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ARTICLE

Protein alignment using cellulose nanocrystals: practical considerations and range of application

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Abstract Cellulose nanocrystals (CNCs) form liquid crystals in aqueous solution that confer alignment to macromolecules and permit the measurement of residual dipolar couplings. CNCs possess many attractive features as an alignment medium. They are inexpensive, non-toxic, chemically inert, and robust to denaturants and temperature. Despite these advantages, CNCs are seldom employed as an alignment medium and the range of their applicability has not yet been explored. We have re-examined the use of CNCs in biomolecular NMR by analyzing the effects concentration, ionic strength, and temperature on molecular alignment. Stable alignment was obtained over wide ranges of temperature (10–70°C) and pH (2.5–8.0), which makes CNCs potentially very useful in studies of thermophilic proteins and acid-stabilized molecules. Notably, we find that CNC suspensions are very sensitive to the concentrations of biological buffers, which must be taken into account when they are used in NMR analyses. These results have led us to develop a general procedure for preparing aligned samples with CNCs. Using the SH3 domain from the Fyn tyrosine kinase as a model system, we find that CNCs produce an alignment frame collinear with that of the commonly used Pf1 bacteriophage alignment medium, but of opposite magnitude.

Keywords Cellulose nanocrystals · Residual dipolar couplings · Liquid crystals · SH3 domains

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Introduction

Residual dipolar couplings (RDCs) result from anisotropic averaging of the orientation-dependent dipolar coupling interaction when molecules are dissolved in an ordered medium, such as a liquid crystal. RDCs provide long-range information on the orientation of inter-nuclear vectors within a molecular frame of reference and are thus valuable in structural and dynamical investigations of macromolecules (Bax 2003; Bax and Grishaev 2005; Lipsitz and Tjandra 2004; Prestegard et al. 2004; Tolman and Ruan 2006). They can be applied to multidomain proteins and macromolecular complexes, where they help to define the relative orientations of individual subunits (Clore 2000; Skrynnikov et al. 2000). RDC data for intrinsically disordered proteins provide unique information on their conformational distributions (Jensen et al. 2009). In addition, RDCs report on intramolecular motions occurring on a wide range of timescales, from picoseconds to milliseconds (Lange et al. 2008; Salmon et al. 2009; Tolman and Ruan 2006; Zhang et al. 2007). In order to extract dynamical information at the level of individual bond vectors, RDCs from multiple ($\geq$5) unique alignment frames are required (Tolman and Ruan 2006). A variety of media are in common use, including phospholipid bicelles, Pf1 bacteriophage, polyethylene-glycol mixtures, distorted polyacrylamide gels, and purple membranes. However, none of these media are universal in terms of the temperatures, pHs, co-solutes and proteins with which they may be employed (Prestegard et al. 2004; Tolman and Ruan 2006; Trempe and Gehring 2003). For instance, phospholipid bicelles form over a fairly narrow temperature range and have delicate phase equilibria (Ottiger and Bax 1998). Negatively charged Pf1 bacteriophage require pH $>$ 5 and can undergo undesirable association with positively charged proteins (Hansen et al. 1998).

Purple membrane fragments are highly sensitive to ionic strength (Sass et al. 1999). It is therefore advantageous to develop a large number of different alignment media in order to meet the particular demands of each system under study.

CNCs are highly crystalline rod-like particles, shown in Fig. 1, that are obtainable in large quantities from cellulosic materials by sulfuric acid hydrolysis (Habibi et al. 2010; Ranby 1951). The sulfate ester surface groups introduced during this process produce a slightly negative electrostatic layer that contributes to the stability of the aqueous suspensions. Above a critical concentration, these colloidal suspensions phase separate into an upper isotropic and a lower anisotropic phase. The ordered phase exhibits optical properties characteristic of chiral nematic liquid crystals (Revol et al. 1992, 1994) and has been shown to orient proteins in solution (Fleming et al. 2000). CNCs are naturally abundant, easily accessible, biodegradable, nontoxic, chemically inert, and thus represent an attractive and inexpensive alignment medium. However, to our knowledge there have been no additional NMR studies employing CNCs in the decade following the original report (Fleming et al. 2000). In order to better understand macromolecular alignment in this medium, we have systematically explored the effects of concentration, ionic strength, and temperature on CNC suspensions. We confirm that protein RDCs are readily obtained using this approach. CNC media show unusual resistance to high temperatures, which makes them well suited to structural and dynamic studies of thermophilic proteins and thermal denaturation of mesophilic proteins. Importantly, the physical properties of CNC suspensions are highly sensitive to ionic strength. We find that careful manipulation of buffer conditions is critical for obtaining high quality NMR spectra and robust RDC measurements. Here, we present a detailed protocol for preparing oriented samples using CNCs and offer guidelines for when this medium can be employed in biomolecular NMR.

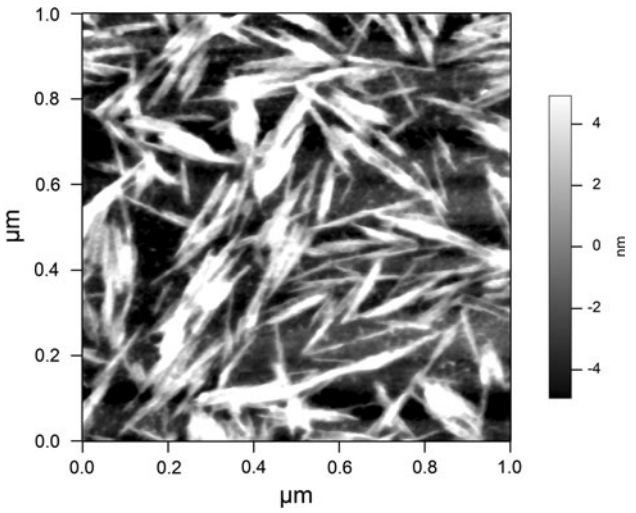

Fig. 1 Tapping mode AFM image of FPInnovations-Paprican cellulose nanocrystals used for molecular alignment in this study

Materials and methods

Protein preparation

Samples of the SH3 domain from the Fyn tyrosine kinase (residues 84–142 of the chicken isoform) were produced in *E.coli* BL21(DE3) using a pET11d-based (Novagen) expression plasmid. Proteins were expressed with ^{15}N enrichment according to the protocol of Bracken and coworkers (Marley et al. 2001). Briefly, cells were cultured in 2 L of LB media until an OD600 of 0.8 was reached. Cultures were centrifuged, washed with M9 salts and transferred to 1 L of M9 minimal media supplemented with 1 g/L $^{15}NH_4Cl$. Protein expression was induced with 1 mM IPTG 1 h after the transfer to M9 and proceeded for 4 h. Cell pellets were resuspended in lysis buffer (50 mM Tris, 2 mM EDTA, 5 mM benzamidine, pH 7.5). Protein purification was achieved with anion-exchange chromatography using diethylamino cellulose DE52 (Whatman) media and a 0–1.5 M NaCl gradient, followed by hydrophobic interaction chromatography using Toyopearl Butyl-650M resin (Tosoh Bioscience) and a 1.2–0 M $(NH_4)_2SO_4$ gradient. The protein eluate was dialysed against buffer with 150 mM NaCl, 50 mM Tris, 2 mM EDTA and 5 mM benzamidine (pH 7.9) and finally purified by size-exclusion chromatography using Superdex-75 resin (GE Healthcare). The total protein yield was approximately 50 mg, and sample purity was confirmed by overloaded SDS–PAGE and NMR HSQC spectra.

Cellulose nanocrystal preparation

Aqueous suspensions of CNC are typically prepared with semi-crystalline cellulose fibres from cotton or wood pulp in a two step process. First, the cellulose chains in the accessible disordered regions of the fibre are broken down and removed by acidic hydrolysis, leaving the inaccessible crystalline regions. These are then dispersed, typically by sonication, giving a colloidal suspension of cellulose nanocrystals, stabilized primarily by surface sulfate groups, which may be left in their acid ($SO_3^-H^+$) form, or converted to their sodium ($SO_3^-Na^+$) form by neutralization with aqueous NaOH. The morphologies of CNCs extracted from wood and cotton fibres are similar (Habibi et al. 2010), and should in principle provide similar levels of molecular alignment. However, we have obtained more consistent CNC preparations starting with wood pulp rather

than cotton filter paper, possibly due to greater variability in the initial condition of the cotton fibres.

A CNC suspension in its acid form (at approximately pH 2.5) prepared from a northern softwood kraft woodpulp was kindly provided by FPInnovations-Paprican (570 St-Jean Blvd. Pointe-Claire, Montreal, QC, Canada; www.fpinnovations.ca). These CNCs are prepared similarly to previously published methods (Revol et al. 1992), but with batches yielding roughly 500 g, as opposed to 1–10 g which are typical of bench-top preparations. In addition, these samples are dispersed using a commercial (Gaulin) homogenizer rather than by sonication, following hydrolysis. The commercial CNCs behave similarly to those obtained from smaller batches (Fleming et al. 2000). The concentration of the FPInnovations-Paprican CNC suspension was 3.36% w/w, measured by weighing the residue after oven-drying 200 µL samples at 105°C. A sulfate group content of 0.22 mmol per g cellulose was determined by conductometric titration. Figure 1 shows an atomic force microscopy (AFM) image of these cellulose nanocrystals. The sample for imaging was prepared from the softwood nanocrystal suspension in its acid form by dilution to a concentration of 0.001% w/w and re-sonication. A drop of poly-L-lysine solution (0.1% w/v, Ted Pella Inc.) was placed on a freshly cleaved mica wafer (1 cm^2); after 3 min it was rinsed with deionized water and the mica was dried with argon. A drop of the cellulose suspension was then allowed to stand on the mica for 1 min and washed off with deionized water and dried again. Images of the CNCs were collected in tapping mode with a Cypher AFM (Asylum Research, Santa Barbara, CA) with a standard Si cantilever (AC160TS, Olympus) having a nominal spring constant of 42 N/m (tip radius <10 nm).

NMR spectroscopy

Spectra were recorded on Varian Unity Inova spectrometer operating at a ^1H resonance frequency of 800 MHz. Protein NMR experiments were performed on 0.2 mM SH3 protein samples containing 10–20 mM sodium phosphate buffer (pH 6.0) and 5–10% ^2H_2O. $^1H/^{15}N$ HSQC spectra were assigned previously for this domain (Mittermaier 2003) and chemical shift values are presented in Supplemental Table S1. RDCs were extracted from $^1H/^{15}N$ IPAP-HSQC experiments performed on isotropic and aligned samples as described previously (Ottiger et al. 1998). ^{15}N R_2 relaxation data (Farrow et al. 1994) were obtained for samples either with or without 9.5% CNC. Relaxation delays of 10, 30, 50, 70, 90, 110, 150, 190, and 210 ms were employed. A 3 second recycle delay was used for all R_2 experiments. NMR spectra were processed using NMRPIPE (Delaglio et al. 1995) and analysed using NMRVIEW (Johnson and Blevins 1994). MODULE software (Dosset et al. 2001) was used to determine the alignment tensor and for comparisons of RDCs with their back-calculated values. The protein structure image was generated using PYMOL (DeLano and Hus 2002).

Results

Preparation of aligned samples

Freshly prepared CNC samples are generally at concentrations of about 2–4% (w/w) and at pH 2.0–2.5. Before initiating NMR studies, the CNC suspensions were adjusted to neutral pH with NaOH, and concentrated to between 9 and 10% by air evaporation, yielding a translucent, viscous liquid. ^2H_2O was added to a final concentration of 10% and used to monitor alignment directly via the residual quadrupolar coupling (RQC) of the water signal in one-dimensional ^2H NMR spectra. Initial tests were performed on 7–8% CNC suspensions at pH 6.0 without the addition of any additional salts, buffers, or co-solutes. Samples were placed in a 18.8 T (800 MHz ^1H Larmor frequency) NMR spectrometer and series of one-dimensional ^2H spectra were collected over a period of several hours. Spectra for unbuffered 7.4% CNC initially showed doublets separated by 4.3 Hz, with well-resolved components with full-widths-at-half-height (FWHH) of about 2 Hz, shown in Fig. 2, indicative of molecular alignment by a homogenously ordered medium. However, this peak pattern was unstable and gradually converted into an irregular multiplet structure over the course of several hours, shown in Fig. 2. NMR samples removed from the

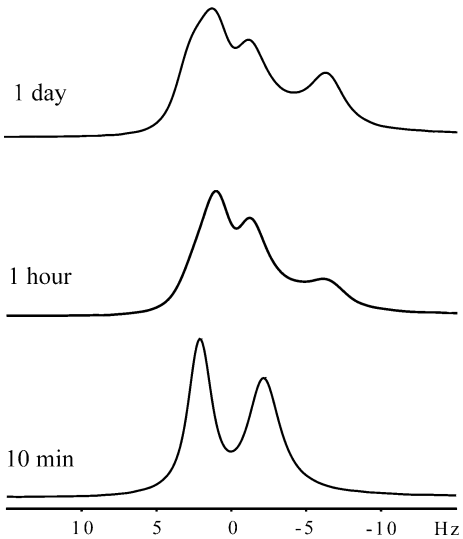

Fig. 2 ^2H_2O NMR spectra for an unbuffered 7.4% CNC suspension (pH 6.5, 30°C) collected 10 min, 1 h, and 24 h after thoroughly mixing the sample

spectrometer at this stage were visibly inhomogeneous. After thorough mixing, the homogeneity of the sample was restored, and ^{2}H NMR spectra reverted to well-resolved doublets. The cycle of separation and mixing could be repeated indefinitely. The reason for this time-dependent loss of homogeneity is not certain, but the nanocrystals are somewhat polydisperse, and this may lead to the separation of some isotropic phase containing the shorter nanocrystals by a fractionation mechanism. Some fractionation by length has been observed for cellulose nanocrystals, with the shorter nanocrystals migrating to the isotropic phase (Dong et al. 1998). Presumably the mixture of isotropic and liquid crystalline phases is both optically and magnetically non-homogeneous, but mixing can regenerate the homogeneous quasi-equilibrium liquid crystalline phase. This time-dependent loss of sample homogeneity would preclude the use of CNC in multi-dimensional protein NMR. Fortunately, we found that addition of salts and/or buffers greatly increased the stability of magnetically aligned samples, as discussed below.

Biological buffers were prepared as concentrated stock solutions of approximately 0.5–1.0 M and added to CNC samples in small volumes to bring the final buffer concentration to between 10 and 30 mM. The addition of electrolytes to CNC suspensions is known to alter their phase composition and chiral nematic properties (Dong et al. 1996). The suspensions exhibited complex behaviour in response to the sudden increases in ionic strength. Unbuffered samples were initially quite viscous. Immediately after the addition of buffer solution there were significant decreases in viscosity. Subsequently, the viscosities of the samples spontaneously increased at rates that depended strongly on the final concentrations of both CNCs and added buffer. Higher concentrations of either component resulted in higher final viscosities and much more rapid changes. For example, 9% CNC solutions almost instantly adopted a stiff, gel-like consistency upon addition of 30 mM sodium phosphate buffer, pH 6.0. In contrast, the same CNC stock suspension gradually became more viscous over the course of 1 h when the same buffer was added to a concentration of only 10 mM. The resultant CNC gels were self-supporting upon gentle inversion of the NMR or 1.5 mL microfuge tube, but exhibited a certain degree of thixotropy or pseudoplasticity and could be induced to flow by vigorous shaking. We observed qualitative differences in the appearance of CNC suspensions under a polarizing microscope upon adding phosphate buffer, most notably the emergence of roughness on the micron scale, as shown in Supplemental Fig. S1. We hesitate to propose a detailed microscopic theory to explain this behaviour, but we note that suspensions of other charged particles have been reported to exhibit superficially similar responses to changes in salt concentration. At low ionic strength, suspensions of charged Boehmite rods form "repulsive gels" with high viscosity at short time scales due to "freezing" of translational diffusion by overlap of the electrostatic double layers (Wierenga et al. 1998). Small increases in ionic strength under these conditions weaken the gel, due to shielding of the electrostatic repulsion. At higher salt concentrations (I = 10–75 mM), Boehmite suspensions form "attractive gels" comprising networks of aggregated rods. Small increases in ionic strength now strengthen the gel by promoting attraction of the rods. In addition, the macroscopic viscosity of these suspensions may be influenced by the translation of ordered domains or "packets" of rods with respect to each other (Wierenga et al. 1998). We therefore hypothesize that the behaviour exhibited by CNCs upon salt addition results from a combination of time-dependent changes in repulsion, aggregation, and microstructuring within the suspensions.

Importantly, samples placed in the NMR spectrometer after completion of the gelation process produced irregular ^{2}H spectra with indeterminate residual quadrupolar splittings. Spectral quality did not improve substantially after extended periods in the spectrometer field. In contrast, samples that were placed in the NMR spectrometer early during gelation and allowed to equilibrate for 2–4 h produced well-resolved ^{2}H doublets whose separations and lineshapes remained stable indefinitely (we periodically collected spectra for several weeks). We attribute this effect to the anisotropy of CNC magnetic susceptibility, which causes them to align with their long axes perpendicular to applied magnetic fields (Fleming et al. 2000, 2001; Revol et al. 1994). In the absence of a magnetic field, the liquid crystalline phase of CNC suspensions shows a range of textures, with no overall uniform orientation. If gelation occurs in this state, then no homogeneous molecular alignment can occur. However, if introduced into the NMR spectrometer prior to complete gelation, the suspension presumably forms a uniform domain with the individual CNCs oriented orthogonally to the magnetic field, and this orientation is locked in by the gelation process. This property of CNCs is quite convenient for producing stable aligned samples. However, it may preclude their use in systems where high ionic strength is required, since gelation may occur so rapidly that samples cannot be placed in the spectrometer before completion of the process. We found that occasionally the CNCs would not fully align, even at moderate ionic strengths and with prompt insertion into the spectrometer. This was corrected by removing the sample from the spectrometer, inverting the sample vigorously several times with shaking, and replacing it in the magnet. This may temporarily loosen the structure of the gel and permit the alignment of the nanocrystals to proceed.

Protein residual dipolar couplings

We used the SH3 domain from the Fyn tyrosine kinase to investigate protein alignment in CNC media. SH3 domains are small mediators of protein/protein interactions that are found throughout eukaryotes and comprise roughly 60 amino acid residues arranged in a β-sandwich fold (Musacchio et al. 1994). Aligned samples containing 7.5–9.5% CNC, 0.2 mM ^{15}N-labeled Fyn SH3 domain, and 10–20 mM sodium phosphate pH 6.0 were prepared by adding concentrated solutions of protein and buffer to CNC suspensions, transferring to the spectrometer, and allowing the samples to equilibrate for 2–4 h, as discussed above. One-bond protein ^{1}H/^{15}N couplings were measured using IPAP-HSQC experiments (Ottiger et al. 1998) performed in the presence and absence of CNCs. HSQC spectra of the aligned samples were of excellent quality and showed no differences in peak position or linewidth compared to the isotropic samples. A region of the IPAP spectrum for an aligned sample is shown in Fig. 3. RDCs were calculated by subtracting data for the isotropic sample, which reflect only scalar couplings, from those of the aligned sample, which correspond to the sum of scalar and residual dipolar couplings. The values of RDCs obtained with a 9.5% CNC preparation varied between −12.5 and +13.2 Hz, which is comparable to the results of the initial study (Fleming et al. 2000). RDC data are commonly interpreted in terms of the orientations of internuclear vectors within a molecular frame of reference and the overall alignment of the molecule as a whole, according to (Dosset et al. 2001),

$$D_{ij} = -S\frac{\gamma_i\gamma_j\mu_0 h}{16\pi^3 r_{ij}^3}\left(A_a\left(3\cos^2\theta - 1\right) + \frac{3}{2}A_r\sin^2\theta\cos 2\varphi\right), \quad (1)$$

where θ and φ are the polar angles of an internuclear vector in the principle axis system of the molecular alignment tensor, A_a and A_r are the axial and rhombic components of the alignment tensor, r_{ij} is the internuclear separation, $\gamma_{i,j}$ are the gyromagnetic ratios, μ_0 is the permeability of free space, and S is the local order parameter, which in this case was assumed to be 1. We fit the RDC data to the X-ray crystal structure of the Fyn SH3 domain (Noble et al. 1993) using (1), extracting A_a, A_r, as well as three Euler angles, α, β, and γ that describe the orientation of the principle axis system of the alignment tensor relative to the frame of reference of the PDB coordinates. Overall, good agreement was obtained between experimental ^{1}H/^{15}N RDCs and those calculated using (1), as shown in Fig. 4. Data for three residues (L3,T14, and S19) deviated significantly from the expected RDC values, and were excluded from the analysis, along with data for the flexible C-terminus (V58 and D59). Leu 3 is located at the N-terminus of the protein while Thr 14 and Ser 19 are located in the RT-Src loop, which is known to be flexible on the nanosecond to

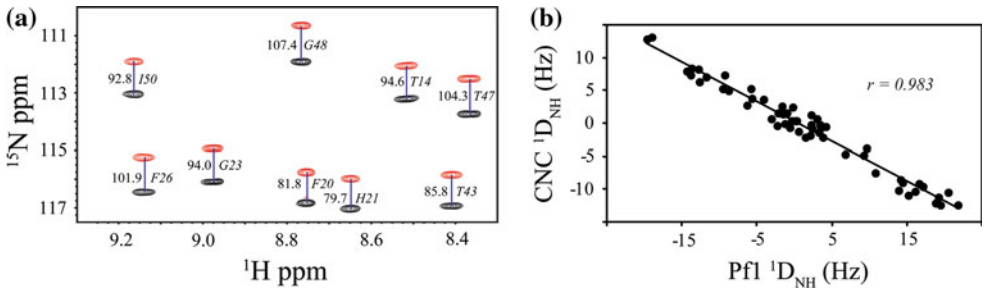

Fig. 3 Region of an ^{1}H/^{15}N IPAP-HSQC spectrum collected for the Fyn SH3 domain in 9.5% CNC (pH 6.0, 30°C) **(a)**. Spectra containing the upfield (*red*) and downfield (*black*) doublet components of the IPAP-HSQC spectra are superimposed. Each pair of peaks is labelled with the total $|^{1}J_{NH} + {}^{1}D_{NH}|$ splitting (Hz) and the corresponding residue number. Comparison of one-bond ^{1}H/^{15}N RDCs collected for the Fyn SH3 domain aligned in 2% Pf1-phage and 9.5% CNC **(b)**

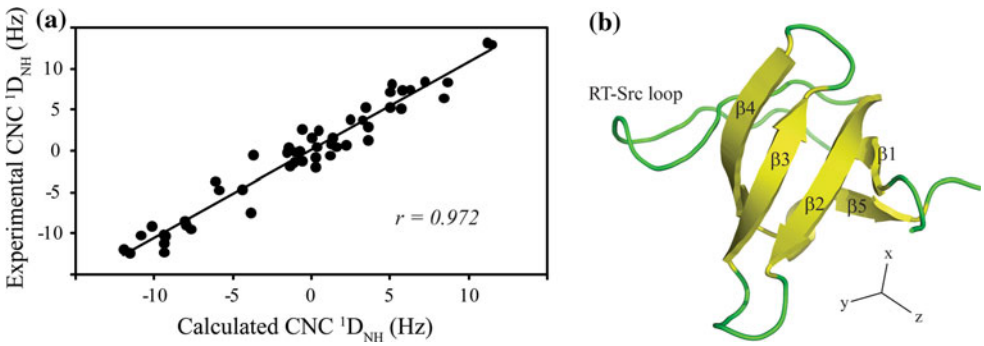

Fig. 4 Comparison of experimental RDCs for the Fyn SH3 domain (9.5% CNC, pH 6.0, 30°C) and those calculated using (1) and alignment parameters extracted from fits to the X-ray crystal structure (PDB entry: 1SHF) **(a)**. Ribbon representation of the protein structure and axes of tensor orientation **(b)**

picosecond timescales (Mittermaier and Kay 2004). Omitting these 5 data points, the pairwise root-mean-squared-deviation between RDCs obtained in 9.5% CNC and those back-calculated from the crystal structure is 1.62 Hz, and the *Q*-value (Cornilescu et al. 1998) is 0.24. This level of agreement is similar to predictions based on the resolution of the crystal structure, 1.9 Å (Noble et al. 1993), and experimental and predicted dipolar couplings obtained with structures at this resolution typically yield Q factors of 0.20–0.25 (Bax 2003).

For the sake of comparison, $^{1}H/^{15}N$ RDCs were also measured for the Fyn SH3 domain using 2% Pf1 bacteriophage as the aligning medium. Interestingly, the CNC- and Pf1-derived RDC values are highly anti-correlated, as shown in Fig. 3. The Euler angles, which relate the alignment and PDB frames, are quite close for the two media, and the rhombicities $R = A_a/A_r$ are likewise similar at 0.54–0.56, as shown in Table 1. This indicates that the molecular orientations produced by the two media are similar, which is perhaps not surprising, given that both liquid crystals are composed of negatively charged rods. The main difference is that the signs of A_a and A_r are opposite, which is likely due to the different orientations adopted by CNCs and bacteriophage with respect to the applied magnetic field. Cellulose crystallites have a large negative diamagnetic anisotropy which aligns their long axis perpendicularly to the applied magnetic field (Fleming et al. 2000, 2001; Revol et al. 1994) while the magnetic susceptibility anisotropy of Pf1 bacteriophage is dominated by the major coat protein helices, leading to parallel alignment with the magnetic field (Torbet and Maret 1981). Interestingly, a liquid crystalline G-tetrad alignment medium comprising negatively charged filaments aligned perpendicularly to the magnetic field also produces RDCs that are anti-correlated with those obtained with Pf1 bacteriophage (Lorieau et al. 2008). A similar sign reversal is also seen for RDCs obtained in stretched versus compressed polyacrylamide gels (Mohana-Borges et al. 2004; Sass et al. 2000).

Concentration dependence

We investigated the dependence of molecular alignment on CNC concentration by monitoring the residual quadrupolar splitting of the water signal in samples prepared with 10–20 mM sodium phosphate buffer at pH 6.0 and 5.7–9.5% CNCs (Fig. 5). We obtained a linear relationship between RQCs and CNC concentration that extrapolates to an RQC of zero, i.e. no alignment, at a concentration of 4.5%. These results are consistent with the RQC previously reported for an 8% suspension of CNC ($\approx$8 Hz) (Fleming et al. 2000). It was not possible to extend this investigation to higher CNC concentrations, since suspensions above about 10% underwent gelation too rapidly to obtain homogeneously ordered media upon buffer addition, as described above. In parallel, we prepared aligned, ^{15}N-labeled Fyn SH3 domain samples containing 7.5, 8.3, and 9.5% CNCs, and monitored the degree of alignment via the axial component of the fitted alignment tensor, A_a, given in (1). A straight line fitted through the A_a values also intersects the *x*-axis, (i.e. no molecular alignment) at a CNC concentration of about 5%, in agreement with the water RQC results. Notably, the disappearance of RQCs and A_a around 5% CNC occurs at the previously determined critical concentration of CNCs required for liquid crystal phase formation in water (Dong et al. 1996).

Temperature dependence

In order to test the thermostability of alignment, one-dimensional ^{2}H NMR spectra were collected between 10 and 70°C, employing a sample containing 9.2% CNCs and 10 mM sodium phosphate buffer at pH 6.0, prepared as

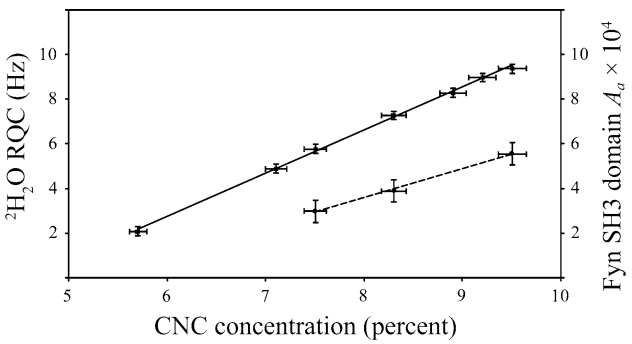

Fig. 5 Dependence of $^{2}H_2O$ residual quadrupole couplings (*solid line*) and absolute values of the A_a component of Fyn SH3 domain alignment (*dashed line*) on CNC concentration (pH 6.0, 30°C)

Table 1 Alignment tensor parameters for the Fyn SH3 domain in 9.5% CNC and 2% Pf1 phage

Liquid crystalline medium	A_a[a]	A_r[a]	Tensor orientation (α, β, γ)[b]
CNC	-5.57×10^{-4}	-2.99×10^{-4}	(−64.0°, 80.6°, −0.3°)
Pf1	9.08×10^{-4}	5.09×10^{-4}	(−69.6°, 78.9°, 0.8°)

[a] Tensor parameters based on fits of fifty $^{1}D_{NH}$ values (omitting data for L3,T14, S19, V58 and D59)

[b] Tensor orientation expressed as the Euler angles between the SH3 molecular frame (PDB entry: 1SHF) and the alignment frame

described above. Well-resolved spectra were obtained throughout the temperature range with narrow linewidths of about 1.5 Hz FWHH, as shown in Fig. 6. The magnitudes of the residual quadrupolar splittings decreased linearly with increasing temperature with a slope of about −0.04 Hz/°C. This temperature profile was completely reversible. In other words, spectra collected at alternating low and high temperatures returned the same RQC values after each temperature cycle. This suggests that the gradual decrease in RQCs is due to temperature-dependent redistribution of the hydration waters rather than due to physical restructuring of the CNC matrix itself (i.e. changes in the liquid crystalline *S*-factor), since in the latter case, the system might be expected to show some hysteresis. Variation of water RQCs produced by temperature-dependent changes in the hydration layers has been observed for suspensions of DMPC/DHPC bicelles, although in this case RQCs increase rather than decrease with increasing temperature (Ottiger and Bax 1998). Thus CNCs can be employed at temperatures up to at least 70°C. Higher temperatures are likely possible, but were beyond the limits of the NMR probe used in these studies. Only a small number of alignment media currently in use are stable over this temperature range, so CNCs are potentially very useful in studies of thermophilic proteins or thermally-denatured mesophilic proteins.

pH dependence

NMR samples containing 9% CNC and 10 mM buffer solutions of sodium acetate (pH 4.0), sodium phosphate (pH 6.0) and Tris (tris(hydroxymethyl)aminomethane, pH 8.0) were studied by one-dimensional ^{2}H NMR. Well-resolved water doublets were obtained at both pH 6.0 and 8.0 with RQCs of 8.3 Hz and 9.1 Hz, respectively. In contrast, the water resonance at pH 4.0 was quite broad with FWHH ≈ 20 Hz, which obscured any residual quadrupolar splitting that may have been present. Similar broadening was observed in a previous study for a sample at pH 5.2, and this was attributed to chemical exchange on a intermediate to slow time scale (Fleming et al. 2000). In order to explore this hypothesis, we collected ^{2}H NMR spectra of 9% CNCs at pH 2.5 in the presence of 5% D_2O and 5% deuteromethanol (CD_3OH). In these samples, the water resonance was broadened almost beyond detection, while that of the deuterated methyl was split into a doublet separated by 8 Hz. This strongly suggests that the broadening of the ^2H_2O peak at acidic pH is produced by rapid exchange of water deuterons with labile CNC deuterons, since the non-exchangeable methyl deuterons are not broadened and reflect molecular alignment. Rapid exchange can produce broadening either by classical Bloch-McConnell effects (McConnell 1958), or by population-weighted averaging of the transverse relaxation rate of water, which is relatively slow, and that of the exchangeable CNC hydrogen atoms, which would presumably be extremely rapid. Possible candidates for the exchangeable CNC groups are surface carboxylic acid moieties introduced during CNC preparation (Araki et al. 1998). Reducing the pH from neutral to acidic would convert the COO^- groups to COOH and COOD, creating a pool of CNC-bound hydrogen atoms capable of rapidly exchanging with those of water. In addition, the surface hydroxyls of the CNCs could potentially contribute to the observed broadening. Hydroxyl hydrogen atoms can exchange with those of water on the timescale of tens to hundreds per second, in a pH-dependent manner (Liepinsh et al. 1992). Most importantly, these results show that CNCs can orient molecules in solution over a wide range of pHs spanning 2.5–8.0, which could facilitate RDC studies of acid-unfolded molecules (Mohana-Borges et al. 2004; Wu et al. 2009).

Compatibility with detergents

The sensitivity of CNC suspensions to ionic strength places some limitations on the detergents that may be employed to enhance protein solubility in aligned samples. In particular, ionic detergents are not amenable to applications with CNCs. We find that even 1% SDS causes rapid gelation of CNC suspensions. In contrast, CNCs are compatible with both non-ionic and zwitterionic detergents. A 7.4% CNC suspension with the non-ionic detergent *n*-octyl-*β*-D-glucoside added to a concentration of 3% yielded a ^2H_2O

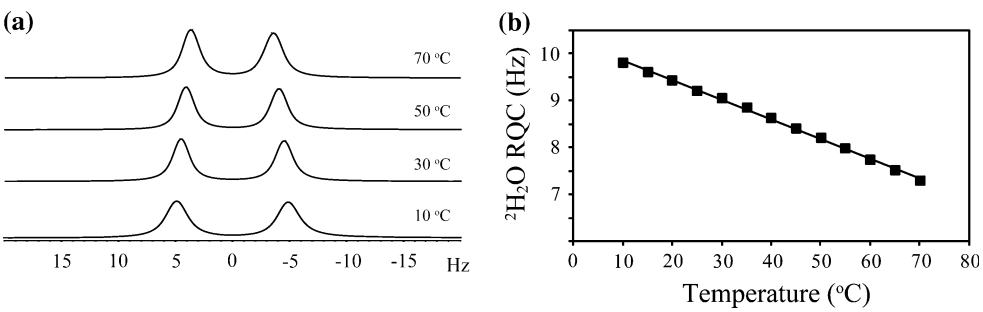

Fig. 6 ^2H_2O NMR spectra collected for a sample containing 9.2% CNC (pH 6.0) at temperatures ranging from 10 to 70°C **(a)**. Linear dependence of the ^2H_2O residual quadrupole coupling on temperature **(b)**

RQC of 5.7 Hz at 30°C. Similarly, a 7.5% CNC suspension with the zwitterionic detergent CHAPS added to a concentration of 2.5% yielded a $^{2}H_2O$ RQC of 5.8 Hz at 30°C. These values correspond closely to the RQCs obtained for CNC suspensions in the absence of detergents (Fig. 5).

Effects on transverse relaxation

It might be anticipated that the high concentrations of CNCs required for protein alignment (7–10% w/v) could slow the rate of protein rotational diffusion and lead to elevated transverse spin relaxation rates, R_2. For example, backbone amide ^{15}N R_2 values for ubiquitin are about 50% higher when the protein is dissolved within the aqueous phase of a 10% polyacrylamide gel compared to when it is free in solution (9.5 vs. 6.1 s^{-1} at 20°C, on average) (Sass et al. 2000). We measured ^{15}N transverse spin relaxation rates for the Fyn SH3 domain in the presence and absence of a concentrated CNC suspension at 25°C. The average ^{15}N R_2 value for positions within β-sheets (residues 3–6, 25–30, 36–41, 47–51, 55–57) is 5.78 s^{-1} in the absence of CNCs. The average R_2 rises slightly to 6.28 s^{-1} when CNCs are introduced to a concentration of 9.5%, as shown in Supplemental Fig. S2. This relatively modest 8% increase in R_2 suggests that CNC suspensions may be suitable for aligning larger molecules where it is important to avoid enhancing transverse relaxation rates in order to obtain high quality spectra.

CNC precipitation

It can be desirable to recover macromolecular samples from an alignment medium once RDC analyses have been performed. The observation of viscous gel-like suspensions formed in the presence of salt suggested that the CNCs could be removed by simple centrifugation in a table-top microfuge under these conditions. We found that the concentrated CNC suspensions used for macromolecular alignment produced pellets comprising 50% of the initial volume after 1 h of centrifugation at 20 k rpm in a typical 20 cm microfuge rotor at 50 mM ionic strength. Better yields were achieved when samples containing 7–9% CNC were diluted with water by factors of 2–3 and NaCl was subsequently added to a final concentration of 100 mM. Centrifugation of these samples as described above produced CNC pellets comprising 10–20% of the total volume. The pellets were sufficiently compact to allow clean withdrawal of the supernatant, and thus separation of the dissolved macromolecule from the CNCs. In principle, several iterations of this procedure would recover in excess of 95% of the dissolved macromolecular from a CNC alignment medium.

Discussion

In this study, we re-examined the use of cellulose nanocrystals (CNCs) as an alignment medium for the measurement of residual dipolar couplings in biomacromolecules. We found that CNCs possess several useful properties that could be valuable for characterizing certain systems. For example, they provide thermostable alignment up to at least 70°C, facilitating high temperature studies of thermophilic molecules. Other media, such as purple membrane fragments (Koenig et al. 1999; Sass et al. 1999) and cetylPrCl/*n*-hexanol/NaCl lamellar liquid crystals (Prosser et al. 1998) are also stable to high temperatures, but may not be applicable in all cases. Purple membrane fragments can interact strongly with some proteins, while the *n*-hexanol medium requires relatively high salt concentrations in excess of 200 mM. In contrast, CNC/protein interactions are minimal even for some positively charged proteins (Fleming et al. 2000) and CNCs require low, rather than high ionic strength. Similarly, we find that CNCs can be used over a very wide range of pHs. Other pH-insensitive alignment media include C_mE_n/*n*-hexanol lamellar liquid crystals (Ruckert and Otting 2000) and DIODPC/DIOHPC bicelles (Ottiger and Bax 1999), neither of which have the thermostability of CNCs. Thus, CNCs have several unique strengths to add to the existing library of NMR alignment media. Conversely, there are some limitations to CNC alignment media. In our experience, the most stringent requirement is that the ionic strength be maintained in the 10–20 mM range. Higher salt concentrations lead to rapid self-association of the CNCs which precludes measurement of RDCs. This limits the use of co-solutes such as guanidinium chloride or sodium sulphate, as well as ionic detergents such as SDS. However, non-ionic and zwitterionic detergents are compatible with CNC alignment media.

Based on our results, we have developed a general approach for preparing aligned samples as follows:

(1) Adjust the pH of approximately 100 mL of freshly prepared CNCs (at approximately 3% w/w) to the desired pH using NaOH.
(2) Concentrate the CNCs to between 9 and 10% by evaporation at 30°C with rapid continuous stirring. The concentration may be determined by weighing the CNC remaining after fully oven-drying approximately 200 µL samples at or above 100°C. 10% $^{2}H_2O$ may be added during this stage to minimize dilution in the following step.
(3) Add concentrated macromolecule and buffer solutions to approximately 600 µL of the concentrated CNC suspension: for example, 40 µL of a 2–3 mM protein solution and 10–20 µL of a 0.5 M buffer solution. Mix very thoroughly. For high-temperature

NMR experiments, briefly degas the sample under vacuum to prevent formation of bubbles.

(4) Transfer into an appropriate NMR tube and immediately insert into the spectrometer. Allow the alignment to fully equilibrate for 2–4 h. This equilibration step need only be performed once. Samples can be stored in the refrigerator for weeks to months, and immediately yield reproducible RDCs upon being returned to the spectrometer.

In summary, cellulose nanocrystals are a robust, versatile and inexpensive alignment medium for NMR residual dipolar coupling studies of macromolecules. We believe that until now, their use has been hampered by their slightly unusual handling requirements. In this study we present a detailed description of their behaviour, offer a general protocol for preparing aligned samples, and discuss the strengths and weaknesses of this medium. This information will hopefully allow cellulose nanocrystals to be fully exploited in future biomolecular NMR studies.

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