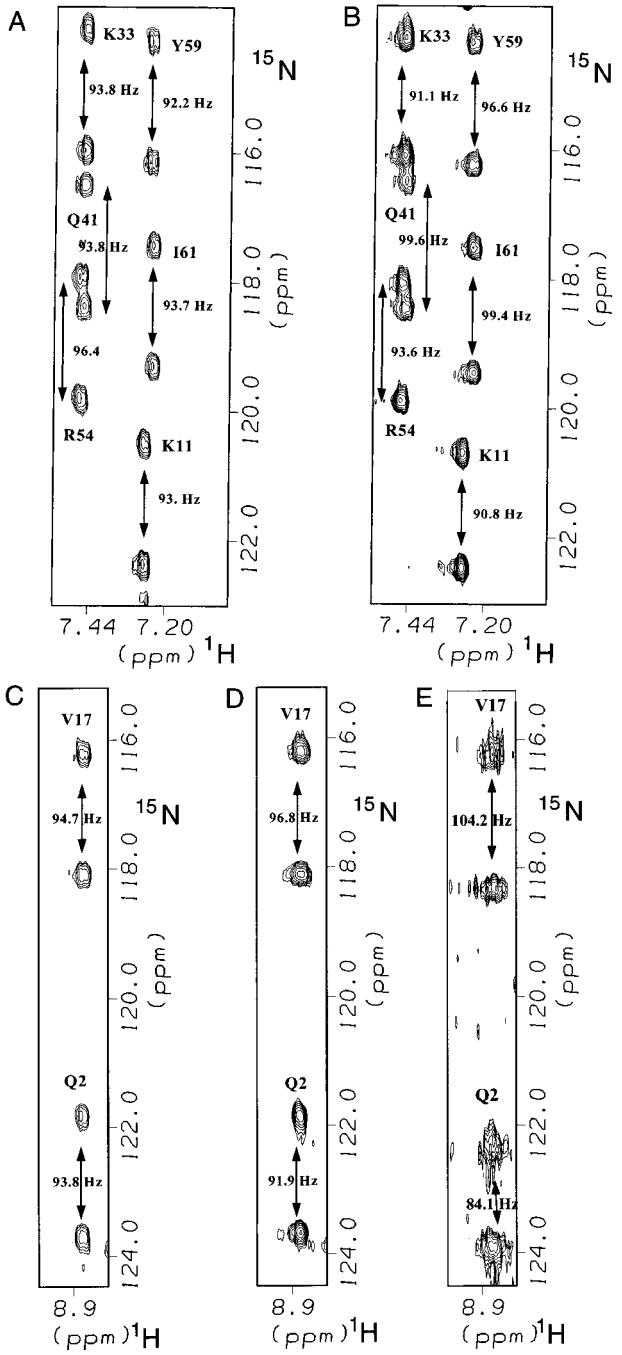

Figure 1. Representative 2D slices from ^{1}H−^{15}N HSQC spectra at 30 °C of ^{15}N-labeled human ubiquitin (0.2 mM) in an isotropic 0.20 M NaCl solution (90/10, H_2O/D_2O) (A) and in a 2% (w/w) 0.20 M NaCl solution of an equal weight mixture of CPCl and hexanol in 90/10, H_2O/D_2O (B). ($J + D$) couplings are shown for two labeled sites under pure 0.2 M NaCl (C), 2% (w/w) dilute liquid crystal (D), and 4.5% (w/w) liquid crystal (E). Spectra were acquired on a Varian Inova 500 using a standard HQSC pulse sequence in which the ^{1}H refocusing pulse was omitted in the t_1 dimension. Spectra from the isotropic and 2% samples were obtained using 40 transients, a sweep width of 1500 Hz in the indirect dimension, and 150 t_1 increments, while 80 transients and 100 increments were used in the 4.5% sample. ^{15}N-labeled human ubiquitin was obtained from VLI Research (Southeastern, PA).

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