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A simple and effective NMR cell for studies of encapsulated proteins dissolved in low viscosity solvents

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Abstract

Application of triple-resonance and isotope-edited-NOE methods to the study of increasingly larger macromolecules and their complexes remains a central goal of solution NMR spectroscopy. The slow reorientational motion of larger molecules leads to rapid transverse relaxation and results in losses in both resolution and sensitivity of multidimensional-multinuclear solution NMR experiments. A recently described technique employs a physical approach to increase the tumbling rate of macromolecules in an attempt to preserve access to the full range of structural restraints available to studies of smaller systems. This technique involves encapsulation of a hydrated protein in a surfactant shell which is subsequently solubilized in a low viscosity solvent. A simple, efficient and cost effective NMR cell that accommodates the moderate liquefaction pressures required in the encapsulation method is described. Application of the method to the 56 kD triose phosphate isomerase homodimer is demonstrated.

The development of multidimensional-multinuclear techniques in solution NMR has allowed macromolecules of increasingly larger size to be examined. However, as the size of a molecule increases, the rate of transverse relaxation also increases. This in turn limits the extent to which extended spin networks can be explored by the NMR experiment. More rapid rates of transverse relaxation also directly lead to broadening of the resonance linewidth ($\Delta\nu_{1/2} = 1/\pi T_2$) so that both resolution as well as sensitivity are compromised. A variety of approaches have been introduced to alleviate the limitations presented by rapid transverse relaxation. For example, deuteration has been successfully used to decrease the dipolar contribution to transverse relaxation (Markley et al., 1968; LeMaster, 1989; Pachter et al., 1992; Grzesiek et al., 1993; Farmer and Venters, 1998), and for example substitution of ^{2}H nuclei ($\gamma^D = 4.107 \times 10^7$ T s^{-1}) for ^{1}H nuclei ($\gamma^H = 26.752 \times 10^7$ T s^{-1}) scales the dipolar interaction by more than a factor of 40. Thus, upon deuteration the relaxation of nuclei with low CSA (i.e., aliphatic ^{13}C nuclei) are virtually decoupled from molecular motion and acquire very long T_2 values. Of course, substitution of ^{2}H nucleus for ^{1}H results in the loss of ^{1}H-^{1}H NOEs that might otherwise be detected and deuteration thereby restricts the major source of structural information available from solution NMR methods. Efforts in a number of groups to produce high-resolution solution structures based on selective reprotonation together with characterization of residual dipolar couplings have yielded promising but not yet definitive results (Venters et al., 1995; Gardner et al., 1997; Mueller et al., 2000).

Transverse relaxation optimized spectroscopy (TROSY) represents a very important strategy by which the rate of transverse relaxation of nuclei with larger CSA values (^{15}N, aromatic ^{13}C) may be decreased (Pervushin et al., 1997; Pervushin, 2000). At the appropriate field strengths, the method leads to substantial increases in resolution and sensitivity whenever nuclei with large CSA values are involved. Nevertheless, effective application of the method to polypeptides obviously requires extensive deuteration,

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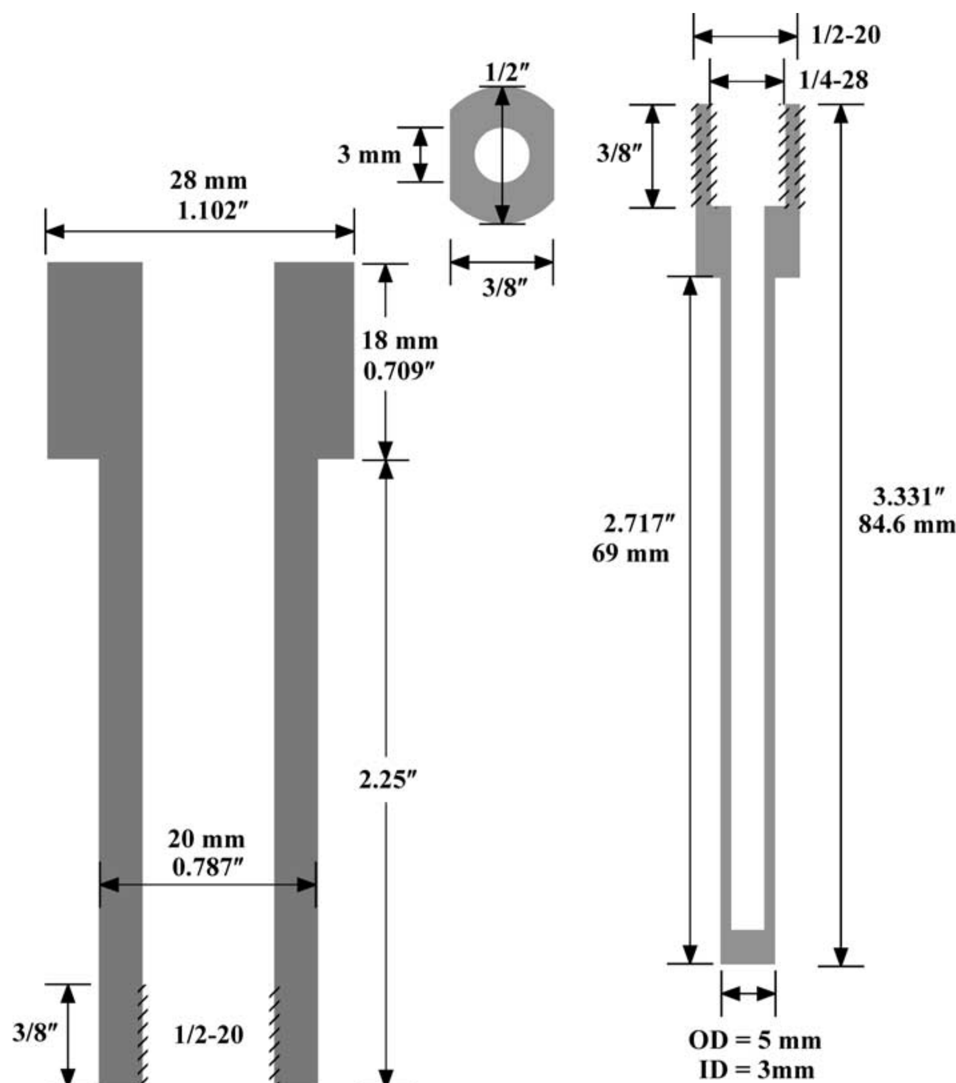

Figure 1. High pressure NMR cell design for reverse micelle samples. The sample tube section (right) was fabricated using standard machining techniques from standard virgin PEEK rod stock (McMaster-Carr Supply), and the turbine section (left) is fabricated from Delrin. The indicated 1/4-28 high pressure fitting is designed to interface to a standard PEEK nut and 1/16″ PEEK tubing (Upchurch Scientific). The sample tube holder is designed to mimic a standard spinner turbine and functions to precisely position the high pressure NMR cell with respect to the probe RF coil. This component does not bear applied pressure and may be manufactured from a variety of inexpensive and more easily machinable materials (Delrin, Nylon, Kel-F).

since the transverse relaxation of the aliphatic ^{13}C nuclei is relatively unaffected by the approach (Eletsky et al., 2001). Although providing a substantial improvement in the ability to assign ^{13}C and ^{15}N resonances, the bulk of NOE-derived information will be lost in practical application of TROSY. A general approach to the challenges posed by rapid transverse relaxation thus remains a central goal of solution NMR methods development.

Recently, we described a method that involves a direct physical approach to the problem of slow global molecular tumbling (Wand et al., 1998). The technique consists of encapsulation of a hydrated protein in a surfactant that forms stable inverted or reverse micelle particles in solution in apolar solvents (n-alkanes in the current case). We demonstrated that the observed changes in the transverse relaxation measured in a series of alkane solvents followed the changes predicted based on the bulk viscosities of the solvents (Wand

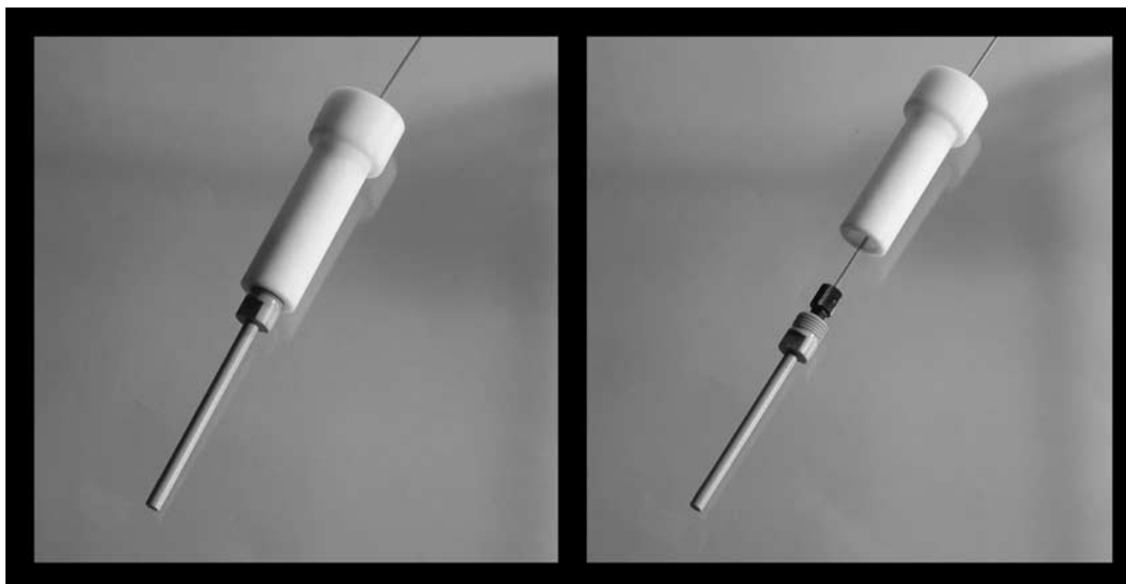

Figure 2. Photographs of the PEEK NMR cell emphasizing finishing details and assembly of the components.

et al., 1998). Bax and coworkers subsequently demonstrated the approach by employing a solvent system based on liquefied carbon dioxide (Gaemers et al., 1999). The method has been further validated to show that the structure of encapsulated human ubiquitin is essentially identical to that present in solution under more standard solution NMR conditions (Babu et al., 2001).

A fundamental requirement of the encapsulation strategy is the need to liquefy an appropriately low viscosity solvent and maintain the system in a stable condition throughout the NMR experiment. The *minimum* liquefaction pressures of n-butane, propane and ethane are 4 bar, 10.5 bar and 47 bar, respectively, at room temperature and the pressure bearing requirement may thus be satisfied with a high pressure NMR cell. The short-chain n-alkanes ($n < 5$) thus liquefy under low to moderate applied pressure and possess bulk viscosities that are sufficiently low to produce an overall increase in the rate of tumbling for an encapsulated protein over that of the same protein in aqueous solution. The bulk viscosities of n-pentane, n-butane, propane and ethane are 200 μPa·s, 158 μPa·s, 97 μPa·s and 35 μPa·s, all of which compare favorable with water, that has a bulk viscosity of 850 μPa·s. Cells with *sufficient* pressure-bearing capacity are not commercially available.

A variety of high-pressure NMR cell designs have appeared over the past 40 years (Horvath and Millar, 1991). The advantages of the cell design over the probe or autoclave design are numerous, most significantly that such designs may be used with standard NMR instrumentation without modification to probes, upper-barrel assemblies, etc., which provides ready access to the full range of current multidimensional-multinuclear experiments. A range of pressure-bearing materials have been employed in the construction of high pressure NMR cells, including glass (Gordon and Dailey, 1961; Wagner, 1980), Vespel/polyimide (Vanni et al., 1978), crystalline sapphire (Roe, 1985; Urbauer et al., 1996; Taylor et al., 1997) and zirconium oxide (Ehrhardt et al., 1999) with the selection of a particular substance generally depending upon the intended operational pressure range. Recently, a NMR cell design employing polyether ketone (PEEK, CA Reg 31694-16-3) has been described that supports pressures sufficient to accommodate studies of supercritical fluids (Wallen et al., 2000). PEEK is a readily available and inexpensive material that may be injection molded or machined to suit a wide variety of purposes. Though capable of withstanding applied pressures up to 400 bar, this particular design (10 mm o.d./3 mm i.d.) is largely unsuitable for high resolution NMR spectroscopy owing to the small sample volume and low filling factor, large outer diameter, and, for the present purpose, a two piece construction that is prone to pressure leakage.

Here we report the design of a single piece pressure cell that is suitable for high resolution NMR spectroscopy of proteins utilizing a 5 mm probe. The

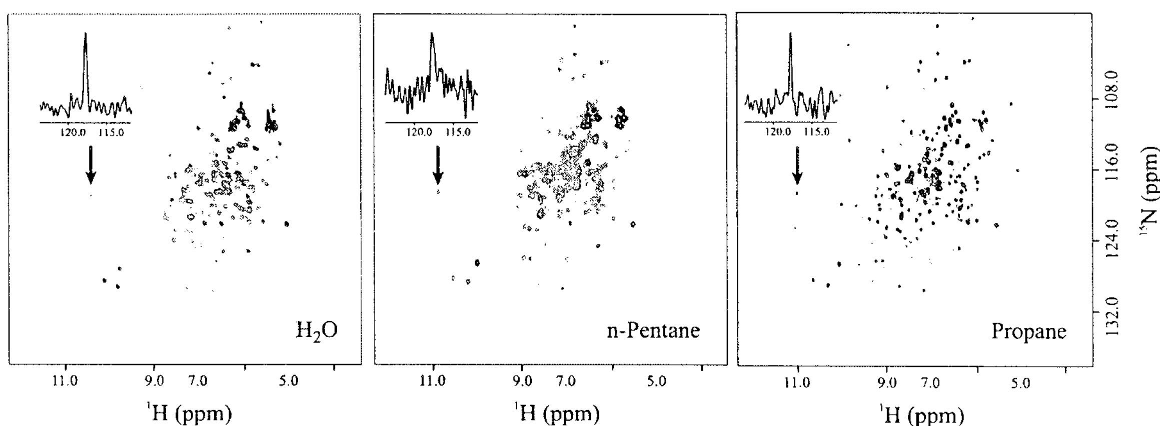

Figure 3. Series of 500 MHz (^{1}H) ^{15}N-HSQC spectra of *T. Brucei* triosphosphate isomerase in free aqueous solution (left) and encapsulated in AOT reverse micelles in n-pentane (middle) and in n-propane (right). All spectra were recorded at 20 °C. The spectral widths in the ^{1}H and ^{15}N dimensions were 8000 Hz (512 complex points) and 2000 Hz (128 complex experiments) respectively. The protein concentrations were $\sim$0.5 mM (monomer) in the H_2O HSQC data; and $\sim$0.3 mM in the encapsulated (propane and pentane) data sets. Insets show cross-sections through the indicated cross peak along the nitrogen dimension.

design is highly suitable for high-resolution NMR studies involving encapsulated proteins dissolved in low viscosity solvents such as n-alkanes. The tube described will also serve general high-pressure NMR applications and withstand applied pressures of up to at least 500 bar. The current design incorporates important improvements over previous implementations, requiring fewer fittings and employing a PEEK high pressure tube section within a Delrin tube holder. This design ensures direct access to standard NMR instrumentation without addition or modification, i.e., conforms to the standard dimensions of a 5 mm NMR tube and spin turbine. The effectiveness of the NMR cell as well as the encapsulation approach is demonstrated using a series of ^{15}N-HSQC spectra recorded for triose phosphate isomerase (TIM) in aqueous solution and encapsulated within dioctyl sulfosuccinate (AOT) reverse micelles dissolved in pentane and propane.

The NMR cell consists of a 5 mm outer diameter and 3 mm inner diameter tube section and a headpiece section that accommodates a single high-pressure fitting consisting of 1/4-28 thread (Figures 1 and 2). The use of a single high pressure port represents an important design improvement since the integrity of the cell is fundamentally dependent upon pressure holding capacity. This high pressure fitting is a standard HPLC-type fitting rated to over 338 bar (5000 PSI). Threads on the outer surface of the headpiece section consist of 1/2-20 thread and allow the main section to be positioned within a collar that is designed to mimic a standard spin turbine and serves to properly position the NMR cell with respect to the NMR probe. This component does not bear applied pressure and may be manufactured from a variety of inexpensive and easily machinable materials (Delrin, Nylon, Kel-F). Associated PEEK tubing and high pressure valves were obtained from commercial sources (Upchurch Scientific).

The PEEK NMR cell is readily fabricated from commercially available rod stock (McMaster-Carr Supply) of raw diameter slightly larger than the desired finished dimensions (3/4″ in the present case). The long thin tube section may be turned down to the desired diameter (i.e., 5 mm) on a standard lathe. The inner diameter (i.e., 3 mm) is produced by fitting the partially finished tube in a jig that holds the tube securely as the inner diameter is drilled. A pilot drill should be run down the length of the rod to generate a guide for a reamer bit that makes the finished diameter cut. Importantly, although PEEK is not a particularly difficult material to machine, slow drill and lathe speeds must be used to minimize heat generated during the machining process that can produce defects in the finished tube.

Protein was encapsulated using a one or two cell high-pressure mixing system as previously described (Wand et al., 1998; Ehrhardt et al., 1999). Briefly, surfactant (AOT) was loaded into a high pressure mixing chamber and sealed. Solvent (propane) was liquefied in the mixing chamber and the resulting surfactant solution was pressurized and allowed to mix for several minutes to assure homogeneity. Separately, hydrated

protein is placed in the NMR cell and the cell is pre-pressurized to within 10% of the final target pressure. The protein-containing NMR cell is then connected to the mixing chamber via PEEK or stainless steel tubing, involving one of more control (ball-type) valves (Ehrhardt et al., 1999). The valve connecting the NMR cell is opened and pressure into the mixing chamber is increased to drive the surfactant solution into the NMR cell. The valve may be closed and the NMR cell is removed to the spectrometer.

NMR spectra were recorded on Varian INOVA systems operating at 500 MHz, 600 MHz or 750 MHz (^{1}H). ^{15}N-HSQC spectra were recorded using a gradient-selected, sensitivity-enhanced pulse sequence. Deuterium lock was achieved using 10% D_2O for the aqueous sample and using 20% d_{15}-n-pentane for the samples made using n-pentane and propane. For simple binary mixtures of n-alkanes, the viscosity of the mixture is approximately equal to the sum of the product of the viscosities of each pure component and its mole fraction (Chevalier et al., 1990).

The high pressure NMR cell described here will safely accommodate the pressures required to liquefy a variety of solvents with appropriate physical properties, withstanding pressures over 500 bar. Such pressures are well above those used to liquefy most common solvents (i.e., liquefaction pressure of C_3H_8 is ~ 10 bar or 150 PSI). In test, specimens with configurations of 5 mm OD and 3 mm ID were capable of withstanding applied pressures of up to 500 bar (7250 PSI), which would enable studies involving liquefied ethane to be executed. It is important to emphasize that the liquefaction pressure is the *minimum* pressure that is required; much higher pressures are anticipated to adequately explore the phase diagram of quaternary solvent-protein-water-surfactant mixtures (see Flynn and Wand, 2001). It is possible that injection-molded pieces or otherwise well-made examples may withstand substantially higher applied pressures. Importantly, failure of the tube most commonly occurs along a manufacturing defect and consists of splitting of the material accompanied by rapid equilibration of pressure. In contrast to the failure of glass, ceramics or crystalline materials that fail with extensive fracturing of the pressure bearing material, PEEK failure is confined to a very small region and thus generally does not lead to collateral damage to the NMR instrumentation.

Although the most important attribute of the NMR cell is clearly its ability to withstand the required applied pressure, spectroscopic compatibility/suitability follows closely. PEEK is chemically inert and non-magnetic and appears to produce no observable NMR signals in solution NMR experiments. Typical examples of the NMR cell described here accommodate very high resolution spectra, with non-spin lineshape resolution of 1% (v/v) chloroform in d_6-acetone recorded at 500 MHz of less than 1 Hz, 8 Hz and 16 Hz measured at 50%, 0.55% and 0.11% of the full peak height – numbers virtually identical to those produced by a standard 3 mm chloroform lineshape sample in the same (5 mm) NMR probe. The results obtained from the chloroform lineshape experiments are entirely consistent with the performance of the PEEK NMR cell under conditions common in biological NMR applications, i.e., 10% D_2O, 90% H_2O, 50 mM buffer, pH 6.0. Suppression of the water resonance by either saturation or non-exciting pulse-sequences (WATERGATE, Jump-Return, etc) is easily achieved.

To demonstrate the effectiveness of the NMR cell design described here as well as the encapsulation method in the larger sense, we have recorded a series of ^{15}N-HSQC spectra for the 56 kD TIM homodimer as shown in Figure 3. The spectrum recorded in water serves as a reference for the encapsulated experiments and was recorded using a standard glass 5 mm NMR tube. The linewidths of resonances present in the aqueous spectrum are those expected for a 56-kD protein. By comparison, the linewidths of resonances in the spectrum of encapsulated TIM dissolved in n-pentane are $\sim 50\%$ broader than those present in the aqueous spectrum and reflect the anticipated increase given the increase in the particle radius due the surfactant, ~ 15 Å, the so-called 'volume penalty' and the bulk viscosity of n-pentane. The viscosity of n-pentane is not low enough to produce a net increase in the tumbling rate of the protein over that of the free protein in water. The improvement in resolution is apparent in the ^{15}N-HSQC spectrum of encapsulated TIM dissolved in propane, and the measured linewidths are $\sim 50\%$ narrower than those present in the spectrum recorded in H_2O. The resonance linewidths thus show the expected enhancement due to the lower bulk viscosity of propane/pentane mixture, which is nearly a factor of eight less than water and about a factor of two lower than pentane. The spectra demonstrate that encapsulation may be used to produce a predictable enhancement of relaxation properties that result in substantial improvement in resolution and sensitivity.

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