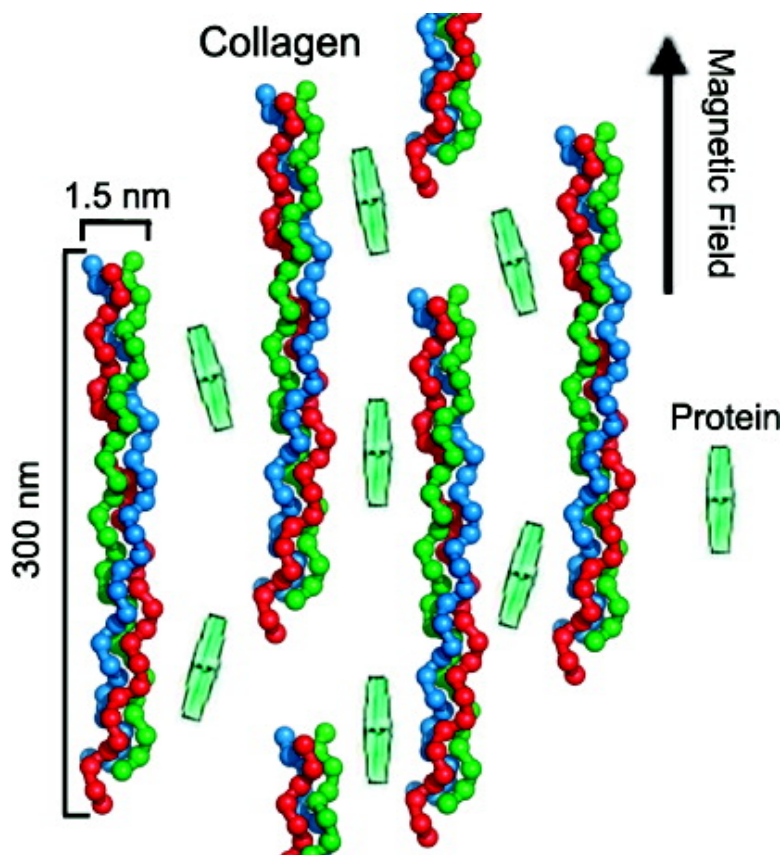## Communication

Weak Alignment of Biomacromolecules in Collagen Gels: An Alternative Way to Yield Residual Dipolar Couplings for NMR Measurements

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Weak Alignment of Biomacromolecules in Collagen Gels: An Alternative Way to Yield Residual Dipolar Couplings for NMR Measurements

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Measurement of residual dipolar couplings (RDCs) between pairs of nuclei has become a routine method for macromolecular structure determinations by solution NMR spectroscopy.[1] Compared to the short-range restraints derived from interproton distances, torsion angles, and *J* couplings, the RDCs can provide unique long-range orientation restraints of internuclear vectors to the molecular alignment frame[2] and, thus, are very useful in improving the accuracy and quality of protein NMR structure. This type of information is essential when the molecule under study is multi-domain.[3] RDCs can also provide important internal dynamic information of biomolecules.[4] To measure the RDCs, a weak alignment of molecule in a magnetic field is required. As such, various technologies for creating the necessary partial alignment in solution have been developed, including direct magnetic align-ment,[5] alignment in different liquid crystals,[6] or dissolving the protein in a highly hydrated, anisotropically compressed polyacry-lamide gel.[7] Recently, new detergent-compatible liquid crystals based on DNA nanotubes[8] and nucleic acid G-tetrad structures[9] have been successfully exploited.

Although currently available alignment media are quite diverse, their application is still molecule specific. Most of them are suitable only to the application of water-soluble protein. Only a few are compatible with the detergents used in membrane protein study. In addition as the effective size gets larger, alignment order will significantly increase and lead to extensive broadening of the NMR resonance signals due to the strong $^1H-^1H$ long-range dipolar couplings. The most effective way to reduce order is to scale down the alignment media concentration. Most media however are not stable at low concentration. Bicelles become unstable when the concentration is lower than 2.5% (w/v),[10] poly(ethylene glycol) is easy to phase separate when the concentration is less than 3% (w/w),[6d] and cetylpyridinium bromide–hexanol mixtures will lose ^2H quadrupolar splitting at lower concentration, e.g., ∼2.5% (w/v).[11] Besides the DNA-based liquid crystals, the poly(acrylamide) gels are the only medium that can provide weak alignment in the presence of detergents.[7] Potential drawbacks relate to the pore size of the gel matrices, which can make it difficult to dissolve larger protein–micelle complexes[12] and which can adversely affect the rotational correlation time.[7b]

Here we describe a new gel system that can weakly align a molecule for solution NMR spectroscopy. The aligned gels were obtained through the polymerization of ordered collagen in a magnetic field. Anisotropy in the collagen gel can be detected in the form of quadrupole splittings in the ^2H NMR spectra. Experi-ments were carried out on uniformly $^{13}C/^{15}N$ enriched glutamine-binding protein (GlnBP), a 226 residue protein. The structure of
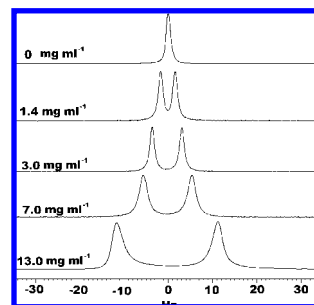

Figure 1. 1D ^2H spectra of collagen gel samples in 200 mM tris, 200 mM NaCl, pH 7.5, 90%/10% H_2O/D_2O with different initial collagen concentra-tions. These spectra were recorded at 37 °C with four scans and a sweep width of 300 Hz on a Bruker DMX 600 MHz spectrometer.

GlnBP has been determined by X-ray crystallography.[13] To date, there are ∼28 different types of collagen described in literature.[14] But only type I and III collagens isolated from tissues can polymerize *in vitro*.[15] In this study, the rat tail tendon type I is selected for preparing the collagen gels. The pH value and temperature are two controlling factors for inducing the collagen polymerization. The polymerization is usually very weak at lower pH value or lower temperature but becomes significantly faster at the pH range 6–8 and temperature range 25–42 °C. The collagen monomer was prepared as described by Saffarian et al.[16] It is kept dissolved in acetic acid at pH value ∼3. We neutralized the collagen solution by adding 2 M Tris/HCl, pH 8.0. The pH value was measured to make sure it is ∼7.5 (can be adjusted by adding NaOH). An appropriate amount of protein, D_2O, and NaCl were added into the collagen solution to make a final sample of 200 mM Tris/HCl, 200 mM NaCl, ∼150 uM protein, and 10% D_2O. This was all carried out at 4 °C. The mixture was put into an NMR tube and placed in a Bruker DMX 600 MHz magnet. To achieve initial alignment of the collagen monomer in the magnetic field, the temperature was kept at 4 °C for 3 h. Subsequently it was increased to room temperature at a rate of 1 °C/10 min. This was followed by elevating the temperature to 37 °C at a rate of 1 °C/15 min for polymerization. The quadrupolar splitting of the ^2H NMR signal of the solvent could be observed when the temperature reached 32 °C. This splitting stabilized in 2 h after the temperature is increased to 37 °C.

As expected, the quadrupolar splitting observed in the ^2H NMR spectrum increased with increasing collagen concentration (Figure 1), indicating that the ordering effect induced by collagen gel can be tuned by adjusting its concentration. After polymerization, the ^2H splitting of the collagen gel becomes stable and totally temperature independent over the entire range tested (5–40 °C). The polymerization is associated by the color change of the sample,
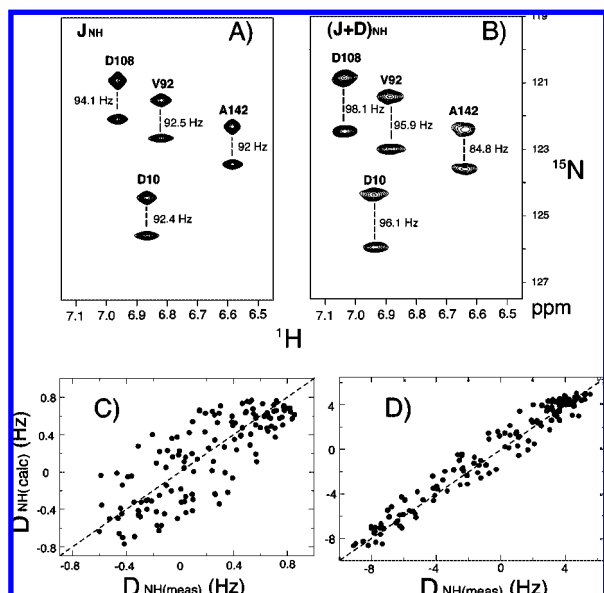
Figure 2. (A) ^{1}H−^{15}N IPAP-HSQC[17] spectra for 150 μM uniformly ^{13}C and ^{15}N labeled GlnBP in 200 mM tris, 200 mM NaCl, pH 7.5 buffer condition. J_{NH} splittings are shown for the isotropic sample. (B) $(J+D)_{NH}$ splittings are obtained from the 13 mg mL^{-1} collagen gel. Spectra containing the upfield and downfield ^{15}N-{^{1}H} doublet components of the ^{1}H−^{15}N IPAP-HSQC spectra are superimposed. Measurements were conducted on a Bruker 600 MHz DMX NMR spectrometer at 37 °C. Correlation between measured ^{15}N−^{1}H RDCs of GlnBP in (C) 1.4 mg mL^{-1} and (D) 13 mg mL^{-1} collagen gel and couplings calculated on the basis of 1.94 Å X-ray crystal structure of GlnBP.[13] Uncertainties in the measured dipolar couplings are (C) 0.4 Hz and (D) 0.6 Hz; the correlation r-factor between the measured and calculated is (C) 0.796 and (D) 0.981.

from clear to slightly opaque white. The opaqueness is correlated to the concentration of the collagen (see the Supporting Information).

Figure 2A and B show ^{1}H−^{15}N IPAP-HSQC[17] spectra for the uniformly ^{13}C/^{15}N labeled GlnBP in the absence and presence of 13 mg mL^{-1} collagen. Figure 2C and D show the correlation between D_{meas} and D_{calc} of GlnBP in 1.4 mg mL^{-1} and 13 mg mL^{-1} collagen gels, respectively. The magnitude of the RDCs observed in 1.4 mg mL^{-1} ranges from −1 to +1 Hz. The relatively large experimental error (0.4 Hz) compared to the magnitude of RDCs causes the dispersion between the measured and calculated RDCs ($R = 0.796$). A 13 mg mL^{-1} collagen gel yielded ∼10 times larger RDCs than 1.4 mg mL^{-1} collagen gel, and the dipolar couplings show good agreement with the X-ray structure ($R = 0.98$).

The collagen gels also showed good pH and detergent tolerance. The collagen gels (1.4 mg mL^{-1}) were still stable after being soaked in a low pH acetate buffer solution (pH = 4) or in a 100 mM DPC solution for several days. This was evaluated by the ^{2}H splitting that could still be observed (Supporting Information).

The collagen molecule is a rod ∼1.5 nm in diameter and 300 nm long, substantially smaller than Pf1 phage particles, which have a diameter of ∼6.5 and 2000 nm in length.[6c] The smaller size and their organization lead to a smaller net susceptibility for the collagen monomer, thus necessitating a longer time to align in the magnetic field. However polymerization essentially locks that alignment so that the sample is permanently ordered after the initial preparation. Previous studies revealed the structure of collagen.[18] It is made up of three left-handed polypeptide strands which are twisted together into a right-handed coiled coil. The numerous hydrogen bonds

between proline and hydroxyproline will form a cooperative quaternary structure that highly stabilizes the collagen helix. This highly stable structure makes collagen gel quite suitable for biomolecular NMR measurements over a wide range of temperature or in various physical conditions, such as low pH, high ionic strength, or in with detergents. At the above concentrations, the collagen gels are very soft. This permits buffer exchange readily through diffusion. This also allows the addition or removal of protein after polymerization in the same manner.

The collagen gel provides a robust and cost-effective alternative to align large biomolecules and membrane proteins in a magnetic field. It can be polymerized at various alignment directions relative to the field, thus affording another mechanism to modulate the size of the observed RDCs.[7b,19]

Supporting Information Available: Photographs of collagen samples before and after polymerization; ^{2}H splitting of collagen samples as a function of temperature, at low pH value buffer condition and in DPC solution; ^{2}H splitting of collagen as a function of the length of polymerization time. This material is available free of charge via the Internet at http://pubs.acs.org.

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