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A simple apparatus for generating stretched polyacrylamide gels, yielding uniform alignment of proteins and detergent micelles*

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Abstract

Compressed and stretched polyacrylamide hydrogels previously have been shown to offer a robust method for aligning proteins. A simple, funnel-like apparatus is described for generating uniformly stretched hydrogels. For prolate-shaped proteins, gels stretched in the direction of the magnetic field yield two-fold larger alignment than gels compressed to the same aspect ratio in this direction. Empirically, protein alignment is found to be proportional to $(c-2.3)^2$ $[(d_o/d_N)^3\text{-}1]$, where d_o and d_N are the diameters of the cylindrical gels before and after stretching, respectively, and c is the polyacrylamide weight fraction in percent. Low gel densities, in the 4–7% range, are found to have minimal effects on macromolecular rotational correlation times, τ_c, and no effect of the compression ratio on τ_c could be discerned over the range studied ($d_o/d_N \leq 1.4$). Application is demonstrated for a sample containing the first Ig-binding domain of protein G, and for a detergent-solubilized peptide.

NMR spectra of small molecules dissolved in organic liquid crystalline media permit measurement of large numbers of intramolecular dipolar couplings that carry very precise information on molecular structure (Saupe and Englert, 1963; Emsley, 1996). However, for molecules with more than half a dozen protons, the strong degree of solute alignment obtained in such media typically results in a vast number of large, non-first-order splittings that make the spectra extremely difficult to analyze. On the other hand, very weak alignment, caused by a molecule's own magnetic susceptibility anisotropy, also gives rise to measurable dipolar couplings (Bastiaan et al., 1987), but only the largest dipolar couplings are detectable and manifest themselves as small, field-dependent changes in the corresponding J splittings. The advantage of such ultra-weak alignment is that the spectrum retains its regular high-resolution simplicity. Tolman et al. (1995) demonstrated that small dipolar couplings, on the order of a few hertz, could be measured for the backbone amides in paramagnetic myoglobin and that these data correlate well with values expected on the basis of its crystal structure. Tjandra et al. (1997) subsequently demonstrated that measurement of a substantial number of ^{15}N-^{1}H and $^{13}C^{\alpha}-^{1}H^{\alpha}$ couplings in a diamagnetic protein-DNA complex can yield a remarkable improvement in the structure of the protein, and permits definition of the relative orientation of structural elements with few interconnecting NOEs. General applicability of this approach greatly increased upon introduction of liquid crystalline media for inducing a tunable degree of macromolecule alignment (Bax and Tjandra, 1997; Tjandra and Bax, 1997; Hansen et al., 1998; Clore et al., 1998; Prosser et al., 1998; Barrientos et al., 2000; Fleming et al., 2000; Ruckert and Otting, 2000), which makes it possible to measure a whole plethora of short-range dipolar interactions, including those between directly bonded ^{1}H-^{13}C, ^{1}H-^{15}N, ^{13}C-^{13}C, and ^{13}C-^{15}N pairs, and also between spatially proximate ^{1}H-^{1}H, ^{1}H-^{13}C and ^{1}H-

*Technical drawings of the apparatus described in this paper are available as supplementary material and may be downloaded from http://spin.niddk.nih.gov/bax/hardware/gel-Funnel

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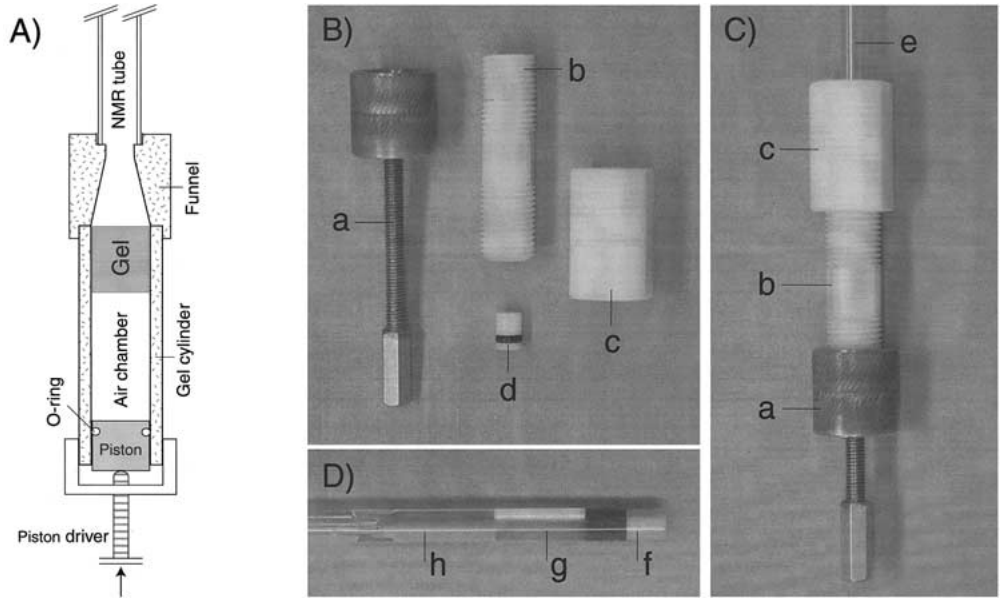

Figure 1. Apparatus for stretching the gel and inserting it in the open-ended NMR tube. (A) Schematic drawing. (B, C) Photograph of the dis-assembled and assembled gel-stretcher. (D) Open-ended NMR tube with the Shigemi plunger above the gel, and the vespel plug and teflon sleeve below the gel. The various components are: (a) Piston driver; (b) gel cylinder; (c) funnel; (d) piston with o-ring; (e) open-ended NMR tube; (f) vespel bottom plug of assembled NMR cell, with teflon sleeve; (g) stretched gel; (h) Shigemi plunger. Detailed dimensions of the gel-stretcher can be downloaded from http://spin.niddk.nih.gov/bax

^{15}N spin pairs (Tjandra and Bax, 1997; Ottiger and Bax, 1998b; Yang et al., 1999; Permi et al., 2000).

An interesting alternative method for weakly aligning biological macromolecules is the use of strain-induced alignment in a gel (SAG) (Tycko et al., 2000; Sass et al., 2000). This method relies on anisotropically compressed polyacrylamide gel, which forms an extremely stable and inert environment for solute proteins and even permits their study under partially or fully denaturing conditions (Shortle and Ackerman, 2001). As we demonstrate here, stretched gels can also be used for aligning detergent-solubilized peptides, thereby avoiding the need for chelating paramagnetics to such micellar systems (Veglia and Opella, 2000).

Previous applications of polyacrylamide gels for aligning proteins primarily used compression in the z-direction, but radial compression has also been demonstrated. This was achieved by rehydrating a dried gel within the NMR sample tube, after first casting it in a larger diameter cylinder (Sass et al., 2000). However, the volume of the rehydrated gel remained considerably smaller than that of the original gel, resulting in a denser gel with larger adverse effect on rotational diffusion, and increasing the problem of magnetic field inhomogeneity across the sample. If radial compression can be obtained without this compaction, the same degree of compression in the radial direction intrinsically yields two-fold stronger alignment than axial compression for prolate shaped solutes. Radial compression is therefore desirable for elongated proteins as it permits the use of lower gel concentrations.

Here we describe a simple mechanical apparatus for making uniformly stretched (radially compressed) gels, without requiring the dehydration step. Solute dipolar couplings are shown to be tunable over a wide range, and the low gel concentrations needed result in minimal adverse effects on solute rotational diffusion and static field inhomogeneity.

Stretching of the gel in the axial direction of the NMR tube is obtained by forcing a cylindrical gel of diameter d_o into an open-ended NMR tube of smaller internal diameter d_N. After the gel has been squeezed into the NMR tube, the pores within the gel on average will be prolate-shaped with their long axis parallel to the NMR tube. The long axis of the pore has a length that is $(d_o/d_N)^2$ larger than the average pore diameter before compression, and the short axes are reduced by a factor d_o/d_N.

The funnel-like device used for radial compression of the gel consists of four pieces: the funnel, the gel cylinder, and the piston, all made of teflon, and a brass

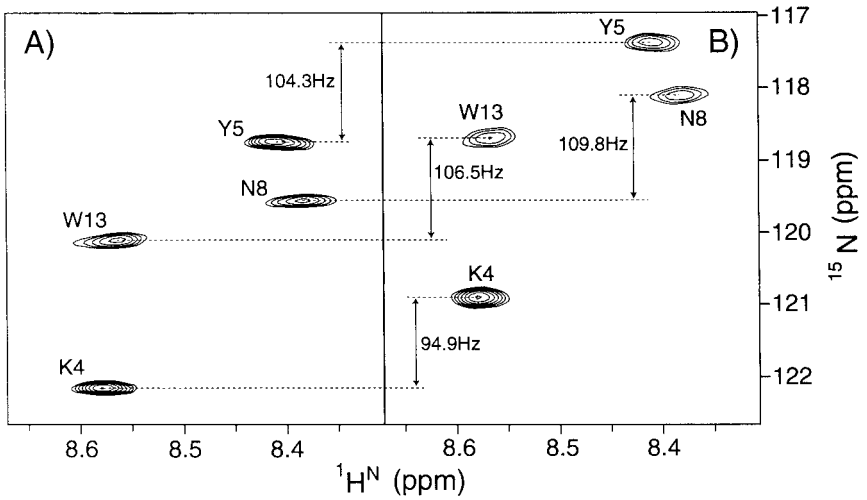

Figure 2. Small region of the ^{1}H-^{15}N IPAP-HSQC spectrum of the peptide PALKYWWNLLQYWSQELKNSAVSL (1 mM) solubilized in 100 mM DHPC, in the presence of 6% polyacrylamide gel, radially compressed by a factor $d_o/d_N = 1.42$, i.e., stretched by a factor 2. In the absence of detergent, the peptide is completely insoluble in water, i.e., the peptide is 100% micelle-bound.

piston driver (Figure 1). Three different diameter gel cylinders, with corresponding funnel and pistons were made, with inner diameters ranging from 5 to 6 mm. Initially, the gel (typically 250–270 μl in volume) is polymerized in the gel cylinder, so that the diameter of the gel is equal to the internal diameter (ID) of the cylinder. The gel is then removed from the cylinder and can be washed in order to dilute the concentrated buffer needed to maintain the desired pH during polymerization (see below). It is then reinserted at the top of the gel cylinder, as shown in Figure 1A, while the bottom end is sealed by the O-ring of the piston, thus creating a closed air chamber between the gel and the piston. Subsequently, the funnel and the plug driver are screwed onto the cylinder (Figure 1A). Rotation of the piston driver then slowly drives the O-ringed piston into the gel cylinder, increasing the chamber pressure, and forcing the gel into the funnel of decreasing ID. A Wilmad open-ended NMR tube (4.24 ± 0.012 mm ID) is mounted on the narrow opening of the funnel (4.1 mm ID) to receive the stretched gel. The tube has been coated with propyltrimethoxysilane, in order to minimize friction between the gel and the wall of the tube. For detergent containing gels such coating is found unnecessary. The bottom of the open-ended tube is subsequently closed with a home-made vespel susceptibility-matched plug, including a teflon sleeve for proper sealing. The top end of the tube is sealed with a regular, susceptibility matched Shigemi (Allison Park, PA) microcell plunger.

All gels were prepared from a stock solution containing 36% w/v acrylamide and 0.94% w/v N,N′-methylenebisacrylamide, which yields an acrylamide/bisacrylamide molar ratio of 83:1. The protein-gel samples were all prepared by first mixing 0.7 mM of the ^{15}N-labeled first-Ig-binding-domain of protein G (further referred to as G/B1), the appropriate amount of acrylamide/bisacrylamide stock solution, 0.08% w/v ammonium persulfate, 50 mM phosphate buffer (pH 5.8), 2 mM sodium azide, and 10% D_2O to make a total volume of 260 μl. Polymerization was initiated by adding and mixing 0.7% v/v N,N,N′,N′-tetramethylethylenediamine (TEMED), and continued overnight to ensure completion of the polymerization process. The latter is found necessary for minimizing gradual expulsion of water from the gel upon compression.

The change in pK of the acrylamide upon polymerization results in a large increase in pH, which in the case of protein G/B1 was compensated by soaking 70 mM of HCl into the sample for every mol of acrylamide. The final pH of all protein G/B1-gel samples is 6.0 ± 0.2. In order to avoid locally very low pH values when adding the acid, an alternate procedure uses a high concentration of phosphate buffer (200–300 mM, pH 5.8) during polymerization, which is subsequently diluted by rinsing the gel after it has polymerized (see below).

For studying the relation between the degree of gel stretching and the magnitude of protein G/B1 alignment, samples containing 7% polyacrylamide gel were

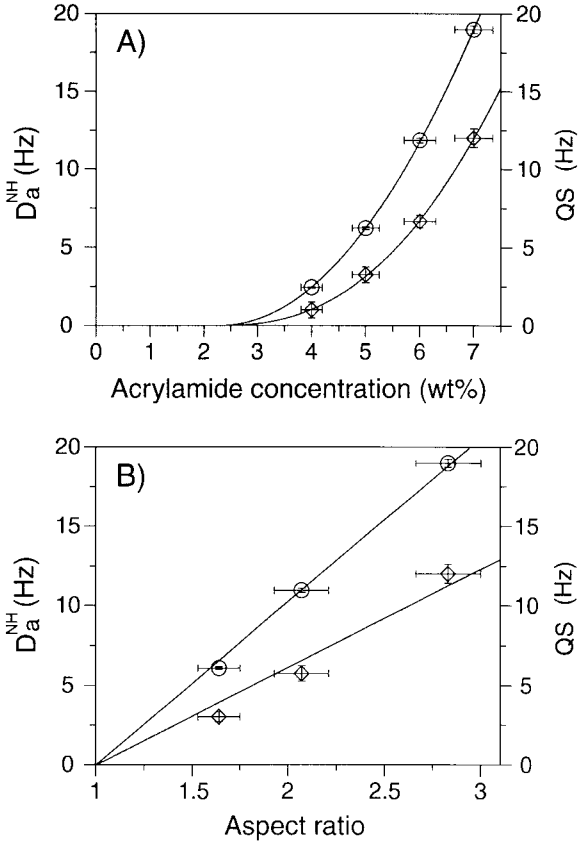

Figure 3. Dependence of G/B1 alignment tensor magnitude (○) and solvent ^{2}H quadrupolar splitting (◊) on (A) acrylamide concentration and (B) the aspect ratio of the compressed gel, $(d_o/d_N)^3$. The curves drawn in (A) correspond to $D_a^{NH} = 0.87 \times (c-2.3)^2$ and $QS = 0.29 \times (c-2.3)^{2.4}$, for $d_o/d_N = 1.42$. The straight lines in (B) correspond to $D_a^{NH} = 10.3 \times [(d_o/d_N)^3-1]$, and $QS = 6.2 \times [(d_o/d_N)^3-1]$, for an acrylamide concentration of 7%.

compressed from initial diameters, d_o, of 6 ± 0.1, 5.4 ± 0.1, and 5.0 ± 0.1 mm to the final diameter, d_N, of 4.24 ± 0.012 mm. The effect of gel density on protein alignment was studied by compressing 4%, 5%, 6%, and 7% gels by the same amount ($d_o = 6$ mm, $d_N = 4.24$ mm).

To illustrate the utility of the gel for aligning polypeptides dissolved in detergent micelles, a 6% gel containing a DHPC-solubilized membrane-associating peptide (PALKYWWNLLQYWSQELKNSAVSL) was compressed from $d_o = 6$ mm to $d_N = 4.24$ mm. The polymerization components are the same as described above for protein G/B1-gel samples, and are mixed with a stock solution containing 1 mM ^{15}N/^{13}C/^{2}H-labeled peptide and 100 mM dihexanoyl phosphatidylcholine (DHPC). For this sample, a 300 mM phosphate buffer (pH 6.0) was used during polymerization to prevent the pH from exceeding 8, where DHPC becomes quite susceptible to hydrolysis (Ottiger and Bax, 1998a). A subsequent wash (~1 hour) of the gel in a pH 6.8 buffer, containing 100 mM DHPC and 2 mM azide, was used to adjust the pH and reduce the ionic strength. Translational diffusion of the peptide-micelle was measured to be extremely slow ($\sim 2\times10^{-7}$ cm^2 s^{-1}), whereas diffusion obstruction of small molecules and ions is found to be minimal. The wash therefore resulted in negligible sample dilution, but brings the buffer condition in the gel close to that of the rinse solution in which it is immersed.

DHPC is in equilibrium between monomeric and micellar forms, and translational diffusion is measured to be 4.5 times faster than for the peptide. The presence of DHPC in the rinse solution is therefore desirable. Instead of polymerizing the gel in the presence of peptide or protein, these solutes can also be diffused into the gel after it has been formed (Tycko et al., 2000; Sass et al., 2000; Ishii et al., 2001). For larger proteins and peptide-micelles this process takes a long time, especially for $d_o > d_N$, and also requires a larger amount of peptide or protein to achieve a suitable sample concentration. Both the co-polymerization and diffusion methods are used in our laboratory, with the choice depending on the system studied.

For application to protein NMR, the three most important requirements for the alignment medium are that (a) the medium must not affect the structure of the macromolecule under study, (b) it must have minimal adverse consequences on the rotational diffusion rate, as manifested in transverse ^{15}N relaxation rates, (c) it must permit adjustment of the alignment over a wide range, such that a variety of differently shaped proteins each can be tuned to the same optimal degree of alignment. Additionally, a convenient way for recovering the macromolecule is desirable. As is briefly discussed below, all these requirements are met by the SAG method.

Acrylamide gels are very inert and generally have very low affinity for protein binding. Although the effect of the medium on solute structure needs to be considered on a case by case basis, experimental evidence available so far suggests that the effect of liquid crystalline media on the structure of most proteins is negligible, even for those with considerable internal flexibility (Goto et al., 2001; Chou et al., 2001). The chemically very inert gels are expected to be equally non-perturbing.

Sass et al. (2000) found that rotational diffusion of ubiquitin in rehydrated polyacrylamide gels is inhibited by an amount that depends approximately linearly on the gel concentration. In the present study, we have evaluated its effect on protein G/B1 and on the detergent solubilized peptide. For G/B1, the ^{15}N $T_{1\rho}$ data in gel-free solution (average of 230 ± 10 ms) is found to be essentially indistinguishable from that in the gel (224 ± 8 ms in 7% gel), independent of the degree of gel stretching used. For structured residues in the detergent-solubilized peptide, the ^{15}N $T_{1\rho}$ decreased from 94 ± 10 ms for the structured region of the peptide in the absence of gel, to 84 ± 10 ms in the presence of 6% gel. Figure 2 shows a small region of the IPAP-HSQC (Ottiger et al., 1998) of the detergent-solubilized peptide. Substantial deviations (up to −21.7 Hz for Tyr^{12}, yielding $^1J_{NH} + {}^1D_{NH} = -114.4$ Hz) in the observed splittings from the isotropic $^1J_{NH}$ coupling are present in this spectrum, indicating significant ordering of the peptide-micelle. A detailed analysis of the peptide structure will be presented elsewhere.

Figure 3 shows the degree of protein G/B1 alignment as a function of both the gel concentration, and the degree of compression. An approximately quadratic dependence on concentration is seen above a 2.3% w/v threshold (Figure 3A). Also, G/B1 alignment is found to scale approximately linearly with the aspect ratio, i.e., with the third power of the radial compression ratio, d_o/d_N. So, the magnitude of the alignment tensor can be written as: $D^a_{NH} \approx 0.47\ (c-2.3)^2\ [(d_o/d_N)^3-1]$ Hz, where c is the acrylamide concentration in weight percent. For reference, the magnitude of the solvent ^2H quadrupole splitting scales according to: $QS \approx 0.151\ (c-2.3)^{2.4}\ [(d_o/d_N)^3-1]$ Hz. After this study was completed, we were kindly given access to data collected by Ishii (Ishii et al., in press), who conducted an analogous study of alignment strength for the same G/B1 domain under conditions of *axial* compression. Although their measurements were carried out at different cross-linking ratios, a roughly similar non-linear dependence on gel density was observed, and an approximately linear dependence on $[1-(d_o/d_N)^2]$. Over the range of compression ratios and concentrations studied, our results are in reasonable agreement with those of Ishii et al. However, for the same gel concentration, cross linking ratio, and aspect ratio, we find the magnitudes of the G/B1 alignment tensor and of the solvent ^2H quadrupole splitting to be approximately two-fold larger for the stretched gel than for the axially compressed gel.

Recovery of the protein from the gel can be accomplished by mincing the gel, followed by suspension for several days in a small volume of distilled water, followed by concentrating the supernatant or, if the protein permits, by lyophilization. Recovery of detergent-solubilized peptides or proteins requires that the minced gel fragments be dissolved in detergent-containing water or in an organic solvent miscible with water, followed by chromatographic separation of the peptide from the detergent.

In summary, the radial-compression apparatus yields gels that are stretched in a highly uniform manner. This variant of the SAG approach therefore offers a robust method for inducing the weak, tunable alignment needed for quantitative measurement of dipolar couplings and chemical shift effects in biological macromolecules. To the best of our knowledge, this is so far the first medium permitting alignment of detergent-solubilized polypeptides. We anticipate that gels will prove to be particularly important for studying this class of molecules, where long-range NOEs are frequently scarce or non-existent.

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