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Preparation of encapsulated proteins dissolved in low viscosity fluids

Mark R. Ehrhardt, Peter F. Flynn & A. Joshua Wand*
The Johnson Research Foundation, 415 Anatomy-Chemistry Building, Department of Biochemistry & Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6059, U.S.A.

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Abstract

The majority of proteins are too large to be comprehensively examined by solution NMR methods, primarily because they tumble too slowly in solution. One potential approach to making the NMR relaxation properties of large proteins amenable to modern solution NMR techniques is to encapsulate them in a reverse micelle which is dissolved in a low viscosity fluid. Unfortunately, promising low viscosity fluids such as the short chain alkanes, supercritical carbon dioxide, and various halocarbon refrigerants all require the application of significant pressure to be kept liquefied at room temperature. Here we describe the design and use of a simple cost effective NMR tube suitable for the preparation of solutions of proteins encapsulated in reverse micelles dissolved in such fluids.

The development of multinuclear and multidimensional NMR spectroscopy now allows the structures of proteins of significant size and spectral complexity to be efficiently determined. Nevertheless, increasing molecular size imposes several important limitations. Since increasing molecular size leads to slower tumbling and correspondingly shorter spin-spin relaxation times (T_2), the standard triple resonance experiments become unreliable at room temperature for proteins larger than 30 kDa. A variety of approaches have been developed to alleviate the limitations caused by T_2 and include use of elevated temperature to promote rotational tumbling, extensive deuteration such that deuterium-decoupled triple resonance experiments are feasible (Grzesiek et al., 1993; Yamazaki et al., 1994; Venters et al., 1995), and the selection of the narrow multiplet component arising from the mutual cancellation of dipole-dipole coupling and chemical shift anisotropy in ^{1}H-^{15}N correlation experiments (Pervushin et al., 1997). As an entirely complementary strategy we have introduced an approach that can directly increase T_2 by reducing the tumbling correlation time (τ_m) of the protein. This is achieved by the encapsulation of proteins in the water cavity formed by reverse micelles in low viscosity fluids (Wand et al., 1998).

The preparation of stable well-behaved solutions of reverse micelles in low viscosity fluids is anticipated to generally require the maintenance of significant sample pressure. This is particularly true for the short chain alkanes such as propane and ethane (Smith et al., 1990), supercritical carbon dioxide (Johnston et al., 1996) or halocarbons (unpublished results) which require pressures approaching 200 bar (∼3000 psi) to achieve optimal solution behavior. These pressures are well beyond the safe operating range of commercial glass NMR tubes. Additionally, preparation of protein-containing reverse micelles is, in our hands, best done in a two-step passive phase transfer process whereby empty reverse micelles are prepared and then subsequently loaded with protein. This requires mixing under defined conditions of temperature and pressure. Several designs of high pressure NMR cells have been reported and have employed a variety of materials including thick walled glass (e.g., Wagner, 1980), Vespel (e.g., Vanni et al., 1978) or sapphire (e.g., Roe, 1985; Taylor et al., 1997) tubes or reinforced quartz capillaries (e.g., Yamada, 1974). Due to the limited solubility of protein containing reverse micelles in low viscosity fluids, we have designed a

*To whom correspondence should be addressed. E-mail: wand@mail.med.upenn.edu

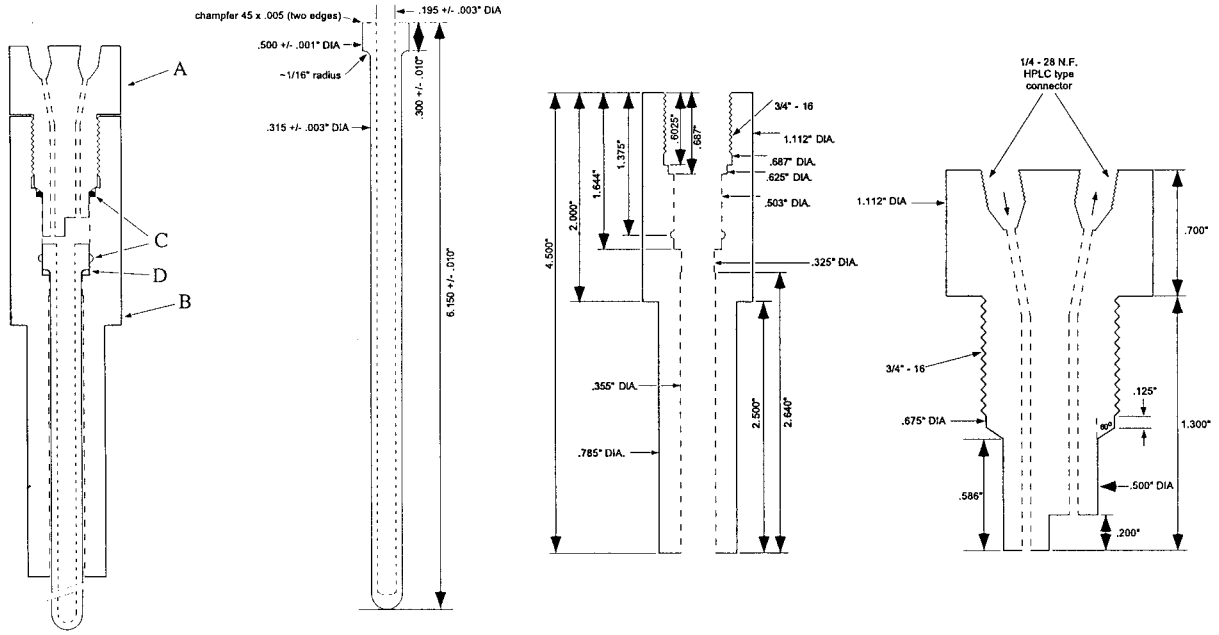

Figure 1. Schematic diagram of a custom NMR tube assembly used to prepare solutions of proteins encapsulated in reverse micelles dissolved in low viscosity fluids under significant hydrostatic pressure. The design is based on the interior dimensions of a standard Nalorac or Varian 8 mm probe and a standard Oxford high resolution shim stack assembly. The tube (E) is manufactured from zirconium oxide (O'Keefe Ceramics) and is joined to a custom manufactured BeCu valve (A and B) using two rubber o-rings (C) and a boron nitride washer (D). No glue or epoxy is required since the embedded o-ring provides sealing at low pressure and the boron nitride washer provides sealing at high pressure. The BeCu valve assembly is designed to mimic the exterior dimensions of an NMR tube spinner. This particular design has a predicted burst pressure of ~5000 psi and has been operated without failure up to 3000 psi. Connections to the NMR cell are made through two standard female HPLC fittings.

relatively large volume (8 mm o.d./5 mm i.d.) tube fitted with a valve assembly allowing for filling with liquid and out-gassing (Figure 1). To safely reach the necessary pressures, the NMR tube is made from 'hot isostatically pressed' zirconium oxide which has a relatively high tensile strength. In this context, zirconium oxide has several advantages over sapphire including lower cost. Tubes of acceptable NMR quality (i.e., giving less than 0.5 Hz linewidth) were custom manufactured by O'Keefe Ceramics (Woodland Park, CO). Valves were constructed from BeCu and machined to accept two standard stainless steel male HPLC fittings (Figure 1). BeCu was found to be sufficiently non-magnetic such that no noticeable effects were observed even at 17.6 T. The tube is sealed to the collar without benefit of glue by the action of the embedded o-ring at low pressures and the boron nitride washer at high pressures. Commercially available o-rings were employed. Boron nitride washers were custom machined. The lower port is used for filling with liquid and the upper port is used for out-gassing. After preparation of the sample both ports are sealed with standard stainless steel valves. This particular design has been operated between 10 and 60 °C and can in principle safely operate at much higher temperatures.

The process by which the hydrated proteins are encapsulated within sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micelles in the apolar low-viscosity solvent consists of three major steps: (i) liquefaction of the apolar solvent (i.e., butane, propane, ethane, etc.); (ii) solvation of the AOT in the solvent; and (iii) transfer or distribution of the hydrated protein into the AOT-solvent phase via encapsulation. Although the techniques described here may be readily adopted by those familiar with experiments at elevated pressure, it should be emphasized that this work involves manipulation of flammable materials and/or suffocants at moderate to very high pressure and utmost caution must be exercised to ensure safe operation.

We have employed two variations of the basic approach outlined above. In both cases the solvent is first liquefied within a pressure cell in the presence of AOT. Concurrent stirring using a small conventional magnetic stir-bar is sufficient to ensure preparation of a homogeneous solution of reverse micelles in the liq-

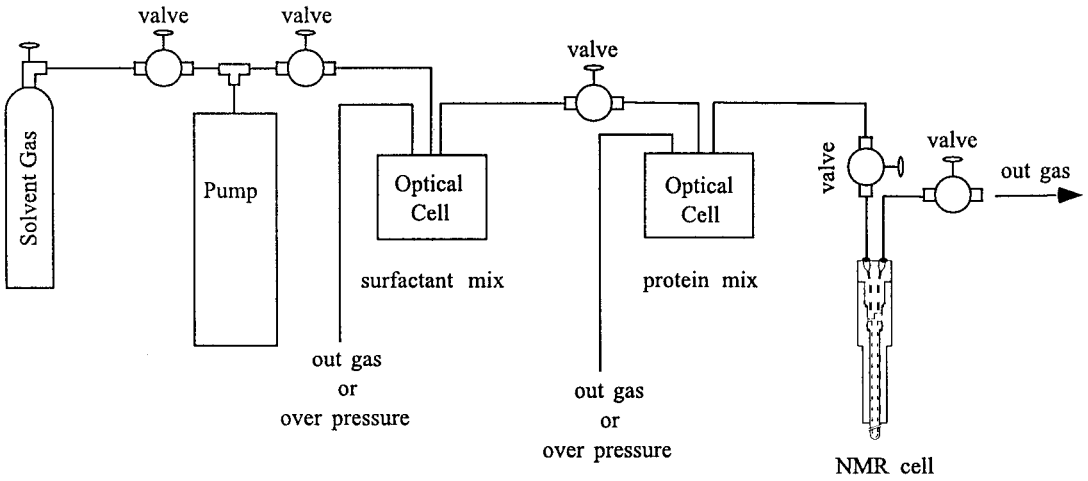

Figure 2. Schematic diagram illustrating two means of sample preparation. Source gas (or liquid) is fed to a standard high-pressure generator (HiPressure) under temperature and pressure conditions leading to liquefaction. Liquid is passed to the first mixing cell containing the desired surfactant. After sufficient mechanical mixing, the solution of dry reverse micelles is then passed to another mixing cell or the NMR tube containing the protein-water mixture. In the former case, mixing can be accomplished by gentle agitation with a magnet stir bar. In the latter case, mixing is accomplished by gentle shaking of the solution in the presence of a capillary filled with lock solvent (D_2O).

uefied solvent. Our cell is based on a standard design (Betts and Bright, 1990) and is equipped with 0.25 inch quartz windows, allowing us to safely visually monitor liquefaction and mixing. In the first scheme the solution of AOT reverse micelles is transferred into a second mixing cell which has been preloaded with the hydrated protein. The solution of AOT reverse micelles and the hydrated protein are then mixed until the desired loading of protein is achieved. In our hands, solutions as high as 0.3 mM in protein-loaded reverse micelles can be prepared using 70 mM surfactant concentrations (aggregation number $\sim$70). Long-term stability (>1 week) appears to be limited, in part, by the purity of the surfactant used. Protein loading can be followed directly by absorbance or fluorescence since the AOT solution is transparent in the near UV and visible regions of the optical spectrum. The protein-solvent-AOT system is then transferred into the NMR cell. In a second scheme, the solvent-AOT phase is delivered directly into the NMR cell, which has been preloaded with hydrated protein. In this variation, the hydrated protein is combined with the solvent-AOT phase using gentle agitation that is aided by a free-standing glass capillary (which also carries D_2O used to lock the spectrometer). In principle the former scheme provides additional control over the process, however, experimental evidence suggests that the results are generally identical and we find both methods readily applicable. As a matter of practical consequence, we have achieved excellent results using the latter method, which encompasses fewer fittings or junctions and which thereby reduces the possibility of leaks, which themselves generally lead to irrecoverable loss of sample.

Liquefaction of the solvent at a given temperature is achieved by raising the pressure of the system above the liquefaction pressure specified at the gas-liquid phase boundary by the pressure-temperature phase diagram for the solvent of interest. Elevated pressure may be generated using a pressure generator (e.g., HiPressure Equipment model 37-5.75-60) or through the use of pressurized inert gas (Ar, He, N_2, etc.). Transfer of the solution of reverse micelles to a second mixing cell or to the NMR cell is readily accomplished by creating a small pressure difference ($\sim$10 psi) between the vessels. In all transfer steps the physical properties of the solvent must be kept in mind to avoid unwanted boiling of the solvent which leads to virtually certain sample loss.

Much of the difficulty associated with making solutions of proteins encapsulated in reverse micelles dissolved in organic solvents for the purposes of NMR spectroscopy arises from the need to keep the volume of the reverse micelle at a minimum. Accordingly, having more than the minimum required amount of water to satisfactorily maintain the structural integrity of the protein unnecessarily increases the size of the reverse micelle. Furthermore, we have found that water:AOT surfactant ratios greater than $\sim$20 lead to unstable reverse micelle preparations. A water:AOT

surfactant ratio of ∼10 has given the best spectroscopic performance with ubiquitin (Wand et al., 1998).

Acknowledgements

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