Communication

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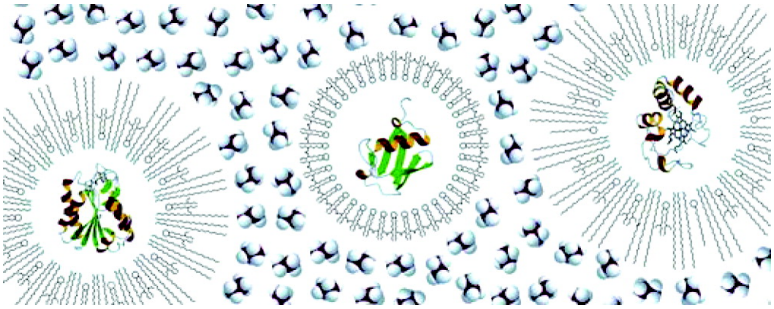

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# High-Resolution NMR Studies of Encapsulated Proteins in Liquid Ethane

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Historically, large, aggregation-prone, and membrane proteins have been difficult to examine by modern multinuclear and multidimensional nuclear magnetic resonance (NMR) spectroscopy. A major limitation presented by these kinds of protein systems is that their slow molecular reorientation compromises many aspects of the more powerful solution NMR methods. Several approaches have emerged to deal with the various spectroscopic difficulties arising from slow molecular reorientation.[1−3] One of these takes the approach of actively seeking to increase the effective rate of molecular reorientation by encapsulating the protein of interest within the protective shell of a reverse micelle and dissolving the resulting particle in a low viscosity fluid.[3]

The preparation of solutions of encapsulated proteins dissolved in solvents of sufficiently low viscosity to obtain short molecular reorientation times has been restricted to the short chain alkanes,[3] liquid carbon dioxide,[4]and recently supercritical xenon.[5] In principle, the short chain alkanes offer the most promise for achieving solutions of lowest viscosity. However, although encapsulated proteins have been solubilized at NMR concentrations in liquid propane with high structural fidelity,[3,6−8] there have been no such reports of proteins solubilized in liquid or supercritical ethane.

The difficulty with ethane arises principally from the low solubility of surfactants, such as AOT. Generally, solubility can be increased with application of elevated hydrostatic pressure.[9] Unfortunately, as we document below, the concentrations of surfactant and water required to solubilize proteins with high structural integrity are such that very high pressures must be used. A study of the dependence of maximal water loading (the molar ratio of surfactant to water) in AOT−ethane on pressure indicates that a pressure in excess of 60 MPa is required to obtain a water loading ($W_0$) of 10.[9] Water loadings of 10−20 are typically employed to encapsulate proteins with high structural fidelity.[3,6−8]

To explore the phase behavior of ethane−surfactant−water−protein systems, samples were prepared in a mixing apparatus of a design adapted from previous work[10] and then transferred to the NMR cell. The NMR cell consists of a high-pressure tube made from alumina-toughened zirconium housed in a custom-built holder with an incorporated high-pressure valve. The valve makes the NMR cell self-contained, thereby eliminating the high-pressure tubing tether present in previous designs from this lab.[11] The tube has an active volume of 200 $\mu$L. Further details of this apparatus will be presented elsewhere. The optimal properties (i.e., nonconductive) of the reverse micelle samples with respect to cryogenically cooled probe performance make up for this smaller active volume relative to conventional glass NMR sample tubes.[12]

To illustrate our ability to prepare, transfer, and study samples prepared in liquid ethane, we present $^{15}$N−HSQC spectra of ubiquitin, cytochrome *c*, and flavodoxin. These proteins have distinctly different pI values and, thus, significantly different surfactant requirements for maintenance of structural integrity upon encapsulation. The encapsulation of flavodoxin (pI $\sim$ 4.5) in cationic CTAB in propane has been previously reported[6] and is employed here for ethane. Cytochrome *c* (pI $\sim$ 11) has been shown to be encapsulated in AOT reverse micelles, but is unfolded under these conditions.[6] The $C_{12}E_4$/AOT surfactant system has been found to be more suitable for cytochrome *c*,[13] but the addition of DTAB improves it even further.[14] Ubiquitin (pI $\sim$ 6.6) encapsulates well in AOT dissolved in propane.[3]

As shown in Figure 1, all three proteins were successfully encapsulated in reverse micelles dissolved in liquid ethane. Relatively high pressures were required to stabilize the preparations. The $^{15}$N−HSQC spectra obtained are closely similar to the corresponding spectrum obtained for each protein dissolved in free aqueous solution. This collection of surfactant systems and proteins

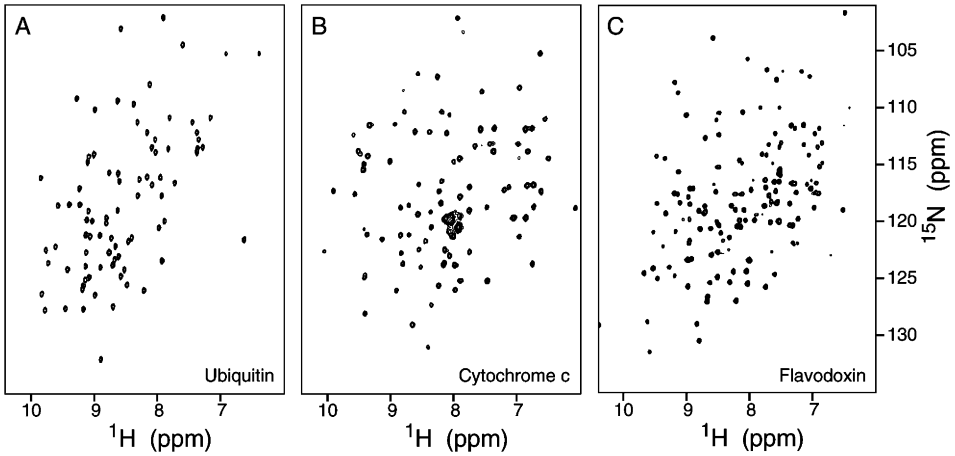


***Figure 1.*** $^{15}$N−HSQC spectra of encapsulated proteins dissolved in liquid ethane. All spectra were collected on a Varian 600 MHz NMR spectrometer equipped with a cryogenically cooled probe. (A) Ubiquitin (250 $\mu$M) in 100 mM AOT at 55 MPa. (B) Recombinant pseudo-wild-type[21] cytochrome *c* (130 $\mu$M) in 150 mM surfactant (70% $C_{12}E_4$/25% AOT/5% DTAB) at 55 MPa. (C) Flavodoxin (180 $\mu$M) in 100 mM CTAB surfactant with 8% (v/v) hexanol at 28 MPa. Spectra were obtained at 298 K, which is below the critical temperature of ethane.


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demonstrates the adaptability of reverse micelles in liquid ethane to a wide range of protein systems.

To evaluate the hydrodynamic performance of the encapsulated protein with respect to rotational correlation, we carried out $^{15}N$ spin−spin relaxation time ($T_2$) measurements.[15] It should be pointed out that at the concentrations of reverse micelles used, the classic translational diffusion equation cannot be used to faithfully extract the hydrodynamic behavior of a macromolecular assembly due to excluded volume effects.[16] For encapsulated ubiquitin in pure ethane, the $T_2$ values of rigid[17] amide sites were on average 8% longer than their counterparts in propane. Although the desired NMR performance of encapsulated ubiquitin in ethane was nominally better than in propane, it was still somewhat diminished from that in water. This is not surprising. Encapsulation of a protein introduces a significant volume penalty for molecular reorientation relative to the free protein.[18]

This penalty is difficult to overcome for small proteins, but becomes relatively less important for larger proteins.[18] In addition, as noted indirectly elsewhere,[5] the viscosity of liquid ethane increases significantly at the pressures required for encapsulation at desirable water loadings.[19] Fortunately, the performance with respect to overall tumbling can be improved even for small proteins. The key is to reduce the pressure required for optimal encapsulation. For example, $T_2$ values for rigid sites in ubiquitin encapsulated in AOT reverse micelles with less water than above ($W_0 \sim 7.5$ at 46 MPa) nominally match those obtained for the free protein in water. Furthermore, the addition of a suitable cosolvent/cosurfactant can lower the required encapsulation pressure significantly. For example, the addition of $CS_2$ (10% v/v) reduces the required pressure to 27 MPa ($W_0 \sim 10$) and results in $T_2$ values 30% longer than those obtained in propane and, importantly, 12% longer than those obtained in water. Thus, despite its small size and accompanying large volume penalty paid for encapsulation,[18] encapsulated ubiquitin has been made to tumble faster than in free aqueous solution. Similarly, for the somewhat larger protein flavodoxin encapsulated in CTAB with the cosurfactant hexanol (8% v/v), the encapsulation pressure in ethane is reduced to 28 MPa. This results in $T_2$ values for rigid sites of flavodoxin[20] that are 25% longer than those obtained in propane and 18% longer than those obtained in water, even though a large volume penalty is present due to the long chain length of the CTAB molecule.

In summary, we have reported the first high-resolution study of proteins in reverse micelles solubilized in ethane. This extends previous work[3] to a solvent with the potential to significantly improve the tumbling time of large proteins that ordinarily could not be studied by traditional NMR techniques. We have also shown that addition of an appropriate cosurfactant can reduce the minimum encapsulation pressure to values where the viscosity of ethane is significantly lower than that of propane. Finally, we have demonstrated that, despite a volume penalty paid for encapsulation, this approach can significantly improve the NMR relaxation properties of even small proteins relative to that obtained in water.

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