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Low-Conductivity Buffers for High-Sensitivity NMR Measurements

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Abstract: The sensitivity of nuclear magnetic resonance (NMR) probes, especially the recently introduced cryogenic probes, can be substantially reduced by the electrical noise generated by conductive samples. In particular, samples of biological macromolecules, which usually contain salts to keep the pH constant and to prevent aggregation, can experience a significant reduction in sensitivity. So far this dependence has forced researchers to minimize the salt concentrations in their samples. Here we demonstrate that the decisive factor is not the salt concentration itself but the conductivity which is a function of both the concentration and the mobility of the ions in solution. We show that by choosing buffers with low ionic mobility, the sample conductivity can be dramatically reduced and the sensitivity substantially enhanced compared to the same measurement with an equal concentration of a standard NMR buffer such as phosphate. We further show that the highest sensitivity gain of one buffer over another buffer is equal to the square root of the ratio of their ion mobilities and describe a simple method to evaluate the effect of a certain buffer on the sensitivity.

Introduction

Compared to other spectroscopic techniques NMR spectroscopy is a relatively insensitive method, requiring concentrations in the micro- to millimolar range. However, NMR provides an enormous amount of detail about the chemical organization and the structure of compounds that—with the exception of X-ray crystallography—cannot be obtained by any other method. Arguably, NMR has become the most important analytical tool in organic chemistry and a very important one in structural biology and biochemistry. On the basis of this importance, improving the sensitivity of NMR experiments has been a major goal for many research groups over the past 30 or more years. The introduction of pulsed Fourier techniques,[1] stronger magnets, better preamplifiers and probes, and pulse sequences such as the sensitivity enhancement method[2−4] have all contributed to an enormous increase in sensitivity. One of the most important contributions has been the recent introduction of cryogenic probes.[5−11] These probeheads increase the sensitivity of NMR experiments 3−4-fold relative to conventional probeheads.[12−14] This sensitivity increase is achieved by cooling the radio frequency (rf) receiver coils to temperatures of 15 to 30 K. At these temperatures the coils have lower resistance, allowing for higher quality factors (Q) (and increased signal amplitude) and of course lower thermal noise. Both of these factors, the higher Q-factor and the lower noise, result in an increase in the signal-to-noise (S/N) ratio and hence sensitivity.

This full sensitivity increase of cryogenic probes, however, is realized only if the sample under study is electrically insulating, for example organic solvents. An electrically conductive sample, such as buffers used in protein structure determinations, will add a resistance to the coil, which can significantly

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Table 1. R_s/R_c Values, Expected Sensitivity Factor *L*, and Dc Conductivity of Several Different Salts, All at 200 mM Concentration

buffer	R_s/R_c	sensitivity factor *L*	conductivity (mS/cm)
pentasodium tripolyphosphate	2.71 ± 0.04	0.22	31.3
potassium chloride	1.93 ± 0.04	0.26	23.3
disodium phosphate (Na_2HPO_4)	1.89 ± 0.04	0.26	22.0
sodium pyrophosphate	1.70 ± 0.04	0.27	20.2
sodium chloride	1.64 ± 0.04	0.28	18.1
PIPES	1.33 ± 0.04	0.30	14.8
β-glycerophosphate	1.31 ± 0.04	0.30	14.9
potassium phosphate (KH_2PO_4)	1.25 ± 0.04	0.31	14.1
TRIS HCl	1.24 ± 0.04	0.31	14.1
BIS−TRIS HCl	1.12 ± 0.03	0.33	13.62
sodium acetate	1.11 ± 0.03	0.33	12.2
sodium phosphate (NaH_2PO_4)	0.95 ± 0.03	0.35	11.0
sodium TAPS	0.90 ± 0.03	0.36	9.55
sodium MES	0.88 ± 0.03	0.36	10.18
sodium MOPS	0.88 ± 0.03	0.36	9.86
sodium TES	0.84 ± 0.03	0.37	9.41
sodium HEPES	0.84 ± 0.03	0.37	9.25
tetrabutylammonium dihydrogen phosphate	0.69 ± 0.03	0.40	9.00
HEPES	0.22 ± 0.02	0.62	0.06
TAPS	0.14 ± 0.02	0.70	0.29
CAPS	0.14 ± 0.02	0.70	0.7
TES	0.12 ± 0.02	0.73	0.25
MOPS	0.10 ± 0.02	0.76	0.04
CHES	0.08 ± 0.02	0.79	0.06
MES	0.08 ± 0.02	0.80	0.15
bicine	0.05 ± 0.02	0.86	0.031
BIS−TRIS propane	0.05 ± 0.02	0.86	0.022
TRIS base	0.03 ± 0.02	0.91	0.1
BIS−TRIS	0.02 ± 0.02	0.93	0.0236
deionized−distilled H_2O	0.01 ± 0.02	0.98	0.0023

reduce the signal-to-noise ratio. Many biological macromolecules must be studied in buffered solutions to keep the pH constant and the molecule in a defined protonation state. Moreover, in many cases additional salts must be added to increase the solubility and to prevent aggregation of the investigated biomolecules. It has been shown by numerous researchers that salt concentrations of 100−150 mM, which are typical for many biological samples, decrease the sensitivity advantage of a cryogenic probe to about a factor of 2 better than that of a conventional probe with the same sample.[9,15] Usually, several different buffers and salts can be used to obtain good solution conditions, providing the NMR spectroscopist with some options. So far, buffers are mainly chosen to minimize interference of their NMR signals with the signals of the biological macromolecules under investigation. A recent survey of buffer conditions used for NMR structure determinations showed that 27% of all structures were determined in unbuffered (or autobuffered) solutions, 50% in phosphate, 10% in acetate buffer, and 9% in TRIS buffer.[16] These buffers either do not contain protons or are commercially available in deuterated forms.

With the introduction of cryogenic probes, optimization of sensitivity has emerged as another criterion for buffer selection. To achieve the highest possible sensitivity and to optimize the advantages of cryogenic probes, the laboratory of Wand has designed a very powerful method that is based on encapsulating proteins in reverse micelles which are themselves dissolved in organic solvents of low viscosity.[15,17−19] Such samples not only show sharper resonance lines due to faster tumbling rates of the proteins but also nearly eliminate the sample noise contribution, thus providing the highest possible sensitivity. Unfortunately, however, this method requires time-consuming sample preparation and is not applicable to all proteins.

Designing buffers that are easily prepared, work with most proteins, are adjustable to the required pH, and preserve the sensitivity of cryogenic probes would be very attractive. To date, the optimization of buffer conditions for achieving high sensitivity has focused on reducing the total salt concentration which of course must be balanced against the need to maintain the conformation and solubility of the macromolecule. However, as we show in this article, the sensitivity depends on the conductivity of a sample, which is a function of both the ion concentration and ion mobility. Using NMR buffers made of ions with low ion mobility should, therefore, provide a way to improve the sensitivity of NMR experiments even if high salt concentrations are necessary, for example to prevent aggregation. In this paper we show that such buffers can indeed be found and that they can increase the sensitivity significantly relative to the currently most widely used buffers.

Experimental Section

Preparation of Buffers. To investigate the influence of different buffers on the sensitivity of NMR experiments, salt solutions were prepared with varying predicted conductivities based on ion type. All solutions were made with 0.22 μm filtered, deionized−distilled H_2O and the highest-grade reagents available (Sigma or Fluka). All buffers in Table 1 were made to a concentration of 200 mM without adjustment of the pH. For the design of buffers at a specific pH, selected salts from Table 1 were titrated with concentrated solutions of different bases or acids as indicated in Table 2. All buffers had a final concentration of the compound indicated in column 1 of 200 mM. The phosphate

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Table 2. R_s/R_c Values, Expected Sensitivity Factor *L*, and Dc Conductivity for Several Buffers, Adjusted to Specific pH Values with Different Acids or Bases

buffer	titrated with	pH	R_s/R_c	sensitivity factor *L*	conductivity (mS/cm)
BIS−TRIS propane	HCl	6.8	1.76 ± 0.04	0.27	19.34
	PIPES		0.84 ± 0.03	0.37	8.75
TRIS base	HCl	8.0	0.88 ± 0.03	0.36	9.60
	TES		0.60 ± 0.03	0.43	5.71
sodium phosphate		7.0	1.63 ± 0.04	0.28	17.34
MOPS	BIS−TRIS propane	7.0	0.22 ± 0.02	0.61	2.40
bicine	NaOH	8.0	0.26 ± 0.02	0.58	2.61
	TRIS base		0.29 ± 0.02	0.56	2.50
HEPES	BIS−TRIS propane	7.0	0.31 ± 0.02	0.55	1.30
	NaOH		0.41 ± 0.02	0.50	2.44

buffer was prepared by mixing Na_2HPO_4 and NaH_2PO_4 solutions according to published tables[20] to a final phosphate concentration of 200 mM. All pH measurements were made with a Mettler-Toledo MP 220 pH-meter.

Measurement of the *Q*-Factor. To determine the rf quality factor (*Q*) of the cryogenic probe loaded with different samples, we connected the probe to a network analyzer. A volume of 700 μL of each solution was placed in a standard 5 mm NMR sample tube and loaded into the cryogenic probe (Bruker 500 MHz TXI CryoProbe). The probe was, for each sample, matched to an HP8752C vector network analyzer, and the *Q* of its ^{1}H channel was measured. Measurement errors for the R_s/R_c values were estimated from $\Delta(R_s/R_c) = [(\Delta Q/\Delta Q_0)^2 + (\Delta Q_0/Q^2)^2]^{0.5}$, where Q_0 is the *Q* of the empty probe. Experimental errors of the *Q*-factor measurements were determined by repeating individual measurements three times.

Conductivity Measurements. The conductivities of all solutions listed in Tables 1 and 2 were determined using an Amber Science Inc. (Eugene, OR) model 1056 conductivity meter at room temperature. The meter was calibrated before each measurement using a 0.005 N solution of KCl (718 ± 1 μS/cm at 25 °C) as a standard.

Signal-to-Noise Measurements. Signal-to-noise measurements were performed on 200 mM solutions of HEPES−NaOH (pH 7.0), TRIS−HCl (pH 7.0), sodium phosphate (pH 7.0), sodium chloride, and pentasodium tripolyphosphate at 25 °C containing 2 mM *p*-aminobenzoic acid (PABA) as a reference. Exactly 500 μL of each solution was loaded into standard 5 mm NMR tubes, and one-dimensional (1D) spectra were acquired with 4 scans. Signal-to-noise ratios of the most downfield doublet of PABA were calculated using the program Xwinnmr (Bruker). Relative peak intensities were also determined on 500 μL, 50 mM solutions of MES−BIS−TRIS (pH 6.0), HEPES−NaOH (pH 7.0), MOPS/BIS−TRIS propane (pH 7.0), and sodium phosphate (pH 7.0) containing 1 mM lysozyme (Sigma) with 4 scans and processed using Xwinnmr. The lysozyme was extensively dialyzed against deionized−distilled H_2O prior to sample preparation. All measurements were carried out on a 500 MHz Bruker DRX NMR instrument equipped with a TXI CryoProbe.

Results and Discussion

The signal-to-noise ratio (S/N) or sensitivity of NMR experiments depends on many different factors that include sample specific parameters such as concentration and parameters linked to the individual pulse sequence and the hardware used during the experiment. The most important hardware components that influence the sensitivity are the probe and the preamplifier. Their contribution depends on the temperature of the coil, T_c, its resistance, R_c, the temperature of the sample, T_s, the resistance added to the coil by the sample, R_s (henceforth referred to as the "sample resistance"), and the noise temperature of the preamplifier, T_a. This dependence can be written in the following equation:[21−24]

$$S/N \sim (T_c R_c + T_a[R_c + R_s] + T_s R_s)^{-0.5} \quad (1)$$

In cryogenic probes the temperature of the coil is in the range of 15−30 K, the preamplifier noise temperature is in the range of 10−15 K, and the coil resistance is small compared to the resistance of conventional room-temperature probes. This makes the first and second terms in eq 1 small and is the basis for the higher sensitivity of cryogenic probes relative to probes with the coil and preamplifier at room temperature. The third term, however, is similar for conventional and for cryogenic probes since it depends primarily on the sample temperature and the sample resistance. From eq 1 it is easy to see that an increase in this third term decreases the sensitivity. Equation 1 also predicts that the relative influence of the third term is stronger for cryogenic probes because the first two terms are much smaller for cryogenic probes than for conventional probes. As we will show below, R_s is proportional to the sample conductivity, and this explains why samples of high conductivity have a stronger impact on the sensitivity of cryogenic probes than on room-temperature probes (however, one should point out that the absolute sensitivity of a cryogenic probe is always higher than that of a conventional probe). For most biological and many chemical applications the sample temperature can only be changed within a narrow range of roughly 30 K or ∼10% of the value of T_s. On the other hand, the sample resistance R_s depends on the exact buffer and salt conditions used in the sample. Salt concentrations in biological NMR samples vary from mM to M or by 2−3 orders of magnitude. On the basis of this large variation, the sample resistance R_s becomes one of the most critical parameters in determining the sensitivity of NMR experiments with cryogenic probes. This sample resistance is the result of the inductive coupling between the sample and the coil. The value of this resistance can be calculated from the energy dissipated in the sample by currents induced in the sample by the rf field. Gadian and Robinson calculated this to be, for a solenoidal coil of radius *a* and *n* turns[23]

$$R_s = \frac{\pi\omega^2\mu^2\sigma n^2 b^4 L}{32(a^2 + (L/2)^2)} \quad (2)$$

where ω is the angular frequency of operation, μ is the permeability of free space, and σ, *b*, and *L* are respectively the conductivity, the radius, and the length of the sample.[25]

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The dependence of the sensitivity of an NMR experiment on the salt concentration has been recognized for a long time, and a lot of attention has focused on minimizing the salt concentration of NMR samples. However, as shown in eq 2, the sample resistance is proportional to the conductivity σ of the sample which in turn is proportional not only to the ionic concentration c but also to the mobility λ of the ions in solution and their respective charge q. For a solution containing different types of ions, their individual contributions are summed up:

$$R_s \sim \sigma = \Sigma c_i q_i \lambda_i \tag{3}$$

Here the index i covers all of the ionic species present. This equation predicts that choosing buffers with low ion mobility λ should allow us to preserve the high sensitivity of cryogenic probes even in the presence of high salt concentrations.

To test this hypothesis we have investigated the influence of several different salt solutions on the sensitivity of a cryogenic probe by measuring the quality- (or Q-) factor of the proton channel of a triple resonance cryogenic probe on our 500 MHz Avance Bruker NMR instrument. Measurements of the individual quality-factors and of Q_0, the quality-factor of the empty, unloaded probe, allows us to calculate the ratio of sample resistance, R_s, to the coil resistance, R_c:

$$R_s/R_c = Q_0/Q - 1 \tag{4}$$

In turn, this ratio can be used to calculate the sensitivity factor, L, defined as the ratio of the sensitivity of the loaded probe and the unloaded probe:

$$L = \frac{(S/N)_{loaded}}{(S/N)_{unloaded}} = \sqrt{\frac{R_c(T_c + T_a)}{R_c T_c + T_s R_s + T_a(R_c + R_s)}} = \left(1 + \frac{R_s(T_s + T_a)}{R_c(T_c + T_a)}\right)^{-0.5} \tag{5}$$

The factor L can vary between 0 and 1 with 1 being the highest achievable sensitivity, i.e, that of a probe with a nonconductive sample, which does not reduce the sensitivity from that of an empty probe. With the temperatures T_s set to 298 K, T_c to 27 K, and T_a to 15 K, this equation can be simplified to

$$L = \left(1 + 7.45\frac{R_s}{R_c}\right)^{-0.5} \tag{6}$$

Table 1 lists the R_s/R_c ratios and the expected sensitivity factors L for all investigated samples. All solutions were at a concentration of 200 mM to increase the accuracy of the rf measurement. Also shown are the dc conductivities measured for each sample. The results in Table 1 demonstrate that huge differences in the R_s/R_c ratios exist, ranging from 2.71 for pentasodium tripolyphosphate to 0.02 for BIS−TRIS. These differences in the R_s/R_c values predict sensitivity differences between the best and the worst solutions of more than a factor of 4, showing a strong influence of the nature of the salt in the sample on the sensitivity. The results suggest that the detrimental effect of high salt concentrations can be counterbalanced by low ion mobilities. However, the conductivity of the sample depends also on the protonation state of the individual buffer. For example, weak acids are mainly protonated and uncharged while zwitterionic buffers carry both a negative and a positive charge and are overall neutral. The results in Table 1 can, therefore, be used only for rough guidance for the creation of buffers that preserve the high sensitivity of cryogenic probes. However, these results also clearly demonstrate the effect of the ion mobility on the sensitivity. Comparison of the results obtained with NaCl and KCl shows that their conductivity and sensitivity factors differ despite both having the exact same ionic concentration (and ionic strength). The sodium salt achieves a higher sensitivity than the potassium salt, in accordance with their relative ion mobilities.[26,27] Stronger effects are seen in a comparison of the phosphate salts: the highest sensitivity is achieved by tetrabutylammonium, followed by sodium and potassium, again following the relative mobilities of these ions.[26,27]

The solutions used in the experiments described above were not adjusted to a particular pH. Most biological and chemical NMR applications, however, require a particular pH value to keep the solute in a defined protonation state. Typically, specific pH values are achieved by titrating a solution of a weak acid or weak base with a strong base or strong acid, usually hydrochloric acid and sodium hydroxide (or, alternatively, specific pH values are achieved by mixing the appropriate amounts of an acid and its conjugate base according to the Henderson−Hasselbalch equation[28]). These titrations, however, add additional ions to the solution. Since the conductivity of the entire sample is the sum of the contributions of the individual ion species, adding ions with a high mobility can have a detrimental effect on the sensitivity. This effect can be seen in the data of Table 1. The sodium and chloride salts of several organic buffers with low ion mobilities of the organic component show a high conductivity and a low sensitivity due to the presence of the sodium or chloride ions with high mobility. This problem can, of course, be avoided if an acid or base with low ion mobility is added. Unfortunately, most acids and bases with low ion mobilities are weak and the titration of a weak acid with a weak base does not necessarily produce a good buffer. However, if the pK_a values of both involved compounds are very similar, solutions with good buffer capacities can be produced. Examples are combinations of the base BIS−TRIS propane (pK_a 6.8) with the acids PIPES (pK_a 6.8) or MOPS (pK_a 7.2) and TRIS base (pK_a 8.1) with bicine (pK_a 8.3). On the basis of the results shown in Table 1, we have selected some of the best compounds and titrated them either with hydrochloric acid or sodium hydroxide or, if the pK_a values of a weak acid and a weak base are close, we also tested those combinations. Table 2 summarizes our results. Clearly, certain combinations show significantly higher predicted sensitivities than others. In particular, the combination of MOPS and BIS−TRIS propane at pH 7 as well as bicine and TRIS base or NaOH at pH 8 should achieve high sensitivities.

Equation 3 shows that the conductivity of a certain buffer also depends on the charge of its ions. At the same molarity, a

(25) Note that while the use of a saddle coil instead of a cylindrical coil changes the form of this equation somewhat, it does not alter the strict linear dependence of the sample resistance, induced by the coil, on the conductivity of the sample.

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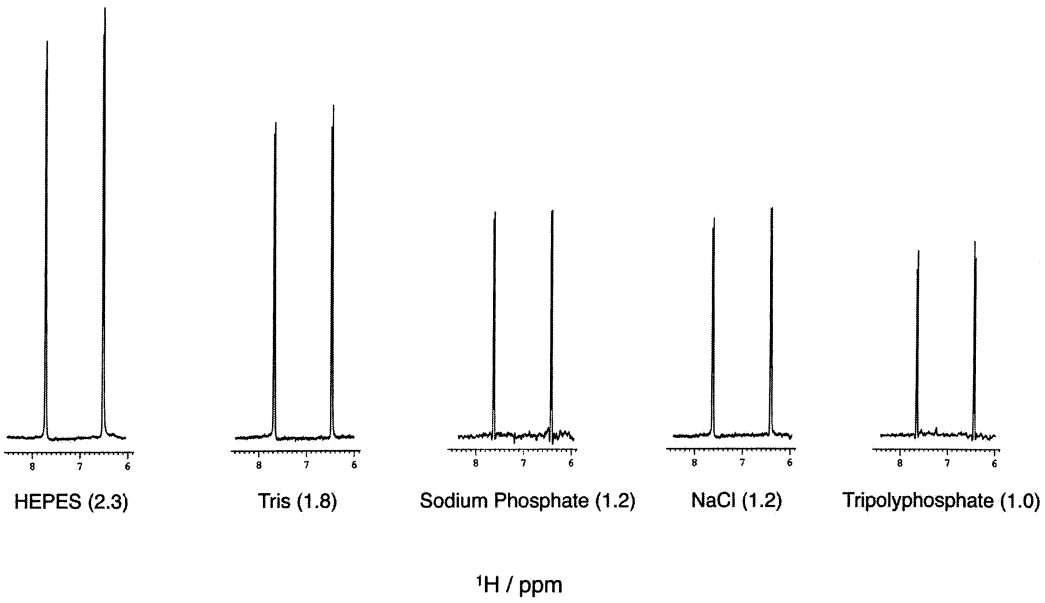

Figure 1. Comparison of the sensitivity obtained with a 2 mM *p*-aminobenzoic acid sample dissolved in 200 mM HEPES buffer (titrated with NaOH), 200 mM TRIS buffer (titrated with HCl), 200 mM sodium phosphate buffer, 200 mM NaCl, or 200 mM pentasodium tripolyphosphate buffer. The first three buffers are identical with the ones used for the measurements in Table 2, and the last two buffers, with the ones used in Table 1. Sensitivities relative to pentasodium tripolyphosphate are given in parentheses. The two phosphate-based buffers showed increased noise around the water resonance that is not observed with the other buffers. Therefore, the noise level was determined between 9 and 10 ppm.

buffer with multiple charges such as phosphate reduces the sensitivity more than a buffer with a single charge and similar ion mobility. To predict the effect of a certain buffer on the sensitivity, the protonation state of its ions must also be considered. At pH values around 2 the main species of a phosphate buffer are H_3PO_4 and $H_2PO_4^-$, while, at the more biological relevant pH of 7, $H_2PO_4^-$ and HPO_4^{2-} are dominant and a further decrease of the sensitivity due to the increased charge is expected. In addition, increased charge also is linked to an increase in the counterion concentration. In particular, if the counterions have a high mobility, they can significantly further decrease the sensitivity. One example is pentasodium tripolyphosphate, which we have included in our investigation as an example of a compound with high conductivity. As mentioned above, all buffer concentrations for the experiments reported in Tables 1 and 2 were 200 mM. We have used this molarity-based comparison and not a normality-based one (normalized to the same amount of charge) because we wanted to compare buffers with similar buffering capacities.

To further investigate if the differences in buffer sensitivities predicted by the data shown above can be detected in real NMR experiments, we prepared 2 mM solutions of *p*-aminobenzoic acid (PABA) in 200 mM solutions of HEPES/NaOH (pH 7.0), TRIS base/HCl (pH 8.0), NaCl, sodium phosphate (pH 7.0), and pentasodium tripolyphosphate (unadjusted pH). Figure 1 shows one-dimensional proton spectra measured with these samples. These spectra confirm that the type of buffer has a dramatic effect on the sensitivity of NMR experiments. Furthermore, comparison of the relative intensities in the spectra with the predicted sensitivity factors L of the used buffers demonstrates a good correlation.

All experiments described above have been carried out at salt concentrations of 200 mM. However, most NMR experiments are performed at different ion concentrations and the relative sensitivity gain of a specific buffer over another type

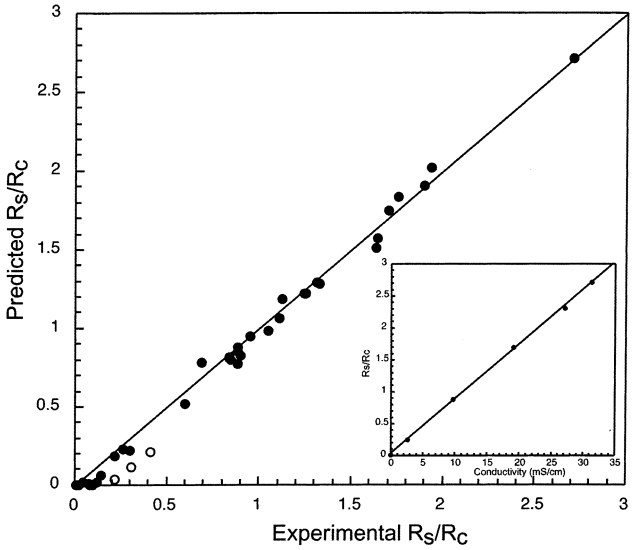

Figure 2. Dependence of the R_s/R_c value on the conductivity of the sample. Pentasodium tripolyphosphate solutions ranging from 8 to 200 mM were used to measure the calibration curve shown in the inserted graph. Virtually identical results were obtained with NaCl samples. On the basis of this calibration curve, the R_s/R_c values for all buffers used in the experiments reported in Tables 1 and 2 were calculated from their dc conductivities. Open circles represent the three HEPES buffers. In addition, the theoretical line with a slope of 1 is also shown.

of buffer also depends on the concentration. The dependence of the relative sensitivity of two buffers can be calculated from the ratio of their sensitivity factors L_1 and L_2:

$$\frac{L_1}{L_2} = \sqrt{\frac{\left(1 + 7.45\frac{R_{s2}}{R_c}\right)}{\left(1 + 7.45\frac{R_{s1}}{R_c}\right)}} \qquad (7)$$

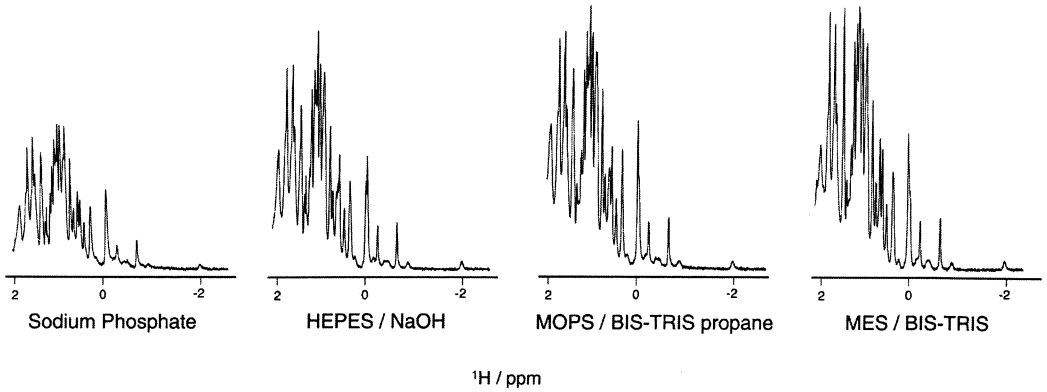

Figure 3. One-dimensional spectra of a 1 mM lysozyme sample measured in 50 mM sodium phosphate, 50 mM HEPES/NaOH, or 50 mM MOPS/BIS−TRIS propane buffer, all pH 7, and in 50 mM MES/BIS−TRIS, pH 6.0. Only the most high-field-shifted regions of the spectra are shown.

For low buffer concentrations the sample resistance of the two buffers, R_{s2} and R_{s1}, decreases. In the limit of very low concentrations the factor $7.45R_{s2}/R_c$ becomes small relative to 1 and can be neglected. In this case, the ratio of L_1/L_2 is equal to 1 and the sensitivity is independent of the nature of the buffer. At high salt concentrations, the sample resistance becomes the dominant factor in eq 7. In this case the factor $7.45R_s/R_c$ becomes large relative to 1, and in combination with eq 3, eq 7 can be simplified (ignoring the effect of the counterion in the sum):

$$\frac{L_1}{L_2} = \sqrt{\frac{\lambda_2}{\lambda_1}} \tag{8}$$

This result shows that for high salt concentrations, the gain in sensitivity of a particular buffer over another buffer becomes independent of the actual ion concentration and reaches a maximum that is equal to the square root of the ratio of the individual ion mobilities.

The results summarized in Tables 1 and 2 show some examples of buffers that can be used to achieve high sensitivity. However, many more potentially interesting buffers exist that remain to be tested. Furthermore, biological samples often require mixtures of different buffers and salts to keep the pH stable and to prevent aggregation. In principle, the measurement of the quality factor of a probe loaded with a specific buffer can be used to calculate the sensitivity factor, which allows us to evaluate the usefulness of a particular buffer. However, in most cases a network analyzer to measure the *Q*-factor will not be readily available. On the other hand, eqs 3 and 4 predict a linear relationship between the R_s/R_c ratio and the dc conductivity of the sample. Since conductivity meters are available in many laboratories, a calibration curve that links the measured conductivity to the R_s/R_c ratio can be used to calculate with eq 5 the sensitivity factor *L*. We have used pentasodium tripolyphosphate and NaCl solutions ranging in concentration from 8 to 200 mM to measure both the quality factor of the probe loaded with these solutions and their conductivity. Figure 2 shows a graph of the R_s/R_c ratio versus the conductivity demonstrating the expected linear relationship. From a linear regression analysis we obtained the following equation with *C* being the conductivity of the sample measured in mS/cm:

$$R_s/R_c = 0.087\ \mathrm{mS^{-1}\,cm\ C} \tag{9}$$

To investigate if eq 9 can be used to calculate the sensitivity factor *L* of a certain buffer, we have used it to calculate R_s/R_c values for all buffers and salt solutions in Tables 1 and 2 on the basis of their conductivity. A plot of the measured R_s/R_c values versus the calculated ratios should yield a straight line with the slope of 1. Figure 2 shows that graph. It demonstrates that indeed a good correspondence between the measured and the calculated values exists, indicating that conductivity measurements can be used to determine the sensitivity of buffers in NMR experiments. Some deviations from the theoretical line exist, mainly in the region with low conductivity buffers. In particular HEPES buffers show a linear correlation with a different *y*-axis intercept. This behavior is due to systematically smaller conductivity values measured by the conductivity meter. We interpret this result as likely being caused by interaction between the HEPES buffer and the electrode used during these measurements. For buffers that show such deviations, an additional calibration curve with this buffer can be determined.

The exact form of eq 9 will also depend on the specific probe. However, a calibration curve could easily be determined with the help of a network analyzer as part of the installation procedure of the probe. Once such a calibration curve is available, the relationship between the conductivity and the R_s/R_c value can be used to optimize buffer conditions that preserve the high sensitivity of cryogenic probes.

Most biological NMR experiments are carried out at buffer concentrations of approximately 50 mM. To investigate the sensitivity gain of two of our best buffers at pH 7.0, MOPS/BIS−TRIS propane and HEPES/NaOH, over the most commonly used NMR buffer, sodium phosphate, we prepared solutions of 1 mM lysozyme in all 3 buffers and measured one-dimensional experiments. The resulting 3 NMR spectra are shown in Figure 3. To avoid problems with differences in the amide proton exchange rates and overlap with buffer resonances, only the extreme high-field end of the spectra were used for an analysis of the relative sensitivities. The sensitivities in the MOPS- and the HEPES-buffered spectra are virtually the same. This is in agreement with theoretical values for both buffers at a 50 mM concentration. The conductivity for the 50 mM HEPES/NaOH buffer is 0.693 mS/cm and for the MOPS/BIS−TRIS propane buffer 0.83 mS/cm. After correction of the HEPES conductivity for its systematic offset and using eq 9 to calculate the R_s/R_c values and the corresponding sensitivity factors, a relative sensitivity of the MOPS-based to the HEPES-

based buffer of 1.09:1.0 is predicted. In contrast, a significant difference exists between these two spectra and the phosphate-buffered spectrum. Using the most high-field shifted resonances, the peak intensity (at equal noise level) is approximately 1.5 times higher in the spectrum with the HEPES and MOPS buffers than in the phosphate buffer spectrum. On the basis of the conductivity of the 50 mM phosphate buffer of 5.71 mS/cm, even higher gains in sensitivity of 1.6 with HEPES and 1.7 with MOPS are predicted. These values are not fully achieved due to the presence of counterions from the protein that lead to a slightly reduced sensitivity gain. This 50% increase in sensitivity relative to the most often used NMR buffer demonstrates that careful buffer selection can lead to significant sensitivity gains even under conditions typical for NMR experiments with biological samples.

Many NMR experiments with protein samples are carried out at slightly acidic pH to reduce the chemical exchange rate of the amide protons with water. A potential useful buffer in the pH range of 5.5−6.5 is MES which has a pK_a of 6.1. To investigate its effect on the sensitivity of NMR experiments, we have titrated a 200 mM sample of MES with sodium hydroxide and with BIS−TRIS to a pH of 6.0. The conductivity of the MES sample titrated with NaOH was 4.65 mS/cm, and that of the sample titrated with BIS−TRIS was 2.85 mS/cm. These values predict a sensitivity of the MES/BIS−TRIS buffer close to that of the MOPS/BIS−TRIS propane buffer. To test this, we have prepared a 1 mM sample of lysozyme in 50 mM MES/BIS−TRIS, pH 6.0, and compared the spectrum to the lysozyme spectra measured in the other buffers. As can be seen in Figure 3, the sensitivity of the MES/BIS−TRIS sample is indeed very similar to the sensitivity obtained with the MOPS/BIS−TRIS propane sample and considerably higher than the sensitivity achieved with the phosphate sample, demonstrating that MES-based buffers are excellent buffers in the slightly acidic pH range.

Although we have tested conductivity, sample resistance, and sensitivity only in a small pH range, we fully expect that similar high-sensitivity buffers can be identified and used at pH values over a range of at least 3−11. Over this range the hydrogen and hydroxyl ion concentrations are equal to or less than 1 mM and, hence, despite their relatively high mobilities, would not substantially increase the conductivity of the sample. As an aid in identifying candidate high-sensitivity buffers, one should note that ion mobility λ and diffusion coefficient D are related by the Einstein relation:

$$\frac{\lambda}{D} = \frac{q}{kT} \tag{10}$$

Here q is the magnitude of the charge of the ion, k is Boltzmann's constant, and T is the absolute temperature. To find or to create new high-sensitivity buffers, researchers should therefore focus on singly charged ions with low diffusion coefficients.

Finally, we point out that the buffers described here are only a very small selection of all possible buffers. While these buffers are optimized for high sensitivity, they may not be optimized for protein solubility. We have used HEPES buffers in the structure determination of two proteins and a protein complex in our laboratory without encountering any solubility problems. In other cases, different buffers or mixtures of buffers and neutral salts might have to be tested, using a systematic method such as the button test.[29]

Conclusions

The results described in this article demonstrate that the exact type of buffer used for NMR experiments, especially in cryogenic probes, can have a dramatic effect on the achieved sensitivity. Specifically, careful choice of buffers can result in sensitivities of well over 50% more than that obtained with the most commonly used buffers. In addition, we have described a simple method that is based on conductivity measurements that allows researchers to identify new high-sensitivity buffers. Although the sensitivity obtained with proteins dissolved in reverse micelles in organic fluids is still superior to that obtained with the best buffers, the simplicity of the buffer method and its robustness make it very attractive in particular for applications that do not allow for lengthy sample preparation procedures, such as high-throughput screening applications.

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