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Reverse Micelle Encapsulation of Proteins for NMR Spectroscopy

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Abstract

Reverse micelle (RM) encapsulation of proteins for NMR spectroscopy has many advantages over standard NMR methods such as enhanced tumbling and improved sensitivity. It has opened many otherwise difficult lines of investigation including the study of membrane associated proteins, large soluble proteins, unstable protein states, and the study of protein surface hydration dynamics. Recent technological developments have extended the ability of RM encapsulation with high structural fidelity for nearly all proteins and thereby allow high-quality state-of-the-art NMR spectroscopy. Optimal conditions are achieved using a streamlined screening protocol, which is described here. Commonly studied proteins spanning a range of molecular weights are used as examples. Very low-viscosity alkane solvents, such as propane or ethane, are useful for studying very large proteins but require the use of specialized equipment to permit preparation and maintenance of well-behaved solutions under elevated pressure. The procedures for the preparation and use of solutions of reverse micelles in liquefied ethane and propane are described. The focus of this chapter is to provide procedures to optimally encapsulate proteins in reverse micelles for modern NMR applications.

Keywords

reverse micelle encapsulation; surfactant systems; NMR spectroscopy; low viscosity fluids; structure determination; molecular reorientation

1. Introduction

A reverse micelle (RM) is a molecular assembly comprised of a nanoscale droplet of water solubilized in non-polar solvent by a surfactant layer (Figure 1A). For water-limited conditions RMs are effectively spherical or sometimes have a slight ellipsoidal shape (Fuglestad, Gupta, Wand, & Sharp, 2016). While all components of an RM system can be adjusted, water loading (W_0 , the molar ratio of water to surfactant molecules) is particularly

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important in controlling the size of the reverse micelle particle (Palazzo, Lopez, Giustini, Colafemmina, & Ceglie, 2003). Control of the hydrodynamic size of the reverse micelle particle is critical for successful solution NMR studies of encapsulated proteins (Wand, Ehrhardt, & Flynn, 1998). For a RM with a W_0 of 20, the aqueous pool of water has a radius of ~ 30 Å, contains ~ 4000 water molecules, and is surrounded by a shell of ~ 200 surfactants. RM solutions are homogeneous and the contents of the RMs exchange rapidly (Luisi, Giomini, Pileni, & Robinson, 1988; Pileni, 1993). Under these conditions, only a single protein molecule is encapsulated within a reverse micelle.

NMR spectroscopy of proteins encapsulated within reverse micelles prepared in low viscosity fluids such as liquid propane was first applied as a strategy to overcome the size limit in protein NMR (Wand, et al., 1998). The size of a RM containing encapsulated protein is much larger than the protein itself and results in a volume penalty for effective macromolecular tumbling of the protein. Rotational correlation time (τ_m) of a spherical particle is given by the Stokes-Einstein relation:

$$\tau_m = \frac{V\eta}{k_B T} \quad (1)$$

where V is the volume of the solute sphere, η is the viscosity of the solvent, k_B is the Boltzmann constant, and T is the temperature. Slowed rotational motion results in generally unfavorable relaxation properties. Fortunately, low-viscosity alkane solvents can be used to ameliorate and even overcome the volume penalty for effective macromolecular tumbling (Figure 1B) (Wand, et al., 1998). Propane and ethane, which are able to support well-behaved solutions of RMs and have very low viscosity, are gases at STP and require modest pressures for liquification. Specialized equipment is required to permit the safe and efficient preparation of samples under pressure and to carry out NMR spectroscopy (Flynn, Milton, Babu, & Wand, 2002) (Nucci, Valentine, & Wand, 2014; Peterson, & Wand, 2005). Transverse Relaxation Optimized (TROSY) methods (Pervushin, Riek, Wider, & Wüthrich, 1997; Salzmann, Pervushin, Wider, Senn, & Wüthrich, 1998; Tugarinov, Hwang, & Kay, 2004) and perdeuteration (Gardner, & Kay, 1998; Tugarinov, Kanelis, & Kay, 2006; Venters, Farmer II, Fierke, & Spicer, 1996) have largely become the mainstream methods for overcoming the size barrier in protein NMR. The TROSY approach is sometimes limited by the increased cost and decreased yields from protein perdeuteration and the loss of the richness of information provided from the abundance of ^1H nuclei. RM encapsulation can often eliminate these disadvantages by allowing fully protonated proteins to be studied.

Recent advances in RM encapsulation technology include the development of optimized surfactants (Dodevski, Nucci, Valentine, Sidhu, O'Brien, Pardi et al., 2014) and a means to control and monitor pH within the RM core (Marques, Nucci, Dodevski, Wang, Athanasoula, Jorge et al., 2014). While nucleic acids have been successfully examined [e.g. (Dodevski, et al., 2014; Workman, & Flynn, 2009)], proteins have dominated and we focus on proteins here. High fidelity RM encapsulation is now generally achievable, with a large variety of proteins with varying properties being successfully encapsulated (Nucci, et al., 2014). This represents major improvements over AOT reverse micelles, which have long

been used to study RM encapsulated proteins. Indeed, it has become clear through high-resolution NMR spectroscopy that nearly all proteins unfold in AOT reverse micelles under most conditions with ubiquitin being the only natural protein that has been documented to remain folded in AOT (Nucci, et al., 2014; Peterson, Pometun, Shi, & Wand, 2005). The reasons for this behavior are discussed below.

RM-NMR affords many advantages in protein studies by NMR in addition to the tumbling advantage. Sensitivity gains from use of cryogenic NMR probes are partially attenuated in high-conductivity (high-salt) samples, thus limiting the allowable ionic strength of the sample (Kelly, Ou, Withers, & Dötsch, 2002). RM samples effectively replace ~98% of the aqueous buffer with alkane, thus eliminating this dielectric penalty, allowing very high salt concentrations to be used in the aqueous RM core (Flynn, Mattiello, Hill, & Wand, 2000). Greatly reduced water content also allows dynamic nuclear polarization (DNP) of proteins in a standard volume solution (Valentine, Mathies, Bédard, Nucci, Dodevski, Stetz et al., 2014) though the general utility of this remains to be demonstrated. In addition, the aqueous core of a RM is a confined space that greatly destabilizes extended or unfolded states and drives proteins to the more compact less extensive state. This property allowed the study of the folded states of a destabilized three helix bundle protein (Peterson, Anbalagan, Tommos, & Wand, 2004), lipid destabilized cytochrome c (O'Brien, Nucci, Fuglestad, Tommos, & Wand, 2015), and the pressure destabilized L99A mutant of T4 lysozyme (Nucci, Fuglestad, Athanasoula, & Wand, 2014). Calmodulin is typically in an extended state without ligand in bulk aqueous conditions, but was observed to populate the compact form in reverse micelles (Xu, Cheng, Wu, Liu, & Li, 2017).

The surfactant shells of RMs have found utility as membrane mimics for use in detailed solution NMR studies of membrane-anchored proteins. Membrane-associated and membrane integral proteins are very difficult to study by conventional NMR in bulk aqueous solution conditions because of poor solution properties. Common membrane models for studying this class of proteins include detergent micelles and bicelles (Fernández, & Wüthrich, 2003; Sanders, & Sönnichsen, 2006; Soong, Xu, & Ramamoorthy, 2010; Zhou, & Dill, 2001). While useful for hosting membrane proteins for favorable solution properties, these protein/membrane model assemblies are very large. Lipid nanodiscs have recently been introduced to limit the size of these assemblies (Bayburt, & Sligar, 2010; Hagn, Etzkorn, Raschle, & Wagner, 2013). Efforts to decrease the size of nanodiscs for protein NMR are ongoing, but these studies generally require protein perdeuteration and TROSY (Hagn, Nasr, & Wagner, 2018; Nasr, & Wagner, 2018). Reverse micelles are dissolved in a low-viscosity solvent that can allow these membrane proteins to be studied via solution NMR without the need for perdeuteration or TROSY. The membrane anchored proteins myristoylated recoverin and myristoylated HIV-1 matrix protein have been encapsulated within RMs and the lipidated tails were observed to anchor into the inner surface of the surfactant shell (Figure 2A) (Kielec, Valentine, & Wand, 2010; Valentine, Peterson, Saad, Summers, Xu, Ames et al., 2010). The integral membrane protein, potassium channel KcsA, was encapsulated into reverse micelles and functional fidelity was confirmed by titration of potassium (Kielec, Valentine, Babu, & Wand, 2009). RM encapsulation of integral channel proteins is characterized by micellar caps that solubilize the hydrophilic regions (Figure 2B) (Binks, Chatenay, Nicot, Urbach, & Waks, 1989). Additionally, protein-lipid interactions

arising within a membrane environment may be observed by titration of surfactant into a protein containing RM (Figure 2C). Examples are HIV-1 matrix protein interacting with PI(4,5)P₂ (Valentine, et al., 2010) and cytochrome c interacting with cardiolipin (O'Brien, et al., 2015).

Structural restraints can readily be obtained in the reverse micelle. In fact, for large proteins dissolved in low viscosity solvents the increased tumbling can enhance the data obtained from NOESY experiments. The faster tumbling will require less (per)deuteration for optimization of T₂ relaxation, which allows for the detection of more intramolecular NOEs. Residual dipolar couplings (RDCs) are obtained by partially aligning a solution in the magnetic field and provide valuable structural restraints (Tjandra, & Bax, 1997). The phase diagram of reverse micelle surfactants is complex and can be manipulated to result in particles with sufficient anisotropic magnetic susceptibility to result in partial alignment and measureable RDCs (Valentine, Pometun, Kielec, Baigelman, Staub, Owens et al., 2006) (Figure 3). Other long distance restraints can also be obtained using paramagnetic relaxation effects (PRE) (Valentine, et al., 2014) and pseudo-contact shifts (PCS) (O'Brien, et al., 2015). High-resolution structures of proteins in different reverse micelle systems have been solved including ubiquitin in AOT reverse micelles (Babu, Flynn, & Wand, 2001), and oxidized horse cytochrome c in 10MAG/LDAO reverse micelles (O'Brien, et al., 2015) (Figure 4). A high-resolution structure of calcium saturated calmodulin (CaM) in RMs was solved showing that it adopts a compacted form (Xu, et al., 2017). Holo-CaM typically adopts an extended form in solution, but the RM structure resembles that of CaM bound to a target peptide (Zhang, & Yuan, 1998).

The core of the reverse micelle is a favorable environment to study protein hydration dynamics. Reverse micelles have been used to study water dynamics and water networks in confined environments (Dokter, Woutersen, & Bakker, 2007; Fayer, & Levinger, 2010; Piletic, Moilanen, Spry, Levinger, & Fayer, 2006). NMR has recently extended this to the study of protein-water interactions via the nuclear-Overhauser effect (NOE) (Nucci, Pometun, & Wand, 2011; Nucci, Pometun, & Wand, 2011; Nucci, et al., 2014). Encapsulation of the protein removes bulk water but maintains the native hydration shell of the protein. The advantage of the reverse micelle is to remove artifacts that arise when measuring protein hydration via the NOE in bulk aqueous solution (Otting, 1997). See Chapter XX by Jorge et al for an in depth description of how to use reverse micelles to measure protein hydration via the NOE.

RM encapsulation provides many spectroscopic advantages over bulk aqueous conditions and offers unique approaches in the biophysical study of proteins. However, care must be taken to ensure that the conditions for encapsulation are optimized to achieve the desired condition. This is most often the “native state” of the protein, which can be generally identified through simple two-dimensional spectra obtained under reference conditions. Misleading behavior of an protein encapsulated under sub-optimal conditions can sometimes occur (Nucci, Marques, Bédard, Dogan, Gledhill, Moorman et al., 2011; Nucci, et al., 2014; Senske, Smith, & Pielak, 2016). This chapter provides guidelines for preparation and optimization of samples of RM encapsulated proteins for NMR spectroscopy. We use three

commonly studied proteins of varying molecular weights as examples: ubiquitin (Ub, 8.5 kDa), interleukin-1 β (IL-1 β , 17.4 kDa), and maltose binding protein (MBP, 40.8 kDa).

2. Sample Composition Considerations

2.1 Aqueous Phase: Protein and Buffer

The choice of protein labeling scheme will depend on the NMR experiments to be undertaken and in general will not differ from standard NMR labeling. One major exception is that perdeuteration is often not needed with RM encapsulated proteins. When encapsulating proteins within reverse micelles, it is important to consider the stability of the protein at high concentration. Because the final overall concentration of protein within reverse micelle samples is necessarily lower than all other components within the sample (surfactant, co-surfactants, alkane solvent), it is often easiest to inject an aqueous solution of protein with concentrations eclipsing several millimolar in order to achieve a final overall protein concentration of 100–200 μ M. This is the so-called “direct injection” method. Some proteins (e.g. ubiquitin, IL-1 β , hen egg-white lysozyme) are amenable to lyophilization. Using the direct injection method, the lyophilized protein can then be dissolved in the necessary amount of buffer to create a reverse micelle sample with the proper water loading with a highly concentrated protein solution (Figure 5). Unfortunately, many proteins are unstable during the process of lyophilization. Some protein systems, while not amenable to lyophilization, are able to be concentrated to >5 mM (i.e. MBP, flavodoxin, cytochrome-c, MSG) to allow for the formation of reverse micelles samples with desirable protein concentrations (Nucci, et al., 2014). It is important to note that “good” solutions are generally not required to employ the injection method for encapsulation. Nevertheless, it is sometimes not possible a given protein to be either lyophilized or highly concentrated.

An alternate strategy for the preparation of solutions of proteins encapsulated in reverse micelles is the “evaporation-injection” method. This approach can usually be used to achieve desirable protein concentrations in reverse micelles samples (Dodevski, et al., 2014; Marques, et al., 2014). In the evaporation-injection approach reverse micelle samples are made from aqueous solutions containing relatively low protein concentrations (Figure 6). The sample is then exposed to a gentle low pressure N₂ gas stream to evaporate the alkane solvent and water within the aqueous nanopool. The appropriate amount of aqueous protein may then be added to reestablish the proper water loading while concurrently increasing the overall protein concentration. This procedure can be repeated until the desired final protein concentration is reached. An example is illustrated in Figure 6. In effect, it is possible to encapsulate almost all protein systems in reverse micelles of concentrations within the desired 100 – 200 μ M range. Though relatively moderate concentrations, the favorable hydrodynamic (Wand, et al., 1998) and dielectric properties (Flynn, et al., 2000) of having alkane as the bulk solvent greatly increases the signal quality of RM NMR and ample signal to noise is achieved to allow essentially all modern protein NMR experiments to be performed.

The NMR performance of reverse micelle samples are much more tolerant to a wide range of buffers than traditional aqueous NMR samples. As discussed above the bulk solvent is a low dielectric organic solvent and the signal-to-noise dependent on the quality factor (Q) of

the probe is relatively insensitive to the small amount of buffer in the sample. Therefore, it is possible to use high conductance buffers and high salt concentrations for optimal protein stability. Reverse micelles are generally tolerant to salt concentration; however, for ionic surfactants (e.g. AOT and CTAB/hexanol) it has been shown that increasing salt concentration decreases the size of the reverse-micelle and ultimately affects the stability of the reverse micelles. However, this is an adjustable parameter that can be screened and will depend on the pH, isoelectric point (PI) of the protein, and W_0 . High salt concentration is less of a concern for the zwitterionic 10MAG/LDAO mixture.

While the choice of buffer is largely dependent on the protein sample and the experiments collected, several considerations remain. Phosphate buffers are commonly used in NMR spectroscopy, but phosphate efficiently catalyzes hydrogen exchange at side chains and should not be used in hydration experiments (Edwards, & Gottfried, 1996). Buffers such as acetate, formate, imidazole, and Tris are reasonable internal indicators of pH but do suffer limitations (Marques, et al., 2014). We generally recommend using the protein itself to monitor the pH. Two-dimensional ^{15}N -correlation spectra of the protein are excellent indicators of the effective pH seen by the protein. Calibration of the pH is achieved by collecting a series of the protein in RMs with varying pH (Figure 7). For 10MAG/LDAO systems, the pH of LDAO is preadjusted before preparation. The aqueous phase in CTAB systems largely drives the pH since the headgroups are non-titratable.

2.2 Surfactants

RMs composed of mixtures of lauryldimethylamine-N-oxide (LDAO) and decylmonoacylglycerol (10MAG) were recently developed with the goal of optimized and generalized encapsulation of proteins and nucleic acids (Dodevski, et al., 2014). LDAO is a zwitterionic surfactant that requires secondary surfactants such as 10MAG at approximately 2:1 (10MAG:LDAO) molar ratio with 10–30 mM hexanol as a co-surfactant. This reverse micelle system has been shown to encapsulate the widest range of proteins thus far with only modest modifications of the composition of the mixture (Nucci, et al., 2014). The titratable LDAO head group provides strict control of the pH of the aqueous core of the RM when near their pK_a of 4.5, with an effective buffer concentration of ~1 M under standard conditions (Marques, et al., 2014). Encapsulation of proteins within 10MAG/LDAO results in the highest structural fidelity as evaluated by chemical shift differences with bulk aqueous protein (Dodevski, et al., 2014). The very low cosurfactant concentration and shorter alkane apolar chains lead to decreased tumbling time in the 10MAG/LDAO systems as compared to either AOT or CTAB/hexanol RM systems. Optimization of a “well-behaved” 10MAG/LDAO reverse micelle solution requires considerations ranging from amounts of one surfactant relative to the other and the amount of hexanol and results nearly universal protein encapsulation system with extremely high structural fidelity and encapsulation efficiency. *We generally recommend the 10MAG/LDAO surfactant system* as the first choice for all RM encapsulated protein NMR applications.

Cetyltrimethylammonium bromide (CTAB) is a cationic surfactant that has extensively been used for reverse micelle-based NMR studies. This surfactant requires the use of high concentrations of a co-surfactant, which is typically a primary alcohol. Hexanol is generally

the cosurfactant used with CTAB RMs for NMR spectroscopy of proteins (Lefebvre, Liu, Peterson, Valentine, & Wand, 2005). The optimal range of hexanol falls between 400–500 mM for most systems with 75 mM surfactant. CTAB/hexanol RMs accommodate a wide range of W_0 values. The CTAB headgroup is not titratable, so the pH stabilization effect that is present in 10MAG/LDAO systems is not present in CTAB/hexanol RMs. In addition, observed chemical shifts of CTAB/hexanol encapsulated proteins are not as close to their bulk aqueous counterparts as 10MAG/LDAO encapsulated proteins. Despite these qualifications, CTAB/hexanol RMs are simpler systems with fewer adjustable parameters to screen as compared to 10MAG/LDAO systems and are useful for many protein NMR applications.

Bis(2-ethylhexyl)sulfosuccinate (AOT) is a surfactant with an anionic head group and has historically been the most commonly studied reverse micelle surfactant system. Because no secondary surfactants or co-surfactants are necessary in order to form reverse micelles with AOT surfactant molecules, it is simple to make AOT reverse micelle samples. The procedure for pre-adjusting the pH of AOT is also relatively straightforward (Marques, et al., 2014). While it is generally simple to make reverse micelle samples with AOT, this surfactant mixture is most often very problematic. While it has been demonstrated that AOT can encapsulate ubiquitin with high structural fidelity (Babu, et al., 2001) and has even resulted in an increase in protein stability in one engineered protein (Peterson, et al., 2004) this favorable result is rare. Even with extensive efforts at optimization, most proteins encapsulated with high efficiency within AOT reverse micelles almost all are largely or completely unfolded (Marques, et al., 2014). *For this reason, we generally discourage the use of AOT reverse micelles for studies of proteins unless detailed effort is undertaken to confirm the structural fidelity of the protein.*

2.3 Bulk Alkane

There are surprisingly few solvents that can support stable solutions of proteins encapsulated in RMs that have sufficiently low viscosity to overcome the volume penalty. The shorter chain alkanes are a prominent family of solvents having these characteristics (Wand, et al., 1998). Pentane, hexane, heptane, and isooctane are liquids at room temperature and pressure and can be employed using commercially available NMR tubes with screw-top caps to prevent evaporation. Sample preparation is straightforward with these solvents. However, only pentane provides even a modest tumbling advantage for larger proteins (>~35 kDa) and a slight penalty for smaller proteins. Hexane, heptane, and isooctane slow tumbling and should only be used in situations where the effects of increased protein spin-spin relaxation can be tolerated. Butane provides a modest tumbling advantage for larger proteins but requires ~50–100 psi pressure to support stable RMs. NMR tubes with pressure ratings of 300 psi equipped with brass vacuum line fittings are commercially available and are recommended for use with butane. Propane and ethane provide great tumbling advantages for proteins but require much higher pressures to retain the liquid state (3000–7000 psi). Specialized NMR tubes and mixing apparatus are required for these very low-viscosity solvents (Flynn, et al., 2002; Peterson, et al., 2005). Despite the greater effort involved, the power of using ethane for enhanced tumbling has been demonstrated on proteins as large as the 81 kDa malate synthase G (Dodevski, et al., 2014; Nucci, et al., 2011).

3. Spectroscopic Considerations

Unlike standard bulk water samples of proteins, solutions of proteins encapsulated in RMs impose a number of spectroscopic issues. Deuterated solvents and surfactants are often required to avoid spectral artifacts arising from residual ^1H signals. Surfactant aliphatic proton resonances are upfield of water and are only a concern for ^1H -detected, ^{13}C -edited experiments. For ^{15}N -edited experiments, protonated surfactants signals are easily suppressed using an echo-antiecho gradient-selected experiment and this type of quadrature detection for the incremented time domain is recommended for this reason. Reverse micelle encapsulation and stability are not affected by deuteration for most surfactants. The only exception is deuterated AOT, which can be unstable. Deuterated versions of the cosurfactants, surfactants and solvents discussed here are all commercially available. If a desired deuterated solvent or surfactant are not available or residual their residual ^1H resonances remain problematic the WET suppression technique can be used with great success (Smallcombe, Patt, & Keifer, 1995).

4. Method for Screening Reverse Micelle Conditions

Optimal RM encapsulation conditions are most easily determined in alkanes that are liquids at ambient pressure i.e. pentane, hexane, or heptane. Due to the tumbling penalty arising from the higher viscosity of longer chain alkanes, we recommend using pentane for all but the smallest proteins. Pentane is volatile and must be handled with care. Proteins and buffers used for demonstration are: 15 mM ^{15}N -Ub in 50 mM sodium acetate, pH = 5.0 for 10MAG/LDAO and AOT RMs with Tris, pH = 8.3 for CTAB/hexanol RMs. 5 mM ^{15}N -IL-1 β in 50 mM sodium acetate, 5 mM DTT, pH = 5.0. 6 mM ^{15}N -MBP in 50 mM sodium phosphate, pH = 7.1 with 7 mM β -cyclodextrin ligand. Ub and IL-1 β were lyophilized and subsequently dissolved in buffer. MBP was spin-concentrated to its final concentration.

4.1 Preparing 10MAG/LDAO Samples

The 1-decanoyl-rac-glycerol (10MAG): lauryldimethylamine oxide (LDAO) surfactant mixture is the recommended system for encapsulated protein NMR studies. Ratios of 10MAG/LDAO may be varied for optimizing encapsulation. A third surfactant, dodecyltrimethylammonium bromide (DTAB) surfactant can also be added or used to replace LDAO for proteins that are otherwise difficult to encapsulate. Typical 10MAG:LDAO ratios can range from 70:30 to 50:50. The pH of LDAO *must* be adjusted before hand. At a pre-adjusted pH of 4.5–5.5, LDAO is a very strong buffer with effective buffer concentration in the RM of > 1 M.

4.1.1 Adjusting the pH of LDAO:

1. Make a stock of both 10MAG and LDAO at the final surfactant concentration by weighing out surfactants into separate microcentrifuge tubes. LDAO is hygroscopic. In order to find the actual mass, divide the mass on the balance by 1.02 to account for the additional mass from water.
2. Calculate the volume of solvent needed to dissolve your surfactants to 75 mg/ml.

3. Dissolve 10MAG in ethanol and LDAO in water. For LDAO add a smaller volume than calculated to account for the additional liquid volume from pH adjustment.
4. pH adjust the LDAO stock using a thin pH probe. Make sure not to overshoot the desired pH value so that the salt concentrations are consistent.
5. Add the remainder of the volume of water to reach the final stock concentration.
6. Calculate the volume of each stock needed to make the final surfactant mixture with the appropriate ratios.
7. Aliquot both stocks together into alkane safe glass vials with Teflon sealing caps. Dilute mixture with water so that the final ethanol concentration is around 15% (v/v) and vortex.
8. Crack the lids and freeze the vials in a dry ice/ethanol bath or liquid nitrogen for at least 15 minutes.
9. Lyophilize the vials overnight to completely dry the mixtures.
10. Alternatively, a large stock of LDAO can be dissolved in water, pH adjusted, and lyophilized. This stock can be used for directly weighing the surfactants rather than lyophilizing each sample.

4.1.2 Completing 10MAG/LDAO Samples

1. Add 500 μL of 10% to 100% D-pentane (Cambridge Isotope Laboratories Inc.) to the dry surfactants and vortex thoroughly. The surfactants may not completely dissolve at this step.
2. Add the volume of aqueous protein solution to the surfactant vial to achieve a W_0 value of 5 to 10 and vortex. The mixture will become opaque white after vortexing.
3. Add a standard amount of hexanol (i.e. 15–20 mM) during the W_0 optimization.
4. Vortex until the solution becomes clear or mildly hazy. This may take several minutes.
5. Transfer to a 5 mm screw cap NMR tube (Wilmad-LabGlass) with a glass Pasteur pipette. Collect the first increment of a ^{15}N -HSQC to assess encapsulation efficiency (Figure 8).
6. Continue to titrate in aqueous buffer in W_0 increments of 2.5 or 5 followed by the collection of the first increment of a ^{15}N -HSQC. Stop when minimal signal improvement between titration steps is achieved.
7. The hexanol concentration can also be optimized. Start with no hexanol or 5 mM hexanol. Assess the encapsulation efficiency by collecting the first increment of a ^{15}N -HSQC.
8. Slowly titrate in hexanol at 2.5 or 5 mM increments. Vortex thoroughly between each addition until the mixture becomes more transparent. Assess the

encapsulation efficiency by collecting the first increment of a ^{15}N -HSQC. Stop when minimal signal improvement between titration steps is achieved.

9. The optimal hexanol concentration for 10MAG/LDAO encapsulation is between 10 and 30 mM.
10. The optimal conditions can be decided after all spectra are collected. Collect a 2D ^{15}N -HSQC or TROSY spectrum to assess the structural fidelity of the encapsulated protein (Figure 9).

4.2 Preparing CTAB/Hexanol Samples

The CTAB/hexanol RM system is less optimal than the 10MAG/LDAO system for reasons discussed above, though it does work well for many protein systems. We highly recommend using the 10MAG/LDAO system for protein NMR, but the CTAB hexanol system also provides a favorable platform for protein encapsulation. If a protein does not encapsulate efficiently in 10MAG/LDAO, CTAB/hexanol provides an additional system to screen.

1. Weigh out the appropriate amount of cetyltrimethylammonium bromide (CTAB) surfactant (Sigma) for a 500 μL sample making sure the surfactants are placed in an alkane-safe tube or glass vial.
2. Add 500 μL of 10–100% D-pentane for lock. For the W_0 optimization protocol, start with a standard amount of hexanol (i.e. 450 mM) added at this step. Vortex thoroughly.
3. Add a volume of aqueous protein solution to the surfactant vial to achieve a W_0 value of 5 to 10 and vortex. The mixture will become opaque white prior to vortexing.
4. Vortex until the solution becomes clear or slightly hazy. This may take up to 5 minutes.
5. Transfer to a 5 mm screw-top NMR tube with a glass Pasteur pipette. Collect the first increment of a ^{15}N -HSQC to assess encapsulation efficiency (Figure 10).
6. Continue to titrate in the aqueous buffer in W_0 increments of 2.5 or 5 followed by the collection the first increment of a ^{15}N -HSQC. Stop when minimal signal improvement between titration steps is achieved.
7. The typical optimal W_0 is 10–15 for smaller proteins and 15–20 for larger proteins.
8. The hexanol concentration can also be optimized. Start with 300 mM hexanol and the previously determined optimal W_0 . Assess the encapsulation efficiency with the first increment of a ^{15}N -HSQC (Figure 10).
9. Titrate hexanol at 50 mM increments. Vortex thoroughly between each addition. Assess encapsulation efficiency with the first increment of a ^{15}N -HSQC and stop when the signal only marginally improves between titration steps.
10. The typical optimal concentration of hexanol for CTAB encapsulation is between 400–500 mM.

11. The optimal conditions can be decided after all spectra are collected. Collect a 2D ^{15}N -HSQC or TROSY spectrum to assess the structural fidelity of the encapsulated protein (Figure 11).

4.3 Preparing AOT Samples

As discussed previously, AOT is generally a poor RM system to encapsulate proteins with high structural fidelity. In our experience, AOT RMs unfold nearly every protein, with ubiquitin being an exceedingly rare exception. *We generally do not recommend AOT.* Proceed with caution and assess structural fidelity and stability over time carefully for any application that uses AOT.

1. Dioctyl sulfosuccinate sodium (AOT) surfactant is first dissolved in water (1 mg/mL). This solution is then titrated to the desired pH, frozen, and lyophilized. This procedure is repeated 3–4 times until the AOT is at the desired pH when re-dissolved in water. This can be done in bulk for large stocks of pH pre-adjusted AOT.
2. Weigh out the appropriate mass of AOT surfactant for a 500 μL NMR sample, making sure the surfactants are placed in an alkane-safe tube or glass vial.
3. Add 500 μL of 10–100% D-pentane for lock or fully deuterated pentane. The surfactant should completely dissolve.
4. Add the volume of protein solution to the surfactant vial to start with a W_0 value of 5. Vortex until solution becomes clear or mildly hazy.
5. Transfer to a 5 mm screw cap NMR tube with a glass Pasteur pipette.
6. Assess the encapsulation efficiency by collecting the first increment of a ^{15}N -HSQC (Figure 12A).
7. Continue to titrate in aqueous buffer in W_0 increments of 5 followed by the collection of the first increment of a ^{15}N -HSQC. Stop when minimal signal improvement between titration steps is achieved.
8. After all spectra are collected, the optimal conditioned can be decided and 2D experiments can be run to examine structural fidelity (Figure 12B, C, and D).

5. Method for Preparation of RM solutions in Propane or Ethane

The low viscosity of ethane and propane potentially provide tremendous tumbling advantages over proteins dissolved in water and RM encapsulated proteins in longer chain alkanes (Figure 1B). This allows very large proteins to be studied without the need for TROSY or perdeuteration. Several challenges must be overcome to prepare RM solutions in liquid ethane and/or propane sample. Both ethane and propane require elevated pressure to be liquids at room temperature. The viscosity of ethane is quite pressure dependent (Figure 1B). Samples cannot be efficiently mixed directly in a high-pressure (HP) NMR tube. Efficient mixing is achieved using a separate mixing chamber (Peterson, et al., 2005). High-pressure rated sapphire windows allow viewing into the mixing chamber allow a visual assessment of sample quality before transferring to the HP NMR tube. A sample preparation

device based roughly on the Peterson-Wand design is commercially available from Daedalus Innovations (Aston, PA). A second challenge is transferring the sample from the mixing chamber to the high-pressure NMR tube. A robust and reliable method is to transfer the sample using high-pressure displacement of a piston through the volume of the mixing chamber to force the sample into the HP NMR tube. Since the pressure pump must be used to equilibrate the sample pressure after transfer an alternate pressure reservoir is used, here termed a booster piston. CO₂ gas is used to drive the booster piston due to its nearly identical compressibility as liquid ethane. The following procedure is a step-by-step guide for making an ethane or propane sample using techniques for the assembly of the sample in a mixing chamber and the transfer of the sample to the HP NMR cell via a booster piston. For simplicity, ethane is referred to in this demonstration. This procedure is directly applicable to propane, but with lower pressures required.

5.1 Safety Considerations

Preparation of reverse micelles in very low-viscosity alkanes uses flammable gasses under high pressures. All high-pressure reverse micelle procedures should be carried out in an exhaust hood. In addition, safety procedures regarding equipment pressure failure should be followed. This includes the use of protective eyewear, the use of an acrylic glass protective barrier when pressure testing and transferring sample to the HP NMR cell, and the use of fittings and equipment that are safety rated for sufficiently high pressures (rated for >14,500 psi; > 1 kbar). Commercial high-volume (i.d. 3.8 mm) ceramic HP NMR cells are available from Daedalus Innovations (Aston, PA). Use of glass or sapphire NMR cells for liquid ethane is strongly discouraged for reasons of safety.

5.2 Preparing Sample Components

Samples are prepared in a closed pressure chamber to maintain the liquid state for ethane and propane. Since this precludes titration or addition of components after application of the pressurized alkane, ethane and propane sample preparation should proceed only after optimization of RM encapsulation conditions in pentane (sections 4.1–4.3). Generally, optimal conditions in pentane apply to ethane and propane sample conditions, though the optimal hexanol concentrations is often ~50% higher in ethane and propane as compared to pentane. If possible, screening multiple samples is recommended. The volume of the mixing chamber is 1.65 ml when assembled and the sample components should be made with this target volume. Typically, these samples are made with 10–20% D-pentane for a lock solvent. Pentane also provides a convenient ambient pressure liquid to pre-solubilize the sample components before introducing liquid ethane or propane. While it is possible to add the surfactant, cosurfactant, and aqueous protein directly to the mixing chamber, sample preparation is more reliable when first made in 10–20% of the mixing chamber volume of D-pentane (165 to 330 µL) before introducing liquid ethane.

5.3 Procedure for Elevated-Pressure Reverse Micelle Encapsulation.

1. Connect a syringe pump with an ample pressure rating to the HP alkane inlet port (a) (Figure 13A). Fill the HP syringe pump with the alkane of interest (ethane for this demonstration). To ensure that the ethane is in the liquid state in

the syringe pump, close the syringe pump valves and pressurize the alkane to 1000 psi.

2. The HP NMR cell must be pressure tested before the sample transfer to ensure proper assembly and cell integrity. Assemble the HP NMR cell according to the diagram in Figure 13B. The pressure test must proceed within an acrylic glass safety box in case of failure. Open the pressure test valve (1) while keeping the HP NMR cell valve closed. This will test the integrity of the connections to the cell. Next, open the HP NMR cell valve. Increase the pressure to 6000 psi (or higher if needed) in steps of 1000 psi, allowing the system to come to rest before applying the next step.
3. If there are no leaks or failures of the HP NMR cell, the pressure test was successful. Refill the syringe pump while open to the HP NMR cell to recover the liquid ethane. Close all valves and disconnect the HP NMR cell.
4. Sample preparation may now proceed. Place the stir bar plate into the mixing chamber (Figure 13C) and a new micro stir bar. Using a new stir bar for each sample is important since the stir bars wear down and have been observed to shed iron into the solution after multiple uses. Position an O-ring into the top of the mixing chamber. An O-ring can only be used once due to the destructive influence the piston during sample transfer. Open the outlet/inlet valve (2) to allow the pressure to escape while inserting the transfer piston.
5. Inject the aqueous phase into the pre-mixed surfactant, cosurfactant and D-pentane solution. Vortex. Transfer into the mixing chamber via pipette.
6. Position the transfer piston into the top of the mixing chamber. Place the cap on the top and tighten the cap bolts. Tighten the bolts in a cross-wise manner to ensure proper seating of the cap. Close the outlet/inlet valve (2) to close the mixing chamber.
7. Adjust the pressure of the syringe pump to 1000 psi. Open the HP alkane valve (3), turn on the stirrer and backlight. Liquid ethane at 1000 psi is now in the mixing chamber and the sample is typically very opaque. Observe the sample through the sapphire viewing window.
8. Increase the pressure in steps of 1000 psi to a total pressure of 3000 psi. Track the appearance of the sample through the sapphire viewing window. Most RM samples encapsulate at pressures > 3000 psi.
9. At above 3000 psi, slowly increase the pressure of the syringe pump in increments of 250 psi, allowing ~2 minutes of stirring in between. Once the reverse micelle sample becomes mostly or completely clear, increase the pressure an additional 200 psi and record this as the **encapsulation pressure**. The additional pressure allows a slight margin of error for the sample pressure when transferring the sample to the HP NMR cell.
10. The volume of the mixing chamber with the piston down and open to the HP NMR cell is 3% larger than the volume of the mixing chamber. To prevent a drop

in the sample pressure upon transfer, increase the density of the sample by 3%. The pressure dependent density of ethane and propane can be found at <http://webbook.nist.gov/chemistry/>. For example, ethane at 4000 psi and 25° C has a density of 0.44175 g/ml. Increasing the density by 3% to 0.45500 requires increasing the pressure to about 5000 psi. This is the **over pressure**. Slowly adjust the pressure of the sample to the over pressure. The reverse micelle sample quality is generally not affected by applying the over pressure, but observation of the sample quality while adjusting the pressure is recommended.

11. In order to effectively transfer the sample to the HP NMR cell, the transfer piston must be driven by 2000 psi above the over pressure. This is the **transfer pressure**, which the gas booster must be adjusted to in order to prepare for the sample transfer.
12. The piston booster must be filled with the chosen booster gas, which is CO₂ for this demonstration. With all other valves closed, connect a CO₂ tank with a regulator set to 1800 psi to the booster inlet port (**b**). Ensure that the alkane chamber of the booster is empty and open to ambient pressure by opening the HP alkane to the booster valve (**4**) and the pressure test valve (**1**). Open the inlet to booster valve (**5**). The booster is now filled with CO₂ liquid at 1800 psi. Close the inlet to booster valve (**5**) and the pressure test valve (**1**).
13. Indirectly pressurize the CO₂ in the booster by increasing the alkane pressure. A separator in the gas booster keeps the CO₂ and ethane apart. Open the HP alkane to booster valve (**4**). Adjust the syringe pump pressure up to the transfer pressure in steps of 1000 psi, allowing the system to come to rest before applying the next step. The CO₂ will compress around 3000 psi, which is accompanied by a sharp drop in pressure that the syringe pump will then correct. After reaching the over pressure, open the piston booster valve (**6**) and adjust the pressure to the transfer pressure. This will bring the booster, the lines, and the sample (via minor displacement of the transfer piston) to the transfer pressure. Close the HP alkane to booster valve (**4**) and lower the pressure in the syringe pump to the encapsulation pressure.
14. Connect the pressure tested HP NMR cell to the outlet/inlet to the mixing chamber and open the cell valves. Turn off the mixing to avoid dislodging the stir bar during sample transfer.
15. Sample transfer to the HP NMR cell may now proceed. Open the outlet/inlet valve (**2**) to transfer the sample. This will allow the transfer piston to displace completely, transferring the sample to the HP NMR cell. Close the piston booster valve (**6**). Open the HP alkane valve (**3**) to allow a final pressure adjustment to the encapsulation pressure by the syringe pump. Close the HP NMR cell valve. Close the outlet/inlet valve (**2**) and disconnect the transfer line from the HP NMR cell. Close all open valves.
16. The sample is now ready for NMR measurement. Raising and lowering the sample into the bore of the magnet requires a non-magnetic bead chain, since the

HP NMR cell is too heavy for the NMR sample lift air. Figure 14 provides examples of 4 proteins encapsulated in RMs with ethane as the bulk solvent.

17. To clean and disassemble the apparatus, first vent the booster gas by opening the vent piston valve (7). The remaining pressurized ethane will raise the transfer piston. Open the waste valve (8) to vent the remaining ethane out of the waste port.
18. The cleaning manifold is typically comprised of three bottles with outlet valves connected to a 10 psi N₂ gas regulator. The N₂ gas feeds the cleaning solutions into the mixing chamber. The cleaning solvents are typically dichloromethane, ethanol, and water. These are connected to allow inlet to the mixing chamber via the cleaning manifold port (d). A N₂ tank set with a regulator set to 60 psi is connected to the N₂ inlet port (c) to push the cleaning solvents out of the mixing chamber.
19. With all valves closed, open the cleaning manifold valve (9). Open the first cleaning solvent bottle outlet valve, dichloromethane. Turn on the stir bar to help solubilization. Briefly open the outlet/inlet valve (2) to clean the transfer line, with a separate container to catch the cleaning solution. Open the waste valve (8) and the N₂ for cleaning valve (10) to expel the cleaning solutions, then close these valves. After the first dichloromethane wash, repeat with the cleaning solutions in the following order: ethanol, water, ethanol, dichloromethane. Close the cleaning manifold valve (9). Disassemble the mixing chamber and swab clean if necessary.

6. Benchmarking Encapsulation

Most screens begin with ¹⁵N-HSQC or ¹⁵N-TROSY spectra because of the relatively inexpensive labeling scheme and relative speed. If aqueous reference spectra are available, the comparison of the chemical shifts will provide insight into the fidelity of the protein structure. For CTAB/hexanol and LDAO/10MAG surfactant mixtures the correlation coefficient (R²) of between RM encapsulated and bulk aqueous ¹H, ¹⁵N, and ¹³C chemical shifts have been shown to be greater than 0.99 with RMSD values less than 0.05, 0.1, and 0.1 ppm respectively (Dodevski, et al., 2014; Lefebvre, et al., 2005; Nucci, et al., 2014; O'Brien, et al., 2015). This should be the threshold for accepting a given encapsulation condition. The chemical shift perturbations in AOT reverse micelles is larger for ubiquitin but structural studies have shown that the backbone RMSD is < 0.5 Å with encapsulated ubiquitin despite the slight changes in chemical shift versus bulk aqueous protein (Babu, et al., 2001). The ¹H, ¹⁵N backbone chemical shifts are more sensitive to the change in environment than ¹H, ¹³C aliphatic chemical shifts that generally show little to no perturbation. The second consideration for assessing encapsulation fidelity is the stability of the sample. It is important to monitor the one-dimensional ¹H and two-dimensional ¹⁵N-HSQC/TROSY spectra for up to several weeks to ascertain that no changes occur to the sample. This includes evaporation of water, organic solvent, protein aggregation or reduced intensity of protein. Exceptionally stable reverse micelle samples are capable of lasting several years, with the average optimized sample remaining stable for several weeks.

7. Conclusions and Outlook

Reverse micelle encapsulation of proteins is a versatile tool for improving NMR performance as well as for biophysical study of proteins. RM systems are highly tunable for the desired application. A recent advance in the 10MAG/LDAO surfactant system has greatly optimized and enhanced the utility of the RM encapsulation of proteins, with CTAB/hexanol RMs also useful for high-fidelity RM encapsulation of proteins. Though historically AOT has been used for protein studies, very few proteins actually retain native structure in this system. RM encapsulation protocols for three commonly studied proteins are presented here as a guide. These proteins span a size range with ubiquitin at 8 kDa, interleukin-1 β at 17 kDa, and maltose binding protein at 41 kDa. As demonstrated by the results presented here, each individual protein requires unique conditions for optimal encapsulation. A variety of alkanes may be used depending on the specific application. Pentane is most recommended, as it is relatively easy to handle at room pressures and imposes only a minor tumbling penalty for small to medium sized proteins, with a tumbling advantage for larger proteins. For large protein applications, ethane and propane may be used with specialized equipment, since these alkanes need to be under pressure to retain the liquid state. Caution should be used however, as pressure sensitive proteins are unstable for long-term as observed for interleukin-1 β and the Lac repressor core domain. Though the effort is relatively high for ethane and propane, these alkanes offer a great advantage in tumbling for large systems, allowing fully protonated proteins greater than 60 kDa to be studied easily by NMR. This guide prepares the user to screen optimal conditions for a variety of NMR applications of RM encapsulated proteins.

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Figure 1.

(A) Schematic of a protein encapsulated in a reverse micelle. Alkane solvent is depicted in gray, surfactant in yellow, water in blue, and the protein in green. (B) Rotational correlation time (τ_m) for a spherical protein of a given molecular weight in water (blue, $\eta \approx 890 \mu\text{Pa}\cdot\text{s}$), or encapsulated in RM prepared in various alkane solvents: pentane (brown, $\eta \approx 220 \mu\text{Pa}\cdot\text{s}$), butane at 50 psi (orange, $\eta \approx 160 \mu\text{Pa}\cdot\text{s}$), propane at 600 psi (green, $\eta \approx 105 \mu\text{Pa}\cdot\text{s}$), and ethane (black) at 3000 psi ($\eta \approx 70 \mu\text{Pa}\cdot\text{s}$) and 7500 psi ($\eta \approx 99 \mu\text{Pa}\cdot\text{s}$) spanning the range depicted in gray. τ_m values were calculated from the Stokes-Einstein equation (Equation 1).

Figure 2.

Reverse micelles as a membrane mimic with (A) A lipidated protein anchored to the inner surface of the reverse micelle, with the attached lipid moiety depicted in red. (B) An encapsulated integral membrane protein via a 'inverted shower cap' arrangement, where separate water pools solubilize the polar water exposed surfaces of the protein. (C) Characterizing the interactions between protein and ligands hosted in the reverse micelle surfactant shell. Lipid (magenta) is titrated into the RM solution and chemical shift perturbations observed. Examples include the study of the HIV matrix protein interacting with PI(4,5)P₂ (Valentine, et al., 2010) and cytochrome c interacting with cardiolipin (O'Brien, et al., 2015).

Figure 3.

(A) RDCs measured via the IPAP experiment for oxidized flavodoxin (Flv) in partially aligned reverse micelles. Data obtained at 750 MHz (^1H). ^{15}N -Flv was encapsulated with 50 mM potassium phosphate, pH= 5.5 in the aqueous phase. RMs were composed of 100 mM LDAO in D-pentane with 320 mM hexanol at a $W_0 = 17$. (B) Histogram of measured RDC values, with $\text{RDC} = [J + D(750\text{MHz}) - J + D(500\text{MHz})]$. (C) Correlation plot of predicted versus observed RDCs based on the determined alignment tensor. (D) Axis of alignment tensor are depicted with the cartoon representation of the RDC refined Flv. Adapted with permission from KG Valentine, MS Pometun, JM Kielec, RE Baigelman, JK Staub, KL Owens, and AJ Wand. Magnetic susceptibility-induced alignment of proteins in reverse micelles. *Journal of the American Chemical Society*. 2006; 128(50):15930–15931. © 2006 American Chemical Society.

Figure 4.

(A) The solution structure of RM encapsulated oxidized horse cytochrome c (Cyt c). The 32 lowest energy structures are overlaid. The internal water molecules are displayed as clusters of blue dots and the heme is displayed in pink. (B) Overlay of the backbone structures of the RM encapsulated Cyt c (green) with the crystal structures of Cyt c solved in low salt (blue, PDB 1CRC; (Sanishvili, Volz, Westbrook, & Margoliash, 1995), high salt (cyan, PDB 1HRC; (Bushnell, Louie, & Brayer, 1990) and a solution NMR structure (orange, PDB 1AKK(Banci, Bertini, Gray, Luchinat, Reddig, Rosato et al., 1997). This research was originally published in the *Journal of Biological Chemistry*. ES O'Brien, NV Nucci, B Fuglestad, C Tommos, AJ Wand. Defining the apoptotic trigger: the interaction of cytochrome c and cardiolipin. *Journal of Biological Chemistry*. 2015; 290:30879–30887. © The American Society for Biochemistry and Molecular Biology.

Figure 5.

Demonstration of direct injection of 300 μM oxidized flavodoxin with its yellow flavin mononucleotide cofactor into 150 mM 10MAG/LDAO (65:35 molar percent ratio) with 15 mM hexanol and $W_0 = 20$ in pentane. Reprinted with permission from I Dodevski, NV Nucci, KG Valentine, GK Sidhu, ES O'Brien, A Pardi, and AJ Wand. Optimized reverse micelle surfactant system for high-resolution NMR spectroscopy of encapsulated proteins and nucleic acids dissolved in low viscosity fluids. *Journal of the American Chemical Society*. 2014; 136(9):3465–3474. © 2014 American Chemical Society.

Figure 6.

Demonstration of the injection-evaporation method. (A) is the reference ^{15}N -HSQC in 500 μM oxidized flavodoxin in bulk aqueous conditions. (B) ^{15}N -HSQC of the protein with a low (0.5 mM) starting concentration (**top**). This is compared to the direct injection method of encapsulating a protein with a high (6.3 mM) starting concentration (**bottom**). Data were recorded at 500 MHz (^1H). Reprinted with permission from I Dodevski, NV Nucci, KG Valentine, GK Sidhu, ES O'Brien, A Pardi, and AJ Wand. Optimized reverse micelle surfactant system for high-resolution NMR spectroscopy of encapsulated proteins and nucleic acids dissolved in low viscosity fluids. *Journal of the American Chemical Society*. 2014; 136(9):3465–3474. © 2014 American Chemical Society.

Figure 7. pH titration of IL-1 β encapsulated in 75 mM 10MAG/LDAO (60:40 molar percent ratio) with 20 mM hexanol and $W_0 = 15$ in pentane. The LDAO was pH adjusted before lyophilization. pH values and the color in the spectrum overlay are: 3.5 (pink), 4.0 (red), 4.4 (orange), 4.6 (yellow), 4.8 (green), 5.0 (blue), 5.2 (purple), 5.6 (gray), 6.0 (black).

Figure 8.

Optimization of 10MAG/LDAO RMs for NMR spectroscopy using the first increment of a ^{15}N -HSQC (Ub) or ^{15}N -TROSY experiments (IL-1 β and MBP). Water loading optimization series for Ub (A), IL-1 β (C), and MBP (E). All W_0 series were performed with 20 mM hexanol. Demonstration of sub-optimal (0 mM) and optimal (20 mM) hexanol concentrations for Ub (B), IL-1 β (D), and MBP (F). Spectra were collected at optimal W_0 , as determined in panels A, C, and E (Ub = 10, IL-1 β and MBP = 15). All 10MAG/LDAO RMs were composed of 75 mM total surfactant in pentane (65:35 molar percent ratio of 10MAG:LDAO for Ub and MBP, 60:40 for IL-1 β). Ub series were collected at 600 MHz (^1H) with 8 scans, IL-1 β and MBP were collected at 750 MHz (^1H) with 16 scans. All spectra were collected at 25°C.

Figure 9.

Two-dimensional ^1H - ^{15}N correlation spectra of proteins encapsulated in 10MAG/LDAO reverse micelles in pentane under optimal conditions. All samples under these conditions exhibit long-term stability (> 2 weeks) (A) ^{15}N -HSQC of 200 μM Ub in 75 mM 10MAG/LDAO (65:35 molar percent ratio), 20 mM hexanol, $W_0 = 10$. Aqueous phase consisted of 50 mM sodium acetate at pH = 5.0. Collected at 600 MHz and 25°C with 1024×48 complex points and 8 scans. (B) ^{15}N -TROSY of 100 μM IL-1 β in 75 mM 10MAG/LDAO (60:40 molar percent ratio), 20 mM hexanol, $W_0 = 15$. Aqueous phase consisted of 50 mM sodium acetate, 5 mM DTT at pH = 5.0. Collected at 750 MHz (^1H) and 25°C with 1024×64 complex points and 16 scans. (C) ^{15}N -TROSY of 180 μM MBP in 75 mM 10MAG/LDAO (60:40 molar percent ratio), 20 mM hexanol, $W_0 = 15$. Aqueous phase consisted of 50 mM sodium phosphate at pH = 7.1. Collected at 750 MHz (^1H) and 25°C with 1024×90 complex points and 16 scans.

Figure 10.

Optimization of CTAB RMs for NMR spectroscopy using the first increment of a ^{15}N -HSQC (Ub) or ^{15}N -TROSY experiments (IL-1 β and MBP). Water loading optimization series for Ub (A), IL-1 β (C), and MBP (E). All W_0 series were performed with 450 mM hexanol. Demonstration of sub-optimal (300 mM) and optimal (400 mM) hexanol concentrations for Ub (B), IL-1 β (D), and MBP (F). Spectra were collected at optimal W_0 , as determined in panels A, C, and E (Ub = 12.5, IL-1 β and MBP = 15). All CTAB RMs were composed of 75 mM CTAB in pentane. Ub series were collected at 600 MHz (^1H) with 8 scans, IL-1 β and MBP were collected at 750 MHz (^1H) with 16 scans. All spectra were collected at 25°C.

Figure 11.

Two-dimensional ^1H - ^{15}N correlation spectra of proteins encapsulated in CTAB reverse micelles in pentane under optimal conditions. All samples under these conditions exhibit long-term stability (> 2 weeks) (A) ^{15}N -HSQC of 200 μM Ub in 75 mM CTAB, 450 mM hexanol, $W_0 = 12.5$. Aqueous phase consisted of 50 mM Tris at pH = 8.3. Collected at 600 MHz and 25°C with 1024×48 complex points and 8 scans. (B) ^{15}N -TROSY of 100 μM IL-1 β in 75 mM CTAB, 450 mM hexanol, $W_0 = 15$. Aqueous phase consisted of 50 mM sodium acetate, 5 mM DTT at pH = 5.0. Collected at 750 MHz and 25°C with 1024×64 complex points and 16 scans. (C) ^{15}N -TROSY of 180 μM MBP in 75 mM CTAB, 450 mM hexanol, $W_0 = 15$. Aqueous phase consisted of 50 mM sodium phosphate at pH = 7.1. Collected at 750 MHz and 25°C with 1024×90 complex points and 16 scans.

Figure 12.

Optimization of AOT for NMR of ubiquitin. (A) The first increment of a ^{15}N -HSQC of ubiquitin in 75 mM AOT RMs. Water loading is increased from 5 (black) to 7.5 (blue) to 10 (orange). (B) ^{15}N -HSQC of 200 μM Ubiquitin in 75 mM AOT reverse micelles, $W_0 = 10$ with pentane as the bulk solvent. Aqueous phase buffer is comprised of 50 mM sodium acetate, 50 mM NaCl at pH = 5.0. Collected at 600 MHz and 25°C with 1024×48 complex points and 8 scans. (C) ^{15}N -TROSY spectrum of 100 μM IL-1 β in 75 mM AOT reverse micelles, $W_0 = 15$ with pentane solvent. *This is a case where the 1D optimization and the initial 2D assessment are misleading; the NMR signal of IL-1 β is completely abrogated within 12 hours.* Aqueous phase buffer is comprised of 50 mM sodium acetate and 5 mM DTT at pH = 5.0. Collected at 600 MHz (^1H) and 25°C with 1024×64 complex points and 16 scans. (D) ^{15}N -TROSY of MBP showing that it is unfolded in AOT reverse micelles as are nearly all proteins.

Figure 13.

(A) A basic schematic of the HP RM synthesis apparatus and cross-sections of (B) the self-contained HP NMR cell and (C) the HP RM mixing chamber. Note that the diagrams are not to scale and serve only as a demonstrative guide for the operation of the apparatus. Fully detailed schematics are published in (Peterson, et al., 2005). An apparatus is commercially available (Daedalus Innovations, Aston, Pennsylvania) or may be constructed.

Figure 14.

^{15}N -HSQC spectra of proteins encapsulated in ethane. (A) 270 μM ^{15}N -ubiquitin (8.5 kDa) in 75 mM 10MAG/LDAO (65:35 molar percent ratio) with 30 mM hexanol, 20% D-pentane $W_0 = 10$ with 4000 psi pressure. (B) 100 μM ^{15}N -IL-1 β (17 kDa) in 75 mM CTAB, 575 mM hexanol, 30% D-pentane, $W_0 = 15$ with 1450 psi pressure. (C) 80 μM of the lac repressor core domain (LacI) homodimer (58 kDa total) in 75 mM CTAB, 550 mM hexanol, 10% D-pentane, $W_0 = 10$ with 4000 psi pressure. (D) 125 μM ^{15}N -MBP (41 kDa) in 75 mM 10MAG/LDAO (65:35 molar percent ratio), 30 mM hexanol, 20% D-pentane, $W_0 = 12$ with 5500 psi pressure. IL-1 β and LacI are unstable under pressure in ethane due to the pressure sensitivity of LacI to unfolding and monomerization (Fuglestad, Stetz, Belnavis, & Wand, 2017) and pressure sensitivity of IL-1 β from an internal hydrophobic cavity (Quillin, Wingfield, & Matthews, 2006). Spectral signal for both of these proteins is abrogated within 24 hours. Note that these are all ^{15}N -HSQC spectra (non-TROSY), which demonstrates the greatly enhanced tumbling properties in ethane.