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Communication

Improving NMR sensitivity in room temperature and cooled probes with dipolar ions

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Abstract

The response of inverse triple resonance cold and conventional probes to ionic strength has been compared under a variety of conditions relevant to protein NMR. Increasing the salt concentration degrades probe performance in terms of sensitivity, and the effect is more severe for cold probes and with increasing magnetic field strength. This is especially noticeable for experiments that involve a spin lock or decoupling, where sensitivity losses compared with pure water can be more than 2-fold. We have investigated the use of glycine as a substitute for salt as a supporting solute for proteins, and we show that it has a minimal effect on probe tuning or performance. Readily available d_5-Gly is a useful co-solute for protein NMR, especially at high magnetic field strengths and on cold probes, as it maintains solubility while not degrading probe performance.

Keywords: Cold probe; Dipolar ions; Protein solubility; Sensitivity

1. Introduction

Recent improvements in NMR hardware and techniques, especially the ultra high magnetic field strengths (>20 T), and cryogenically cooled probes have greatly enhanced sensitivity. Cooled probes (CP)[1] rely on the large decrease in thermal noise in the primary detector circuit that can be obtained by cooling to low temperature (typically around 25 K), including both the coil and the preamplifier [1,2]. In principle, this can give rise to a very large enhancement in the signal-to-noise ratio (SNR). However, the necessary thermal isolation between the liquid samples and the detector coil leads to a substantially poorer filling factor compared with a conventional room temperature (RT) probe. Typically, there is a 4- to 6-fold net gain in SNR for non-lossy samples.

The SNR can be markedly reduced in aqueous biological samples where supporting electrolytes are used as buffers to maintain biological function and increase solubility. The dielectric losses due to ionic conductance are substantial. Recently, the relationship between sample conductivity and sensitivity was established experimentally [3]. To maximize the SNR of the system, one should either avoid ions, or at least use ions of low conductance [3]. For biological samples, this is usually impractical, as many proteins are actually salted in at moderate concentrations of simple salts, a thermodynamic phenomenon that has been known for at least 60 years [4,5]. For example, the solubility of horse carbonmonoxyhemoglobin increases 6-fold in the range 0 to 300 mM NaCl [5]. Unfortunately, for NMR the dielectric losses at 300 mM NaCl are rather severe, such that the performance of even RT probes can degrade 2-fold or more at high magnetic field strengths. For many purposes this is unacceptable

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[1] *Abbreviations used:* RT, room temperature; CP, cold probe; SNR, signal-to-noise ratio.

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as it implies an increase in acquisition time of more than 4-fold compared to a non-lossy sample. Furthermore, the sensitivity of the SNR to salt, and the frequency dependence of that sensitivity is more severe for cooled probes than for RT probes (cf. Table 1) which suggests that the performance of cooled probes at very high magnetic field strengths (e.g., $\geqslant$18.8 T) will be less than optimum for typical biological samples. Hence, there is a need to maximize the performance of such probes, or at least find conditions where the deleterious effects of salt can be avoided.

Dipolar ions are an attractive alternative to salts for maintaining protein solubility while having no other deleterious effects. Many such ions are actually present at high concentration in biological tissues, where they act as osmolytes (or compatible solutes), and generally do not directly interact with the macromolecules [6] (and see below). Zwitterions at a pH near their isoelectric point do not contribute to the solution conductance, and therefore should not contribute to the detector noise in the sample. Furthermore, it is well known that dipolar ions also solubilize and stabilize proteins, albeit at much higher concentrations than simple salts [5–9]. Clearly, one should use a dipolar ion that is fully deuterated to avoid swamping the receiver. There are many possible such dipolar ions that could be used. We have focused on d_5-Gly because it has a number of favorable characteristics, including high water solubility (ca. 2.5 M), large dielectric increment ($\partial D/\mathrm{d}[\mathrm{Gly}] = 22.6$ D/M [5]), biological compatibility, low molecular weight ($M_r = 75$), and ready availability in perdeuterated form at low cost. Even in ^1H_2O solutions, the rapid exchange of the amino protons leads to a broad, weak resonance near 8 ppm, that dos not interfere with solute resonances (see below). Here we report a comparison of sensitivity measurements on different systems for both RT and a cold probe at 14.1 and 18.8 T, and show that indeed d_5-Gly is a useful replacement for common salts in biological systems.

2. Methods

Hen egg white lysozyme (3× crystallized, Sigma, St. Louis) was dissolved in 18 MΩ water at 50 mg/ml, and dialyzed exhaustively two times against 1 L of 18 MΩ water for 48 h at 4 °C. The solution was then aliquotted into 4 equal volumes, lyophilized, and redissolved in 0.63 ml each of four solvent systems: 18 MΩ water; 18 MΩ water containing 0.77 M d_5-glycine (Cambridge Isotope Laboratories, MA); 18 MΩ water plus 0.1 M NaCl, 0.02 M sodium phosphate (pH 7); and 18 MΩ water plus 0.2 M NaCl, 0.02 M sodium phosphate (pH 7). Seventy microliters of D_2O was added to each sample before loading into a 5 mm NMR tube (Wilmad pp535-7). Similarly, lysozyme samples were prepared in D_2O for assessing performance of the cold probe for heteronuclear NMR experiments. D-Glucose samples were prepared similarly from a stock solution in D_2O aliquotted and lyophilized, then redissolved in 0.75 mL of 100% D_2O.

Conductivity and pH measurements were made using a combination Accumet meter (Fisher Scientific) standardized against appropriate NIST-traceable solutions.

NMR spectra were recorded on 4 channel Varian Inova spectrometers at 14.1 and 18.8 T using inverse triple resonance HCN probes. All spectra were recorded at 298 K. 1D spectra were recorded using a simple presaturation sequence for the glucose samples, with a recycle time of 5 s with and without steady-state pulses. For the lysozyme samples, the water was suppressed with the Watergate method [10] with optimization of the solvent frequency, pulse spacing and gradient strength. The acquisition time was 1.5 s, and the recycle time was 4 s. Watergate TOCSY and NOESY experiments were recorded on the lysozyme samples with mixing times of 50 ms, for 10 h with acquisition times of 0.256 s in t_2 and 36 ms in t_1. The TOCSY spin lock strength was calibrated at 8 kHz, and a recycle time of 2 s.

Natural abundance gradient ^{13}C–^1H HSQC experiments were carried out on the glucose and lysozyme

Table 1
Comparison of probe performance for different solvent conditions

Sample	P90/μs (SNR)		
	HCN_RT(600)	HCN_CP(600)	HCN_RT(800)
Glucose/D_2O	6.70 (120)	6.08 (375)	5.70 (215)
Glucose/Gly	6.81 (120)	6.25 (318)	5.90 (192)
Glucose/0.08 M Na^+	7.80 (102)	7.18 (232)	6.73 (225)
Glucose/0.2 M Na^+	8.30 (99)	7.80 (183)	7.30 (166)
HEWL/H_2O	6.20 (11)	6.20 (33)	5.80 (13)
HEWL/Gly	6.30 (11)	6.40 (28)	5.90 (12)
HEWL/0.1 M Na^+	7.55 (8.5)	7.85 (17.2)	7.41 (9.0)
HEWL/0.2 M Na^+	8.26 (7.7)	8.85 (15.7)	nd

Glucose or hen egg white lysozyme (HEWL) solutions were prepared as described in Methods. The 90° pulse widths are compared, with the SNR values for a single pulse in parentheses. The glucose solutions are in D_2O, and the HEWL are in 90% H_2O/10% D_2O. HCN_RT is the HCN triple resonance room temperature probe, HCN_CP is the HCN triple resonance cold probe. nd, not determined.

samples to assess heating effects from decoupling. The acquisition time in t_2 was set to 0.13 s and the recycle time was 2 s.

3. Results

Even moderate concentrations of sodium chloride have a significant effect on probe tuning and raw sensitivity. Table 1 shows the 90° pulse widths at constant transmitter power for glucose and lysozyme samples on different probes at different magnetic field strengths. Thus, compared with water, the 90° pulse width for 200 mM NaCl increased about 25% on the RT (600) probe 30–40% on the cold probe, and about 30% on the RT (800) probe. In single pulse 1D experiments, these losses translate to similar decreases in SNR on the RT probes, and rather more on the cold probe, i.e., up to a factor of 2-fold at 14.1 T. In contrast, 0.5 M glycine has a small (<3%) effect on the probe tuning, and correspondingly small effects on the raw SNR.

The 90° pulse widths correlate with the conductance of the solutions, which increases in the progression: water (2.3 μS) < 0.5 M Gly (22 μS) < 1 M Gly (39 μS) < lysozyme in H_2O (90 μS) < 0.09 M NaCl (14,600 μS).

Fig. 1 shows low field 1D spectra of lysozyme in 90% H_2O/10% D_2O solutions. Under these conditions there is no significant contribution from aminoproton resonances of glycine owing to exchange broadening. The slight differences in the spectra are due to salt-dependent effects on exchange and minor pH differences between samples. Solvent-dependent changes in linewidth were corrected for in the determination of the SNR values in Table 1.

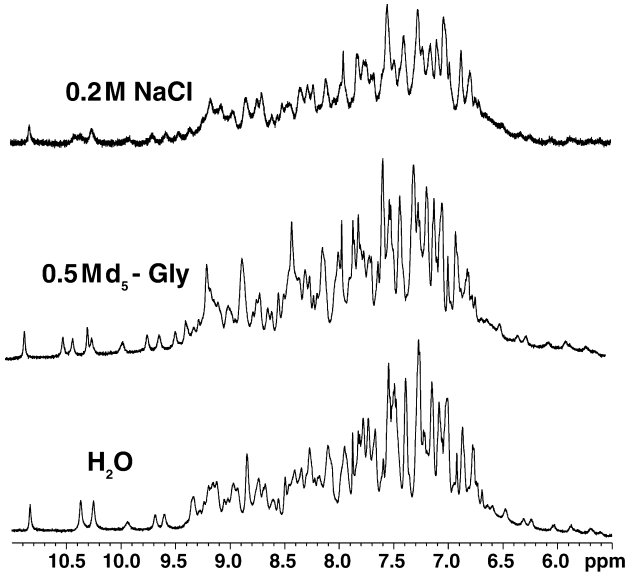

Fig. 1. D Spectra of lysozyme. Hen egg white lysozyme samples were prepared as described in the text. Spectra were recorded at 14.1 T using an HCN cold probe (CP). Spectra were recorded using Watergate [10] at 20 °C with an acquisition time of 1.5 s and a recycle time of 5 s. Thirty-two steady states were used, and the displayed spectra are the result of one transient. The spectra were transformed by zero-filling once and transforming with no window function. The spectra are displayed under the following solution conditions: water (bottom), 0.5 M d_5-Gly (middle), and 0.2 M NaCl (top).

3.1. Effects of decoupling

We have recorded ^{13}C–^{1}H HSQC spectra of glucose at natural abundance with and without ^{13}C decoupling during the acquisition time to assess the influence of decoupling during the acquisition time to assess the influence of decoupling. At 14.1 T the presence of 0.2 M NaCl decreased the SNR, averaged over all cross-peaks in the spectrum, to 89 ± 12% on the RT probe, compared with 54 ± 11% on the cold probe. In contrast, 0.5 M d_5-Gly had little effect on the SNR (<5% decrease in SNR on both probes). Comparing coupled with decoupled spectra revealed that decoupling in the cold probe in the presence of 0.2 M salt was marginally poorer than on the RT probe. The intensities of 20 cross-peaks were also measured on the natural abundance, decoupled HSQC spectra of the lysozyme samples. The ratio of the SNR in D_2O was 0.87 ± 0.04 for the glycine versus D_2O, and 0.35 ± 0.03 for 0.2 M NaCl versus D_2O. Thus, HSQC spectra with heteronuclear decoupling are substantially affected by salt on cold probes, which is largely overcome by replacement with glycine.

3.2. 2D NMR

For biological samples, 2D NMR experiments in H_2O may provide a more appropriate real world comparison. We have used NOESY and TOCSY to compare the performance of the probes in different solution conditions.

Table 2 shows that all probes show significant sensitivity loss as the ionic strength is increased. The decreased performance is greater at 800 MHz than at 600 MHz, and is most severe on the cold probe. The performance is much worse in the TOCSY experiment with a 50 ms spin lock and an 8 kHz B_1 field strength, both of which are typical values for moderate sized proteins. This degradation in performance greatly ameliorated by replacing the salt with 0.5 M d_5-Gly.

At zero added salt, the cold probe showed a 2.8-fold sensitivity enhancement in both the NOESY and TOCSY experiments,; the values for 0.5 M d_5-Gly are essentially the same. However, in the presence of 100 mM Na^+ the enhancement on the cold probe is significantly diminished. For NOESY, the enhancement was 2-fold, whereas for TOCSY it was only 1.5-fold under the same solvent conditions. The presence of 0.5 M Gly maintains the intrinsic sensitivity enhancement.

Table 2
Comparison of probe performance in 2D experiments with lysozyme samples

Solvent	NOESY	TOCSY
HCN_RT(600)		
Gly	1.04 ± 0.1	0.97 ± 0.1
0.1 M Na^+	0.53 ± 0.09	0.33 ± 0.02
0.2 M Na^+	0.46 ± 0.07	0.25 ± 0.02
HCN_CP(600)		
H_2O	2.73 ± 0.3	2.74 ± 0.1
Gly	3.0 ± 0.4	2.73 ± 0.2
0.1 M Na^+	1.08 ± 0.18	0.47 ± 0.06
0.2 M Na^+	0.78 ± 0.13	0.45 ± 0.05
HCN_RT(800)		
0.1 M Na^+	0.44 ± 0.04	0.26 ± 0.02

Spectra were recorded with 300 real increments in t_1, using flipback Watergate NOESY (mix = 100 ms) and TOCSY (mix = 50 ms, $B_1 = 8$ kHz) experiments as described in Methods. Data tables were padded with zeroes to 8192 by 2048 complex points, and apodized using unshifted Gaussian functions and 1 Hz line broadening in both dimensions. SNR measurements were made on ten isolated cross-peaks on slices parallel to F2. Values have been normalized to the spectrum in water recorded on the RT probe, and are quoted as means ± SEM. HCN_RT is the HCN triple resonance room temperature probe, HCN_CP is the HCN triple resonance cold probe.

For comparison, we have recorded the same experiment on a RT probe at 18.8 T. This shows that in the presence of 0.1 M Na^+, the sensitivity compared with pure water was reduced 56% in the NOESY and 74% in the TOCSY. The lower performance in the TOCSY experiment is probably due to heating effects during the spin lock period, as this is more noticeable at higher ionic strength.

4. Discussion

At low ionic strength, our data show that, as expected, the NMR sensitivity scales with the magnetic field strength. As the ionic strength is increased, there is a significant reduction in sensitivity even for simple experiments, as pointed out by Kelly et al. [3]. Furthermore, this ionic strength-dependent sensitivity loss increases at higher magnetic field strengths, and is more pronounced for the cooled probes than for the conventional probe. We have found that the simple compatible osmolyte, glycine, is a reasonable substitute for a supporting electrolyte in terms of maintaining protein solubility. Salt affects the SNR of the cold probes more than conventional probes because they contribute to thermal noise in the sample, which dominates the noise in cold probes [3]. As glycine does not contribute to the solution conductivity near its isoelectric pH, a >3-fold enhancement of sensitivity is obtained using the cold probe compared with the conventional probe.

Glycine, and presumably other biological dipolar ions, is effective at increasing protein solubility at 0.5–1 M by thermodynamic activity effects, and also because it increases the dielectric constant of water substantially. Glycine, and other compatible solutes, also stabilizes proteins [7,9] without direct binding. Perdeuterated glycine is readily available and relatively inexpensive. Its low molecular weight (75) also means that only small quantities are needed to make concentrated (0.5–1 M) solutions. Chemically it is very simple, and less likely to interact with proteins than more complex, large solutes [6]. These results are important for NMR of large proteins, which cannot be dissolved at high molar concentration, so that sensitivity is at a premium. For this it is desirable to use very high magnetic field strengths to take advantage of TROSY [11–13] and cold probe technology. To optimize sensitivity, one should avoid dielectric losses from ions, which can be achieved by using dipolar ion substitutions, such as glycine. The relative improvement due to replacement of salt by glycine at higher magnetic field strengths should be more pronounced given the general response of sensitivity to ionic strength with increasing magnetic field strength. Thus, we would expect this approach to be even more useful at 900 MHz.

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