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Characterization of polyacrylamide-stabilized Pf1 phage liquid crystals for protein NMR spectroscopy

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Abstract

A new polymer-stabilized nematic liquid crystal has been characterized for the measurement of biomolecular residual dipolar couplings. Filamentous Pf1 phage were embedded in a polyacrylamide matrix that fixes the orientation of the particles. The alignment was characterized by the quadrupolar splitting of the ^{2}H NMR water signal and by the measurement of ^{1}H-^{15}N residual dipolar couplings (RDC) in the archeal translation elongation factor 1β. Protein dissolved in the polymer-stabilized medium orients quantitatively as in media without polyacrylamide. We show that the quadrupolar splitting and RDCs are zero in media in which the Pf1 phage particles are aligned at the magic angle. This allows measurement of J and dipolar couplings in a single sample.

Abbreviations: aEF1β, archeal translation elongation factor 1β; LC, liquid crystal; PA, polyacrylamide; PSLC, polymer-stabilized liquid crystal; RDC, residual dipolar coupling; REDOR, rotational-echo double resonance; RQS, residual quadrupole splitting.

One recent development in biomolecular NMR is the addition of residual dipolar coupling (RDC) restraints to the structure determination process. As opposed to the local nature of J-coupling and NOE data, residual dipolar couplings provide long-range information on the orientation of interatomic vectors. In solution, dipolar interactions can be partially recovered by the use of anisotropic media. Various types of dilute liquid crystals (LC) have been used for this purpose, including lyotropic lipid bicelles (Tjandra and Bax, 1997; Losonczi and Prestegard, 1998; Ottiger and Bax, 1998, 1999; Cavagnero et al., 1999), lyotropic cetylpyridinium/hexanol lamellar phase (Prosser et al., 1998; Barrientos et al., 2000), filamentous bacteriophage particles (Clore et al., 1998; Hansen et al., 1998a, b), purple membrane (PM) fragments (Koenig et al., 1999; Sass et al., 1999), cellulose crystallites (Fleming et al., 2000) as well as strained polyacrylamide gels (Sass et al., 2000; Tycko et al., 2000). The large variety of media provide alternatives in case of incompatibility between a particular medium and a macromolecule (due to precipitation, aggregation, disruption of the liquid crystal phase, etc.) and allows independent sets of RDCs to be measured (Al-Hashimi et al., 2000).

Here, we present a new nematic liquid crystal medium consisting of a colloidal suspension of Pf1 bacteriophage particles embedded in a cross-linked polyacrylamide gel. This type of liquid crystal is called a polymer-stabilized liquid crystal (PSLC), as the macroscopic anisotropy of the medium is preserved by the polymer network in absence of an external director. This is conceptually related to the embedding of PM fragments in polyacrylamide gel and allows independent control in NMR experiments over the orientation of the liquid crystal and the external magnetic field (Sass et al., 2000). Similar control

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over director orientation was achieved by sample spinning of bicellar mesophases (Tian et al., 1999; Zandomeneghi et al., 2001). By ^{2}H NMR, we show that phage particles are fixed by polyacrylamide concentrations above 3% and that the residual quadrupolar coupling can be scaled by the macroscopic orientation of the sample. Using the archeal protein aEF1β (Kozlov et al., 2000), we show that the Pf1 PSLC yields the same residual dipolar couplings as unpolymerized samples and allows independent measurement of scalar and dipolar couplings in a single sample.

Pf1 bacteriophages were grown and purified according to the literature (Hansen et al., 2000). The concentration of phage stock was 90 mg ml^{-1}, as estimated from the optical density of diluted phage solutions at 270 nm, using an absorption coefficient of 2.25 ml mg^{-1} cm^{-1}. Polyacrylamide was prepared from a stock solution of 29% w/v acrylamide and 1% w/v N,N′-methylenebisacrylamide. The PSLC was prepared as following. The required amount of acrylamide mix was first diluted with D_2O or a H_2O/D_2O/protein mix and the polymerization started by the addition of 0.1% w/v ammonium persulfate (APS) and 0.1% w/v TEMED (N,N,N′,N′-tetramethylethylenediamine). Pf1 phage was added to a concentration of approximately 20 mg ml^{-1} and rapidly mixed with a vortex. Under these conditions, polymerization required approximately 20 min to complete which allowed time to centrifuge the viscous suspension to remove bubbles. The still unpolymerized sample was immediately transferred to a 100 μl glass capillary and positioned either vertically or at the magic angle (55°) with respect to the B_0 field, inside the bore of a 11.75 T magnet. NMR spectra of samples in capillaries were obtained by mounting the capillary vertically inside a standard 5 mm NMR tube. Spectra were recorded on a Bruker DRX500 spectrometer equipped with a ^{1}H/^{13}C/^{15}N triple resonance probe and a ^{2}H lock channel.

When suspended in a magnetic field greater than 7 Tesla, filamentous Pf1 phage align spontaneously to form a liquid crystal with a unique director (Hansen et al., 1998a). A good indicator of the alignment state of a liquid crystal is the residual quadrupolar splitting (RQS) of the water deuterium resonance. The splitting originates from deuterated water molecules transiently interacting with the aligned phage particles. The RQS is scaled by the second-order Legendre polynomial

$$P_2(\phi) = (3\cos^2\phi - 1)/2 \qquad (1)$$

where φ is the angle between the liquid crystal director (i.e., phage orientation) and the B_0 magnetic field (Tian et al., 1999; Zandomeneghi et al., 2001). In general, scaling of first-order quadrupolar, CSA and dipolar interactions can be described by multiplying the Saupe order matrix (Saupe and Englert, 1963) by $P_2(\phi)$, with the matrix defined with respect to the LC director.

Three different conditions for preparing PSLC samples in capillaries were examined. The birefringence photomicrograph of a sample polymerized outside of the magnetic field showed a mottled appearance due to the presence of multiple liquid crystal domains with different orientations. To record a ^{2}H spectrum, the capillary was mounted vertically in a 5 mm NMR tube and a powder spectrum recorded (Figure 1A). Simulation of the spectrum with a RQS of 20 Hz and a linewidth of 9.5 Hz reproduced the experimental lineshape.

Birefringence photomicrographs of two samples polymerized in the magnetic field were similar and showed a single LC domain across the entire volume of the sample (Figures 1B and 1C). However, the ^{2}H spectra strongly differed. The sample polymerized vertically (0°) showed the residual quadrupolar doublet expected for vertically aligned phage (Figure 1C). The sample polymerized at 55° relative to the B_0 field showed a singlet, as the RQS is scaled by $P_2(55°) \approx 0$ (Figure 1B). The ^{2}H spectra were stable over time, demonstrating that the polyacrylamide matrix had permanently fixed the phage orientation.

To determine the minimum concentration of polyacrylamide necessary, several samples were prepared with acrylamide concentrations of 0, 1, 2, 3, 4, 5, 7%. All samples were polymerized in the magnet with the capillary axis at the magic angle. Following polymerization, ^{2}H spectra were acquired every 30 sec with the capillaries mounted vertically. A stable singlet was observed for samples with 3, 4, 5 and 7% polyacrylamide. Slow recovery of the full RQS was seen for the sample with 2% polyacrylamide. The magnetic field-induced realignment was slowed by the aqueous gel, but the original orientation was not maintained. The realignment was exponential with a half-life of approximately 10 min. The samples at 1% and 0% polyacrylamide showed immediate recovery of the RQS. The Pf1 phage has a diameter of 70 Å and a length of about 2 μm (Bradley, 1973). At low polyacrylamide concentration, the phage rods presumably slide back and forth by Brownian movement through the gel pores which, on average, are larger than the

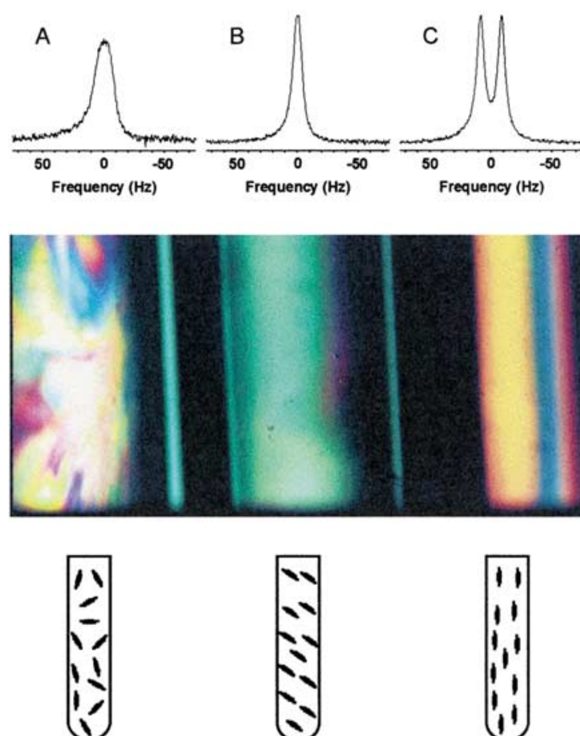

Figure 1. ^{2}H NMR spectra (top), birefringence photomicrographs (center) and schematic representations (bottom) of polyacrylamide-stabilized Pf1 phage liquid crystals (5% PA, ~20 mg ml^{-1} Pf1). (A) Unaligned sample polymerized in the absence of an external magnetic field. The LC domains are randomly oriented and the ^{2}H NMR spectrum is a powder pattern. (B) Sample polymerized with the capillary oriented at the magic angle (~55°) with respect to the magnetic field. A single LC domain is observed and the ^{2}H NMR spectrum is a singlet with a linewidth of 10 Hz. (C) Control sample polymerized with the capillary parallel (0°) to the magnetic field. A single LC domain is again observed, but the ^{2}H NMR spectrum shows residual quadrupolar splitting of 18 Hz, also with 10 Hz linewidth. The ^{2}H spectra were rescaled vertically.

phage diameter, and reorient with the magnetic field. Gels with polyacrylamide concentration less than 3% are very soft and the observed reorientation could also be due to gel distortion.

A minimum polyacrylamide concentration of 3% is thus required to maintain the alignment of the phage, independently of the magnetic field. It is preferable to use at least 4% polyacrylamide, to ensure there is no slow realignment over very long periods of time, and to maintain a minimum rigidity for subsequent manipulation of the gel. Higher concentrations of polyacrylamide will decrease translational diffusion and increase the correlation time of the protein (Sass et al., 2000). An increase in correlation time leads to a decrease in T_2, in addition to the line broadening effect of multiple ^{1}H-^{1}H RDCs. Under fast polymerization conditions, it is advantageous to use the highest field possible, to ensure magnetic field-induced alignment is much faster than the polymerization. This is particularly important considering that the orientation is much slowed once the acrylamide is partially polymerized. It is possible to slow the rate of polymerization by lowering the amount of ammonium persulfate, but then the amount of unpolymerized acrylamide increases, producing undesirably large proton signals in the 7–8 ppm region.

The new alignment system was tested on the archeal translation elongation factor 1β, a 89 amino acids polypeptide, whose structure has been solved by NMR spectroscopy (Kozlov et al., 2000; PDB accession number 1GH8). The structure of aEF1b was further refined using RDC's measured in Pf1 media without polyacrylamide using CNS (Brünger et al., 1998). ^{15}N-labeled protein aEF1β was prepared as previously described (Kozlov et al., 2000). For NMR samples, protein was either added to Pf1 phage alone or Pf1 PSLC as described above. Experiments were carried out in 150 mM NaCl, 30 mM sodium phosphate, 0.1 mM NaN_3, pH 6.3, 10% v/v D_2O at 303 K. Two capillaries of ^{15}N-labeled protein in the PSLC medium were prepared: one with the liquid crystal director aligned with the magnetic field and the other with director at magic angle. A control, unpolymerized sample (LC) was also prepared with 25 mg ml^{-1} Pf1 phage in a standard 5 mm NMR tube.

Two-dimensional ^{1}H,^{15}N-HSQC spectra were acquired with 100 F1 complex points, apodized and zero-filled to 2K points in both dimensions before Fourier transformation. The proton 180° pulse in the middle of the t_1 ^{15}N evolution period was removed to allow the heteronuclear ^{1}H-^{15}N coupling to evolve. ^{1}H-^{15}N scalar coupling constants were measured in an isotropic protein solution and subtracted from the experimental $J + D$ couplings measured in an anisotropic medium to give the RDC values. The RDC data for the LC and PSLC aEF1β samples were well reproduced by calculations based on the RDC-refined protein structure (Figures 2A and 2B). In calculating the alignments, fourteen ^{1}H-^{15}N couplings were removed because they increased dramatically the r.m.s.d. between experimental and predicted RDC (Table 1). These arise from residues in loops, mobile regions and the protein termini, with lower order parameters and thus with local alignment tensors different from the global alignment tensor (Tolman et al., 2001). In addition, six couplings in the PSLC medium could

Table 1. Alignment tensors for aEF1β in Pf1 and polyacrylamide-stabilized Pf1 derived from experimental ^{15}N-^{1}H residual dipolar couplings[a]

Liquid crystal	$^{1}D_{a}^{NH}$ (Hz)	D_r/D_a	α (deg)	β (deg)	γ (deg)	r.m.s.d. (Hz)	No. of measured dipolar couplings
Pf1 (∼25 mg/mL)	−15,0	0,15	144,9	52,4	−62,3	1,00	70
Pf1 (∼18 mg/mL) + 5% PA	−11,5	0,15	148,0	55,3	−64,6	1,96	64

[a]The five alignment tensor parameters were evaluated from a least-square minimization of the rmsd between observed and calculated ^{15}N-^{1}H residual dipolar couplings, relative to the coordinates of the structure of aEF1 β refined using RDC's in the unstabilized medium. Phage concentrations were calculated from the RQS measured in parallel aligned samples.
PA, polyacrylamide.

not be measured because of low peak intensity and/or spectral overlap.

The orientation of the alignment tensors in the vertically aligned PSLC and control LC samples were similar to each other and to the orientation of the moment of inertia tensor of the protein (α, β, γ = 148°, 53°, −74°), which indicates that the nature of the alignment is mainly mechanical and not influenced by the polyacrylamide matrix. Linear regression analysis between dipolar couplings in the two media gave a correlation coefficient (*r*) of 0.97. The larger magnitude of the LC tensor is attributed to the higher concentration of Pf1 phage. The error in coupling measurements from the capillary PSLC systems was ±1 Hz and less than ±0.5 Hz for the LC sample. The difference in errors is due to differences in filling factors and shimming between the capillary tubes and standard NMR tube. In both cases, the r.m.s.d. between calculated and experimental dipolar couplings is larger than the error in the coupling measurements. We attribute this to small-amplitude backbone dynamics (Tolman et al., 2001).

One application of the present system is the measurement of J and J + D couplings in a single sample. Because the RDC's are scaled by $P_2(\phi)$ (Equation 1), where φ is the angle between the PSLC director and the magnetic field, RDCs in the sample with the phage particles aligned at magic angle were close to zero (Figure 2C). A small positive slope was observed because the angle was slightly smaller than 55°. Scalar couplings in PSLC (or LC) samples can also be measured under conditions of magic-angle spinning. Preliminary experiments have shown that PSLC samples with phage initially aligned with B_0 (at magic-angle with the rotor axis) are mechanically stable under slow MAS (less than 3000 Hz) and that the residual quadrupolar coupling in ^{2}H spectra can be reversibly suppressed.

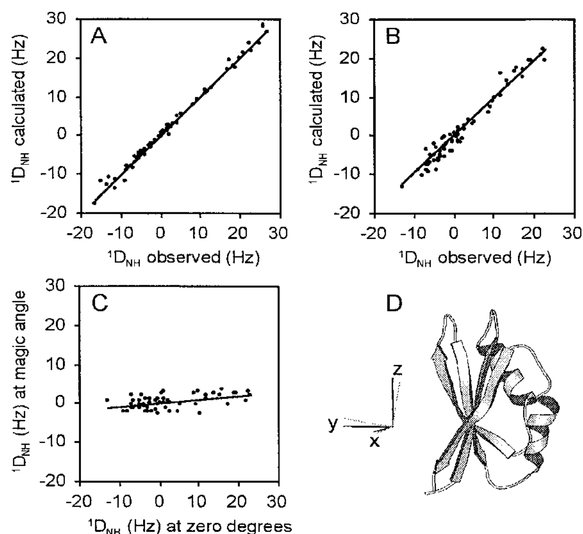

Figure 2. (A) Correlation between calculated and measured ^{1}H-^{15}N RDCs for aEF1β in 25 mg ml^{-1} Pf1. The correlation coefficient (r^2) is 0.99. (B) Correlation between measured and calculated RDCs for aEF1β in the PSLC medium (∼18 mg ml^{-1} Pf1, 5% PA) aligned parallel (0°) to B_0. Linear regression applied to the data gives a correlation coefficient of 0.95. (C) Correlation of RDCs measured in PSLC media aligned at 55° and 0°. The slope of the correlation is 0.095, equivalent to an angular error of ∼4° in the orientation of the capillary. (D) Structure of aEF1β shown with the orientation of the principal components of the alignment tensors (black) and the moment of inertia tensor (grey). The figure was generated with MOLSCRIPT (Kraulis, 1991).

A second, more interesting application is H-X dipolar recoupling or REDOR experiments under slow MAS. When the PSLC medium is rotated around an axis at the magic angle, the angle φ between the director and magnetic field oscillates. This causes the RDCs to vary as a function of the rotor phase. The integral over a complete rotor cycle is zero which is the basis of MAS suppression of time-averaged RDCs as well as CSA and first-order quadrupole interactions. Application of 180° pulses on one of two nuclei involved in a RDC will interfere with the MAS averaging. For

a single domain PSLC (analogous to a single crystal), the π pulses must not only be speed-synchronized, but also phase-synchronized with the rotor. Up to 47% of the full RDC (obtained with phage aligned along the magnetic field) can be recovered if recoupling pulses are applied whenever the scaling factor goes through zero. An advantage of this method is selective control over the dipolar couplings manifested. For example, homonuclear proton-proton RDC's could be eliminated by MAS while heteronuclear couplings are selectively reintroduced. This would open the door to high resolution NMR studies of more strongly aligned macromolecules.

In conclusion, we have shown that Pf1 phage can be embedded in a polyacrylamide matrix and form a polymer-stabilized nematic liquid crystal phase, with fixed director orientation. Protein dissolved in the medium orients quantitatively as in LC Pf1 media without polyacrylamide and the RDCs can be scaled by varying the angle between the PSLC director and the magnetic field.

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