Cellulose Crystallites: A New and Robust Liquid Crystalline Medium for the Measurement of Residual Dipolar Couplings

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Received March 2, 2000
Revised Manuscript Received April 11, 2000

The use of residual dipolar couplings has revolutionized the refinement of NMR-derived solution structures of biological macromolecules,[1] particularly in circumstances where distance information, obtainable from nuclear Overhauser effect (NOE) data, is sparse. Moreover, dipolar couplings provide long-range structural information that is not available by measuring distances between adjacent atoms. Particularly powerful applications of these include structural studies of multidomain proteins, macromolecular complexes, and deuterated molecules.

Residual dipolar couplings can be measured if a weak molecular alignment is achieved in the presence of the magnetic field. Some macromolecules, for example, DNA, have sufficient magnetic susceptibility anisotropy that a small amount of alignment is inherent during the NMR experiment.[2] More generally, it is necessary to induce additional alignment by dissolving the sample in a medium that becomes highly ordered when placed within a magnetic field. A small amount of alignment is transmitted to the molecule of interest via steric or interactional forces, often producing residual dipolar couplings that are both tunable and large enough to be accurately measured for structure calculation. A general expression for the residual dipolar coupling between nuclei X and Y (D_{XY}) is given by:

$$D_{XY} = S\gamma_X\gamma_Y[A_{axial}(3\cos^2\theta - 1) + {}^3/_2 A_{rhombic}(\sin^2\theta \cos 2\varphi)]/r^3_{XY}$$

S is the generalized order parameter, γ_X and γ_Y are the gyromagnetic ratios of the nuclei, r_{XY} is the internuclear distance, θ and φ describe the orientation of the vector within the coordinate system of the alignment tensor, A. The magnitude of the alignment tensor can be estimated from the distribution of measured dipolar couplings, while its orientation can be allowed to vary during the structure calculation.[3] Significant improvements in the precision and accuracy of NMR structures, supplemented with residual dipolar couplings, have been recently demonstrated.[4]

A number of suitable alignment media have been established but none have been ubiquitously applicable. Lyotropic liquid crystals composed of disk-shaped phospholipid particles, known as bicelles,[5] have been shown to be particularly useful. However, these systems have narrow temperature ranges and rely on a very delicate phase equilibrium that is often disrupted by the presence of soluble macromolecules. It has also been shown that the nematic phase of filamentous phage particles can produce a modest degree of alignment.[6] These systems tend to be charged and can often interact with oppositely charged species.[7] This can either destroy the nematic phase, removing measurable dipolar couplings or induce excessive order, which produces unacceptably broad lines. Furthermore, generating large quantities of the virus-based media can be impractical. Membrane fragments containing integral proteins have been demonstrated as viable alternatives, but appreciable interactions with the solute are also observed, and high ionic strengths are necessary to alleviate these.[8]

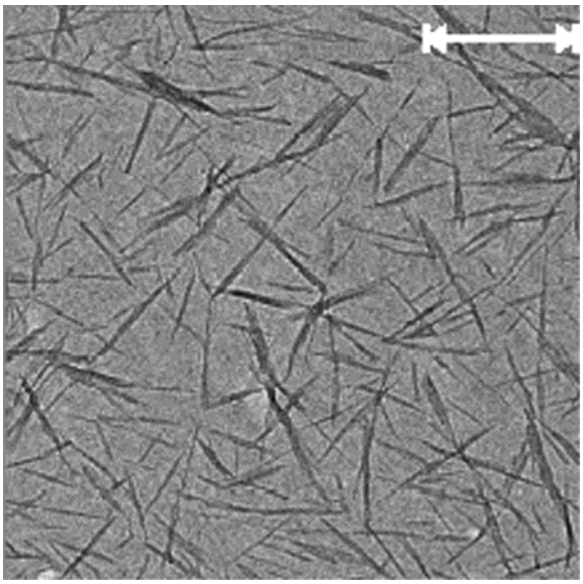

Figure 1. Positively stained electron micrograph of cotton cellulose crystallites taken from the anisotropic phase, illustrating particle dimensions. The scale bar corresponds to 400 nm.

In this paper, we demonstrate that a cellulose crystallite suspension can provide suitable alignment for the measurement of residual dipolar couplings in soluble macromolecules. Moreover, they are readily produced in large amounts, are highly stable, and show no significant binding to the soluble molecule of interest. Cellulose crystallites are prepared by careful hydrolysis of natural cellulose fibers (in this case Whatman filter paper) with sulfuric acid, followed by concentration and extensive dialysis.[9] After standing for several hours the suspension separates into an upper isotropic and a lower anisotropic phase (critical concentration >4.5%), which is free from precipitant. For NMR studies cellulose crystallites were prepared according to the literature methods.[10] Figure 1 shows an electron micrograph of a dried cellulose sample taken from the anisotropic phase; each crystallite is typically 200 ± 70 nm long by 10 nm wide, giving an aspect ratio of approximately 20:1. Individual crystallites possess a large negative diamagnetic anisotropy, which gives rise to a perpendicular alignment of their long axis relative to an applied magnetic field.[11]

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10.1021/ja000764e CCC: $19.00
Published on Web 05/12/2000

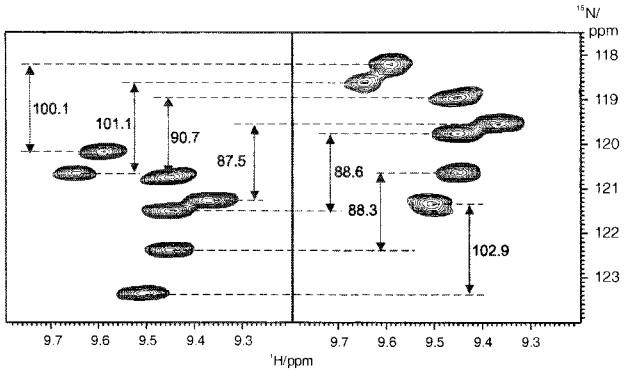

Figure 2. Representative regions of the C′ decoupled ^{1}H−^{15}N IPAP-HSQC[13] spectra for 1 mM Int190 in the anisotropic solution. Downfield (left) and upfield (right) peaks in 8% w/w cellulose crystallites, 20 mM sodium acetate, pH 5.2 at 310 K. The magnitude of the splitting is indicated in Hz.

This stands in contrast to the frequently tested bicelle and phage systems, which align parallel to the field. In an 11.7 T magnet a freshly prepared 8% w/w sample of cellulose crystallites will align fully within 4 h.[12]

The measurement of one-bond dipolar couplings is demonstrated for the 20 kDa ^{15}N/^{13}C-labeled protein, Int190. Int190 is a cell-adhesion fragment from intimin from enteropathogenic *Escherichia coli*; it has a pI of 9.1 and contains an extensive protein-binding surface.[14] Exhaustive attempts to measure residual dipolar couplings in intimin using established liquid crystalline technology have failed. This is presumably because the extreme positive charge and significant surface hydrophobicity interfere with the alignment system. Accordingly, it provides a stiff test for the applicability of cellulose crystallites. Since cellulose crystallites have a slight overall negative charge, it was envisaged that maintaining nematic phase in the presence of positively charged macromolecules might prove troublesome. Despite this, Int190 readily dissolves in the suspension up to concentrations of 2 mM, even at low pH, providing NMR spectra comparable in line-width to those obtained in isotropic aqueous solution. The addition of salt to destabilize unwanted electrostatic interactions was found to be unnecessary in the case of Int190, indicating that the soluble molecule is practically unaffected by the negative charge of the cellulose particles.[15] Moreover, there are no discernible chemical shift changes for the protein in the presence of the cellulose medium, which implies the structure remains unaffected. The robustness of cellulose crystallite suspensions has been previously ascertained; varying ionic strength and counterion type had little detrimental effect on the ability to form the liquid crystalline phase.[16]

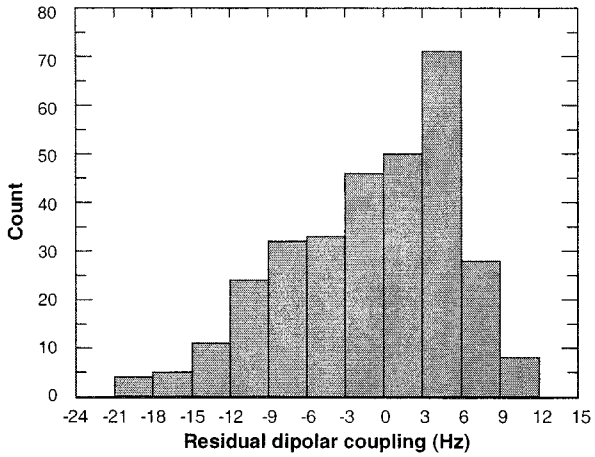

Figure 3. Histogram of $^{1}D_{NH}$ and normalized $^{1}D_{NC'}$ values measured for Int190 in 8% w/w cellulose crystallites, 90% H_2O, 10%D_2O, 20 mM sodium acetate, pH 5.2 at 310 K. ^{1}H−^{15}N IPAP-HSQC experiments with and without carbonyl decoupling were employed. Average values from duplicate experiments are presented.

Figure 2 shows a region from the C′ decoupled ^{1}H−^{15}N IPAP-HSQC[13] spectrum of ^{15}N/^{13}C-labeled Int190 in the anisotropic cellulose suspension containing 20 mM sodium acetate, pH 5.2. The alignment induced by the cellulose crystallites produces significant deviations from isotropic $^{1}J_{NH}$ values. It is also possible to adjust the magnitude, as the crystallites can be concentrated or diluted before magnetic alignment.[17] In our experience, approximately 8%w/w cellulose provides an ample range of dipolar couplings while maintaining excellent line shape. Figure 3 summarizes the distribution of one-bond dipolar couplings for well-resolved amides within Int190; $^{1}D_{NH}$ and $^{1}D_{NC'}$ (normalized with respect to $^{1}D_{NH}$). The values range from −18 to +12 Hz in the 8% w/w cellulose sample and resemble a characteristic powder pattern. In addition to the innate stability of cellulose suspensions the residual dipolar couplings remained constant for the entire measurement period, and data were reproducible six months later.

In conclusion, we demonstrate that a suspension of cellulose crystallites will induce the modest alignment necessary for the measurement of residual dipolar couplings in biological macromolecules. Furthermore, cellulose suspensions display several desirable characteristics, which are not available in any one existing system: they are readily produced in large amounts, highly stable, unperturbed by variable solution conditions and show no significant binding to soluble macromolecules. Other polymers that form liquid crystalline suspensions, for instance poly(tetrafluoroethylene) (PTFE) whiskers,[18] may also be suitable this purpose.

Acknowledgment. The authors are indebted for the financial support of the Wellcome Trust, EPSRC, BBSRC and NSERC. We also thank Brent Gowen for help with the electron microscopy.

JA000764E

(12) Adjudged by monitoring J_{NH} splittings for Int190 and the HDO quadrupolar splitting (∼8 Hz). Significant broadening for the HDO resonance was observed, due to chemical exchange on an intermediate to slow time scale.

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