Optimal Use of Cryogenic Probe Technology in NMR Studies of Proteins

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Nuclear magnetic resonance (NMR) spectroscopy is an inherently insensitive technique, and this insensitivity is often a fundamental limitation to its application in a variety of contexts in physics, chemistry, and biology. Over the past several decades a variety of approaches have been employed to improve the signal-to-noise performance of solution NMR experiments. Examples include the introduction of the Fourier transform approach[1] and its attendant advantages such as quadrature detection[2] and digital filtering,[3] the use of higher magnet field strengths, and a general improvement in electronics. The use of incoherent and coherent polarization transfer[4] also greatly improves the sensitivity of heteronuclear NMR spectroscopy and, when combined with a facile and broad library of pulse sequences, provides a powerful battery of analytical NMR methods.[5] The general sensitivity of the NMR experiment can also be enhanced by collection of both oscillatory components of the NMR signal.[6] Numerous post-acquisition processing strategies have also been introduced.[7] Because of these and numerous other advances, state-of-the-art multinuclear multidimensional NMR experiments can be routinely carried out on samples in the ∼1 mM concentration range. Nevertheless, many systems of interest are of limited solubility or of limited availability. This is particularly true for biopolymers such as proteins and nucleic acids. Thus, a further extension of the sensitivity of the NMR method to the submillimolar sample concentration regime is highly desirable. For the purposes of the following discussion we represent the sensitivity or signal-to-noise (S/N) of an NMR probe as a function of its filling factor (η), the effective quality factor of the probe (Q_{eff}), the effective noise-temperature of a room-temperature sample in a coil (T_{eff}), and the noise-temperature of the receiver (T_{A}).[8]

$$\frac{S}{N} \propto \left[\frac{\eta Q_{eff}}{T_{\mathrm{eff}} + T_{\mathrm{A}}}\right]^{1/2}$$

$$Q_{\mathrm{eff}} = \frac{Q_{\mathrm{c}}Q_{\mathrm{s}}}{Q_{\mathrm{c}} + Q_{\mathrm{s}}} = \frac{\omega_0 L}{R_{\mathrm{c}} + R_{\mathrm{s}}};\ T_{\mathrm{eff}} = \frac{T_{\mathrm{c}}Q_{\mathrm{s}} + T_{\mathrm{s}}Q_{\mathrm{c}}}{Q_{\mathrm{c}} + Q_{\mathrm{s}}} \quad (1)$$

Here, Q_{c} is the directly measured quality factor of the empty coil and Q_{s} is the contribution of the sample to the effective quality factor, ω_0 is the resonance frequency, L is the inductance of the circuit, R_{c} and R_{s} are the equivalent resistances respectively of the coil circuit and sample, T_{s} is the sample temperature, and T_{c} is the coil temperature.

It has long been realized that cooling the receiver coil to cryogenic temperatures would significantly reduce the noise voltage associated with signal detection. The first practical implementation of a cryogenic probe showing substantial gain in sensitivity was made by Styles et al.[9] Little further progress was made with cryogenic probes until the discovery of high-temperature superconductors. Probes using thin-film superconductors were first introduced for imaging applications[10] and later for high-resolution spectroscopy.[11] Unfortunately, the performance of the cryogenic probe is exquisitely sensitive to conductivity of the sample rendering their utility in the context of samples containing high (∼100 mM) concentrations of salts problematic. This is obviously particularly pertinent in the context of multinuclear multidimensional NMR studies of proteins and other biopolymers. Inductive losses result from the dissipation of power due to the induction of current in the conducting sample.[8] Dielectric losses result from the passage through the conducting sample of the electrical lines of force arising from the distributed capacitance of the rf coil.[8] This dependence of Q_{s} on the conductivity of a cylindrical sample of radius r_{s} and conductivity σ is conveniently expressed by Equation 2.

$$Q_{\mathrm{s}} \propto [\eta\omega_0 r_{\mathrm{s}}^2 \sigma]^{-1} \quad (2)$$

Simply stated, the high Q of a cryogenic probe is rapidly degraded by the effective resistance caused by inductive and dielectric losses being introduced into a circuit of otherwise very low resistance. Accordingly, the conventional probe is relatively insensitive to

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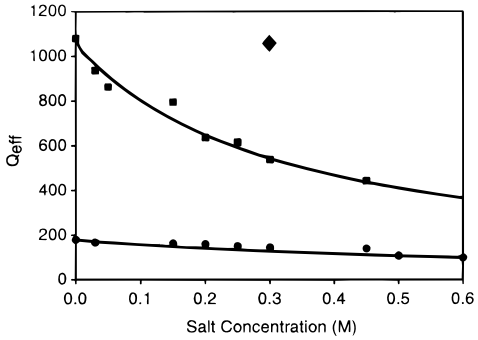

Figure 1. Effective probe quality factor (Q_{eff}) as a function of salt concentration. Measured Q_{eff} values for a conventional 400 MHz probe (●) and a cryogenic 400 MHz probe (■) are shown. Solid lines indicate the fitted theoretical curves (see eqs 1 and 2). The effective probe quality factor obtained for ubiquitin dissolved in 50 mM acetate buffer and 250 mM NaCl encapsulated in AOT reverse micelles dissolved in liquid pentane is shown with a solid diamond (♦). These data indicate that the full performance of the cryogenic probe is recovered for lossy protein samples when encapsulation in low dielectric solvent is employed.

conductive samples as compared to a cryogenic probe. This is illustrated in Figure 1 for a 400 MHz cryogenic probe and its room-temperature counterpart.

A simple solution to this unsatisfactory loss of performance comes with the realization that the losses arise from the bulk properties of the solvent and have little to do with the protein solutes themselves. Ideally, then, one would simply like to replace the ~99% of the volume of the sample occupied by bulk solvent and dissolved ions with a solvent of low conductivity. Here we demonstrate that this can be achieved using an approach that was recently introduced as a means for reducing the tumbling correlation time (τ_m) of proteins: the encapsulation of proteins in the water cavity formed by reverse micelles in low viscosity fluids.[12] To illustrate the approach, we have used the protein ubiquitin encapsulated within sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse-micelles prepared in *n*-pentane. Isotopically ^{13}C,^{15}N-enriched protein was solvated by aqueous buffer containing 50 mM sodium acetate, pH 5, and 250 mM NaCl and then encapsulated with AOT reverse micelles, as described in detail elsewhere,[13] to a concentration of 0.3 mM. We have previously shown that the native structure of the protein is maintained and that the water pool of the interior of the reverse micelle is a good model for bulk aqueous environment.[13] The Varian cryogenic probe used in this study employed coils cooled to 25 K. The first stage of the preamplifier was also cooled to reduce its noise contribution. The loading of the reverse micelle sample did not significantly affect the effective *Q* of the cryogenic probe. Indeed, essentially the full performance of the probe is recovered (Figure 1) and results in the anticipated gains in S/N of one-dimensional ^1H spectra (data not shown). This allows very high quality two-dimensional ^{15}N HSQC spectra to be obtained in as little as 7 min at a field strength of 7 T (400 MHz ^1H) (Figure 2).

It is important to confirm that the maintenance of a high *Q* also actually translates into a gain in sensitivity in the context of a multidimensional NMR spectrum. This is because true thermal noise may be dominated by so-called correlated noise which can arise from instability occurring over the time course of data collection. Signal-to-noise ratios[14] were obtained for resolved cross-peaks in ^{15}N HSQC spectra along the frequency axis corresponding to the acquisition time domain (^1H). Corresponding traces of the HSQC spectra of encapsulated ubiquitin dissolved in pentane obtained using the cryogenic probe at 400 MHz (^1H) were compared to those obtained using a conventional probe at 500 MHz (^1H) (data not shown). A conventional 500 MHz probe (ethylbenzene sensitivity of 1012:1) rather than a 400 MHz probe (ethylbenzene sensitivity of 450:1) was employed to ensure that correlated noise did not artificially dominate the conventional probe data, that is, the 500 MHz probe could be expected to give data of reasonable quality in a similar amount of time as the cyrogenic probe (ethylbenzene sensitivity of 1878:1). Ratios of ^1H-trace S/N for the cryogenic probe versus the conventional 500 MHz probe averaged 1.5 ± 0.2. This indicates that the sensitivity of the experiment is largely dominated by thermal noise. Therefore greater than 80% of the potential gain afforded by the cryogenic probe over a conventional probe is actually achieved in the context of a multidimensional NMR experiment.

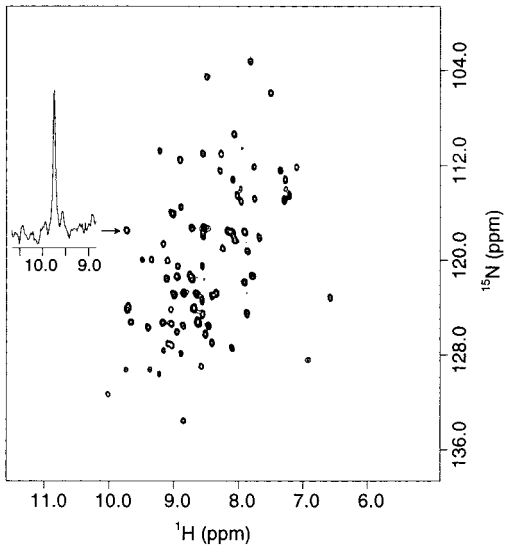

Figure 2. Contour plot of the ^{15}N HSQC spectrum of recombinant human ubiquitin encapsulated in AOT reverse micelles dissolved in *n*-pentane. The protein was encapsulated at a concentration of ~0.25 mM in sodium acetate buffer (50 mM) containing 0.25 M NaCl. The spectrum was recorded at 400 MHz (^1H) using a cryogenic probe with the sample chamber temperature equilibrated at 20 °C. The spectral width in the direct ^1H-dimensional was 8000 Hz (20 ppm) digitized into 512 complex points (64 ms acquisition time); the spectral width in the indirect ^{15}N-dimension was 2200 Hz (54.3 ppm) using 76 complex points (34.5 ms acquisition time). Total acquisition time for the experiment was approximately 7 min. A standard polynomial-based linear-prediction algorithm was employed during data processing that effectively extended the acquisition time in the ^{15}N-dimension to 58 ms. The inset shows a ^1H cross section through the cross-peak indicated by the arrow and illustrates the typical S/N of the spectrum.

The combination of cryogenic probe technology with encapsulation of proteins within reverse micelles dissolved in organic solvents is perhaps a perfect marriage of methods. On one hand, the enormous sensitivity of the cryogenic probe is made accessible to lossy samples such as protein solutions of high ionic strength by use of the reverse micelle approach. On the other hand, the apparent limitation on protein loading to ~0.25−0.5 mM solutions of reverse micelles is overcome by the use of the high sensitivity cryogenic probe. Since the reverse micelle approach is not limited to proteins, one can imagine that the study of other biopolymers such as nucleic acids will be aided by the approach described here.

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