Sample preparation

Adapted from (1)

1. Introduction

Over the last few years, there have been rapid and sustained advances in the hardware and software associated with the NMR analysis of biological macromolecules. Larger magnets, faster computers, and new and improved pulse sequences have been combined with isotopic labelling to make NMR the unrivalled method for answering biological questions at the atomic level in the solution state. These dramatic advances have perhaps tended to obscure the importance of correct preparation of the sample, which tends to be a rather poorly documented subject. The purpose of this chapter is to aid you in the correct choice of conditions for preparation of your vital and hard-won sample. This will then allow you to obtain the maximum benefit from the sophisticated spectroscopic methods described in other chapters in this book. The importance of correct handling of the sample can not be over-emphasized, as no amount of expensive spectrometer and computer equipment can compensate for an intrinsically poor sample. A Formula One racing car will not break any lap records running on two-stroke fuel!

2. Requirements for an NMR sample

2.1 Sample quantity

The fundamental problem in the biological applications of NMR is its intrinsically poor sensitivity. The intensity of (area under) the NMR signal is proportional to the amount of material within the 'sensitive volume' of the spectrometer. For high resolution NMR, the homogeneity of the magnetic field must be extremely good. In practice, this means that the sample is confined to a small volume within the central bore of the magnet, commonly in a tube measuring 5 mm o.d. The different probe designs used by the various instrument manufacturers mean that the optimum height of the liquid sample within the tube, and hence the optimum sample volume, varies somewhat. A filling length of 40 mm often recommended for modern high-sensitivity probes requires the sample volume of about 550 μl for 5 mm tubes with walls 0.4 mm thick. For significantly lower sample volumes, Shigemi or 3 mm tubes can be used. In order to improve the signal-to-noise ratio, the signal from the sample is commonly averaged many times in the course of an experiment. The signal-to-noise ratio increases as the square root of the number of times the signal is averaged. Thus for a sample concentration of 0.5 mM, it will take four times as long to obtain a spectrum with a given signal-to-noise ratio as for a 1 mM concentration. Very weak samples may require an impractical length of time in the spectrometer to obtain a satisfactory spectrum. For structural studies, a solution concentration of the macromolecule of interest of at least 100 μM is required for simple sample checks, and 0.5-1.0 mM for the complete set of multi-dimensional experiments, assuming the use of a high-field spectrometer equipped with a cryoprobe.

Taken together, these concentration and volume requirements mean that amounts of the order of ten milligrams of a small protein or nucleic acid will be required. Whilst the technique is by its very nature non-destructive, the sample must spend tens of hours in the spectrometer, often at elevated temperatures, and this can lead to some breakdown of even the most perfectly prepared sample. Some material will also be lost during preliminary experiments designed to optimize the sample conditions. Thus, the amount of material available may well be the limiting

factor in the application of NMR to a specific system. It is not within the scope of this chapter to discuss ways in which these material limitations may be overcome, but the application of recombinant DNA technology has enormously increased the ease with which large amounts of protein may be isolated, while oligonucleotides may be produced synthetically in large quantities.

2.2 Solubility

If ample quantities of material are available, solubility may become the limiting factor. This is mainly the issue with proteins; nucleic acids are typically well soluble in water. Techniques are described later in this chapter which can be used to identify the conditions for maximum solubility of the biological macromolecule of interest.

2.3 Stability

The macromolecule must be stable during the course of the NMR experiment. Air oxidation, microbial contamination, and hydrolytic breakdown must all be eliminated to maximize the lifetime of the sample in the probe. Even after all necessary precautions have been taken, there remain molecules which are intrinsically unstable, for which it may be impossible to obtain high resolution spectra. To determine the stability of a particular macromolecular solution, the 'nativeness' of the sample should be tested before and after a long term data acquisition-for example by enzyme activity assay. In practice, the best way of determining if your sample is in the same state at the end of the experiment as at the beginning is to run a quick 1D ^{1}H NMR spectrum or, for ^{15}N-labelled proteins, a ^{1}H-^{15}N correlation spectrum. An identical spectrum means an identical sample.

2.4 Molecular weight

Increasing the size of a molecule has two main effects on its NMR spectrum:

(a) The complexity of the spectrum increases. A larger molecule has more chemically and magnetically distinct nuclei than a smaller one. Each unique nucleus will contribute at least one line to the NMR spectrum, so the total number of lines will be proportional to the molecular weight (see *Table* 1). As the number of lines in a given frequency interval increases, the greater the overlap between them, and the harder it is to distinguish the resonances of individual residues.

(b) The linewidths of the individual resonances increase. Linewidth is proportional to the correlation time of the molecule. Larger molecules have longer correlation times and therefore broader lines. Broad lines are both intrinsically less easily observed than narrow ones and more subject to overlap.

The rotational correlation time, τ_c, is the time taken for the particle to rotate through an angle of one radian (approximately 57.3°). For a spherical particle, an upper limit to this value can be approximated by the Stokes-Einstein equation,

$$\tau_c = \frac{4\pi\eta a^3}{3kT}$$

where *a* is the radius of the particle, η is the solvent viscosity, and *k* is Boltzmann's constant

The complexity of the spectrum can be reduced by isotope labelling and employing editing techniques. The problem of broad lines and spectral overlap can be alleviated by multi-dimensional NMR techniques. As of October 2009, the largest monomeric proteins for which extensive sets of assigned ^{1}H, ^{13}C, and ^{15}N resonances have been deposited in BRMB databank are Maltose binding protein complexed with β-cyclodextrin with 370 residues, M_r=42000 (2) and Malate synthetase G with 723 residues, M_r=80000 (3), concentration 0.8 mM, for fully protonated and deuterated samples, respectively.

Table 1. Average number of unique resonances expected for biopolymers of various molecular weights

				Number of magnetically distinct nuclei				
	No. of resides	**M (x10^{-3})**	**Amount required[a]**	**$^{1}H^{b}$ $^{2}H_2O$**	**$^{1}H^{c}$ H_2O**	**^{13}C**	**^{15}N**	**^{31}P**
Protein	50	5.5	2.8	210	270	240	52	0
	100	11.0	5.5	420	540	470	105	0
	200	22.0	11.0	840	1080	940	210	0
DNA[d]	10	3.4	1.8	90	100	100	40	10
	20	6.7	3.4	175	200	195	75	20
	50	16.8	8.5	440	500	490	190	50
RNA[d]	10	3.5	1.8	80	90	95	40	10
	20	7.0	3.5	155	180	190	75	20
	50	17.5	8.8	390	450	475	190	50
Carbohydrate	5	1.0	0.5	30	35	35	2	0
	10	2.0	1.0	60	70	70	4	0

Note. All the data in this table is dependent on the exact sequence of the biopolymer involved. For proteins, the statistical distribution of the amino acids in known proteins was used. For RNA and DNA, an equivalent number of each of the four bases was assumed. The data for carbohydrates is extremely dependent on sequence and the numbers quoted should be used as guidelines only.

[a] Amount required (mg) for 500 μl of a 1 mM solution.

[b] Assuming that for proteins, all β-protons are non-equivalent, as are γ-protons for Glu and Gln residues; γ, δ, etc., protons for Lys, Arg, and Pro are equivalent, and that the aromatic rings of Tyr and Phe residues are flipping freely.

[c] Only exchangeable protons bound to nitrogen are additionally included.

[d] Calculated for single stranded oligonucleotides.

3. The NMR tube

3.1 Choice of NMR tube

Get a good one! High resolution NMR requires high resolution tubes. Although these may

appear to be quite expensive, they can be reused many times if properly maintained. The cost of the tube reflects the dimensional tolerances to which the side walls have been ground and polished. Cheap tubes have low tolerances and do not possess perfect cylindrical symmetry. This is most obviously manifested as unacceptably large side bands in the spectrum when the tube is spun in the spectrometer; it is common practice to obtain two-dimensional spectra of macromolecules without sample spinning, but the low tolerance tubes should still be avoided as they limit the attainable field homogeneity.
The traditional tube has a round bottom, but this can be wasteful of sample, as the part of the tube where the side walls are not parallel should not be within the receiver coil. The hemispherical dead volume can be filled with a PTFE spacer, but flat-bottomed tubes are much more practical.

For tubes greater than 5 mm diameter, the bottom can be made flat and perpendicular to the side walls, resulting in the absolute minimum dead volume. For 5 mm tubes, this process is technically more difficult, and there tends to be slight variation in the actual shape of the bottom of each tube, even within the same batch. This necessitates adjustments in the exact placing of each tube within the probe of the spectrometer to achieve optimal resolution. The standard length of an NMR tube is seven inches. Other sizes are available, but may not then fit specialized pieces of maintenance equipment.

3.2 Cleaning the NMR tube

NMR tubes should not be used straight from the pack without cleaning, as they may contain chemicals associated with their manufacture. Most tubes (especially those >5 mm in diameter) can be cleaned by simple soaking in cleaning solutions and rinsing. Particular care should be taken, however, not to use cleaners containing metal ions, which, if not rigorously removed will give rise to paramagnetic line-broadening. This is especially true of chromic acid, which should be avoided at all costs, in cleaning glassware used in sample preparation as well as with the NMR tube itself. If soaking in a strong acid is really necessary, metal-free sulfuric acid preparations are commercially available. Strong acid should not, however, be used as the first resort, as it can lead to formation of stubborn precipitates of biological macromolecules. These may be removed by long pipe cleaners (though special care should be taken to avoid scratching the glass, which will degrade spectral resolution), or by soaking in a proprietary detergent solution after extensive washing with water. A small amount of a chelator, such as EDTA, can be added if contamination by metal ions is thought to be a problem. For tubes of 5 mm diameter, these operations can be difficult to perform, due to the high surface tension of aqueous solutions. However, devices are available which overcome this problem by using an applied vacuum to direct a spray of cleaning or rinsing solution against the bottom of the NMR tube.

Organic solvents are not normally necessary for cleaning NMR tubes used for biological samples. Laboratory grade acetone or other solvents contain impurities which will be deposited on the inside surface of the tube. If these solvents must be used for particular cleaning applications, it should not be as the final step, and particular care should be exercised in rinsing out all final traces. The NMR tube washer in is particularly convenient for this.

NMR tubes should be stored, capped, in a dust-free environment where they will not become scratched. Scratches on the outside of the tube are just as detrimental to resolution as those on the inside.

3.3 Drying the NMR tube

It is impossible to get an NMR tube completely dry; the glass will always retain a layer of adsorbed water on its surface. The use of very high temperatures to remove this last trace of moisture will cause the glass to creep, losing its cylindrical perfection and degrading its high specifications. Adequate drying can be accomplished by blowing a stream of filtered dry nitrogen gas against the bottom of the tube, by keeping the tube, inverted, in a dust-free oven at 50℃ (no higher), or by applying a vacuum to the tube, either by storing it in a vacuum desiccator or lyophilizer chamber, or directly via a specially designed valve. A combination of these three techniques should suffice for most purposes. Where it is necessary to remove the residual bound water, say due to very low sample concentration and/or overlap of signals of interest with the water proton resonance, the tube can be pre-treated with deuterium oxide. Exhaustive pre-rinsing with ^2H_2O will substitute most of the bound water, or alternatively in-tube lyophilization is possible. With careful handling, it is possible to freeze a column of ^2H_2O inside a 5 mm tube and then lyophilize it in a large vacuum container or using a special fitting made to hold NMR tubes for degassing. It is possible that the advantage gained in removal of the last trace of water will outweigh the finite risk of breaking the tube during the freezing process.

3.4 Sample volume

As discussed in Section 2.1, the volume of the sample in the NMR tube is critical for good sensitivity, resolution, and lineshape. Since the amount of the sample is often limiting in biological NMR, we want to use the minimum volume of sample that we can, without compromising sensitivity. This volume is the amount required just to cover the receiver coils in the probe (see *Figure* 1), without giving rise to any 'end effects'. The change in magnetic susceptibility at the solvent/glass (bottom of the tube) and solvent/air (meniscus) interfaces will cause loss of resolution. If the sample volume is limiting, various devices can be employed to fill the remaining space within the coils. The most popular approach in biomolecular NMR is using Shigemi tubes (Figure 2), which are made of glass doped to mach the susceptibility of the solvent. The filled end of the tube and the insert substitute for the outer parts of the solution allowing to achieve good magnetic field homogeneity with sample volumes as low as 250 μl. The active part of the sample has to be carefully centred within the position of the probe coil.

4. Sample handling

This section deals with the techniques required to actually prepare your sample for the NMR tube. Given the appropriate amount of the macromolecule in a particular buffer, we generally require to reduce the sample volume to the minimum necessary for the NMR experiment, change the sample conditions (pH, ionic strength, buffer components) to those desired, and filter the final solution. Methods for performing each of these steps are described below.

4.1 Methods for concentration

One of the simplest methods for reducing the volume of the sample, is to dry it down and then to reconstitute it in the appropriate volume of solvent. This can be the last step in NMR sample preparation, if the sample components have been previously adjusted to take account of subsequent concentration changes.

Lyophilization can be used if the material can withstand the rigours associated with this technique, i.e. the increasing concentration of the macrosolute and buffer ions, and the extremes

of pH occurring during the removal of the last traces of water. Not all proteins can be successfully lyophilized, especially those with surface thiol groups. Nucleic acids, on the other hand, usually tolerate the conditions during lyophilization well. Lyophilization of small volumes of biological samples is most conveniently performed in microcentrifuge vials, preferably those with a screw cap and rubber O-ring often used for cryo-storage. The efficiency of lyophilization is proportional to the surface area of the frozen sample, so freeze-drying in such vials can be slow. This is a problem where the sample has a high ionic strength and the rate of evaporation is not high enough to keep the sample frozen. Frothing can occur in these cases, which will lead to loss of some of the sample. Such samples are better handled in a vacuum centrifuge (see below). Samples with lower ionic strength should produce the final pellet in a small, narrow container, from which it can be efficiently removed, after dissolution in just the amount of solvent necessary for the NMR tube and no more. Sample loss is thus minimized in these cases. An additional advantage of using small tubes that can be centrifuged is that, after addition of solvent and mixing/vortexing, the solution can be spun to the bottom of the tube for removal by pipette or syringe.

One problem with lyophilization concerns the freezing of the sample, which-apart from causing denaturation in some cases-can lead to the adsorption of atmospheric water vapour on to the frozen surface of the sample, unless strict precautions are taken to prevent this occurring. This becomes a problem when a $^{2}H_2O$ sample is being prepared and strict exclusion of water is necessary. Handling of the sample within a dry atmosphere can overcome this to some extent and the frozen sample vial can be contained within a manifold whilst on the lyophilizer. When the sample is dry, the manifold is isolated and removed to a dry environment for opening and subsequent sample preparation.

The danger of the sample 'bumping' whilst on the lyophilizer can be reduced by ensuring that other users do not put large volume flasks on at the same time. A lyophilizer dedicated to NMR samples is a good idea. Vacuum centrifuges (such as the Savant Speed-Vac) eliminate the possibility of 'bumping' completely and produce a final product that is dense and convenient to handle, without the difficulties of static charge associated with the fine powdery samples often produced by standard lyophilization. A slight problem of the vacuum centrifuge is that the lyophilized sample may become 'glassy' and difficult to redissolve. It is important to realize that lyophilization of a sample containing certain buffers may lead to large pH changes, even after reconstitution. This occurs because the neutral form of buffer ions such as ammonium, acetate, bicarbonate, and formate are volatile. Evaporation of the solvent at or above room temperature avoids some of the problems of freezedrying, although this will only be successful for the more robust samples. It is especially useful for oligosaccharide samples and may also be used for oligodeoxynucleotides and a limited number of protein samples. A heated centrifuge is the ideal piece of equipment for accomplishing this task, for the same reasons as stated above.

Simple concentration of the sample may not be the most appropriate method for reducing its volume to that required, if the initial sample conditions are far removed from those necessary for the NMR experiment, or if lyophilization or evaporation would damage the biomolecule. Under these conditions, ultrafiltration should be used instead. The stirred cell ultrafiltration has been used most widely in the past and is still the most convenient way of handling large initial volumes. The large surface area of the membrane and continual stirring ensure a smooth, continual, relatively fast filtration. A major drawback is the difficulty of handling smaller volumes in a large cell. It may be necessary to redilute the concentrated sample to ensure maximum recovery from the cell, followed by transfer to a smaller cell and refiltration; this is time-

consuming and increases the amount of handling. The most serious drawback of the stirred cell is the possibility of filtering all of the solvent off and leaving an intractable and potentially denatured sample smeared over the surface of the membrane. The spun concentrator is capable of handling small volumes efficiently and has a dead stop to prevent filtration to dryness. Centrifugal recovery of the concentrate minimizes losses at the membrane and on the reservoir wall.

Both types of concentrator are available with different membrane types and molecular weight cut-offs. Adsorptive losses can occur with these membranes and are dependent on the concentration of the solute, its hydrophobicity, the temperature, and the time of contact with the solution. Low starting concentrations lead, in general, to a lower recovery. Hydrophobic membranes tend to sequester a certain amount of material irreversibly, but this can be minimized by prior treatment with a solution of bovine serum albumin, followed by thorough washing. All ultrafiltration membranes retain macromolecules according to their molecular size and shape, so the nominal molecular weight cut-off may not hold true for all macrosolutes. A cut-off should be chosen to be considerably less than the weight of the macromolecule involved, despite the increase in concentration time that this implies. All the components of the concentrator should be pre-rinsed with buffer prior to use. It is especially important to wash a small amount of buffer through the membrane to remove any trace of preservative (e.g. glycerine, azide).

4.2 Desalting and buffer exchange

4.2.1 General techniques

Repeated passage of a solution of a biological macromolecule through an ultrafiltration cell or spun concentrator with intermediate redissolution in water will effectively desalt the sample. Two successive 10:1 concentration steps will remove 99% of all the low molecular weight species. The sample can be transferred into a solution of specific buffer, ionic strength, or pH by repeated concentration/dilution with the ultimate solution. This is the best way of preparing the NMR sample in the first instance.

Changing the buffer conditions of an existing NMR sample is more difficult if dilution is to be avoided. The sample may be passed through a short column packed with a size exclusion gel which has been previously equilibrated with the new buffer. Small pre-packed disposable columns are commercially available, (e.g. Pharmacia's NAP columns), or these can be simply constructed from a disposable plastic syringe, filter, syringe needle, and the appropriate adsorbent. A bed volume of 1-2 ml is required for a 500 μl sample. The protocol is the same with commercial or home-made columns.

Spun columns are also available for buffer exchange (e.g. Isolab Inc.'s Quik-Sep columns). These have the advantage that more of the macromolecule can be recovered, but less operator control is possible whilst dealing with the small volumes involved. All methods involving the use of size exclusion gels will dilute the sample to some extent.

4.2.2 H_2O to ^2H_2O exchange

^1H NMR spectra of biological macromolecules are frequently run in ^2H_2O solution. This is partly historical, in that methods for suppression of the 110 M proton resonance in water have not been as efficient in the past as they are currently. However, spectra run in ^2H_2O continue to have an

important place in NMR studies of proteins. The resonance lines for all the exposed, labile (N-, O-, and S-bound) protons disappear as they are exchanged for deuterons. The spectrum of the remaining non-labile protons is simplified by the loss of spin-spin coupling to the labile protons. In nucleic acids, ^2H_2O solution greatly simplifies the detection of sugar protons, especially H2' and H3', whose signals would be otherwise suppressed together with the water resonance.

Importantly, the dynamic range problem is effectively decreased by the reduction of the intensity of the largest signal, allowing observation of small signals above the noise level.

The intensity of the residual water line is, of course, dependent on the efficiency with which the labile protons are exchanged, and on the isotopic enrichment of the ^2H_2O used. Suppliers of ^2H_2O label their products by the percentage deuterium content, and it is important to appreciate exactly what these figures imply for the NMR experiment. 99.96% ^2H_2O has a water content equivalent to 44 mM protons, which means that for a normal biological sample made up in this solvent, the water resonance will still be easily the most intense line in the spectrum. Even in 99.996% ^2H_2O (4 mM protons), the water line will be at least of equivalent size to those of the macromolecule.

It is worth noting that the viscosity of ^2H_2O is around 1.2 times that of H_2O at room temperature; this will affect the correlation time of the macromolecule and lead to a proportionate broadening of the lines in the NMR spectrum. It is also important to remember that the freezing point of pure ^2H_2O is 3.8℃. Samples which have been run close to th eir freezing point in H2O solution may actually freeze whilst in ^2H_2O solution; simple observation of the deuterium lock level will determine whether your sample is still fluid or not.

The methods for deuterium exchange are basically those described in Section 4.2.1, but with additional safeguards where maximum exchange is required. Lyophilization or evaporation and redissolution in ^2H_2O is effective for oligonucleotides and oligosaccharides, since all of their labile protons will exchange extremely rapidly. This is especially true for oligonucleotides at acid pH, where the intrinsic exchange rate is greater than at basic pH. Repeating the process twice should suffice to make the concentration of labile protons in the macromolecule the same as that in the solvent. For proteins, with their additional tertiary structure, not all of the labile protons are exposed to the solvent and some may have very slow exchange rates, due to their involvement in hydrogen bonds. In addition, the shell of water surrounding a protein molecule can be considered to be an integral part of the protein structure and is very difficult to remove without damaging the protein itself. For some proteins it has been necessary to warm the ^2H_2O solution to 40℃ or even 70℃ for periods from a few minutes to several hours to achieve complete exchange. For maximum possible exchange with ^2H_2O, either extremely acidic (pH <2.5) or, more realistically, basic pH (>7.0) should be used. The stability of the protein of interest at different temperatures and pH values will need to be determined before the correct choice of conditions for deuterium exchange can be made.

Where lyophilization or evaporation is not possible, repeated concentration and dilution with ^2H_2O buffers or the use of size exclusion gels pre-equilibrated with ^2H_2O is recommended.

The removal of the maximum amount of water is especially important for oligosaccharides. Not only they are often only available in small quantities, but a large number of important resonances in their 1H NMR spectra are close to that of water. Because of this, it may be necessary to work in a glove box, reducing 'proton contamination' from atmospheric water, and to repeatedly flash evaporate the oligosaccharide with ^2H_2O of the highest isotopic enrichment available. In extreme

cases, it may be necessary to dry the sample cryogenically, e.g. by suspension in an evacuated sealed chamber immersed in liquid nitrogen.

4.3 Methods of filtration

After preparation of the NMR sample in a small vial, the next step is to transfer it to the NMR tube. Since high resolution NMR can be compromised by the presence of suspended dust or fibres, these contaminants have to be removed prior to recording the spectrum. Where the exclusion of water vapour or oxygen is vital, the following procedures can be performed in a dry box.

The vial containing the sample may be spun in a bench centrifuge to pellet the contaminants, which also suffices to remove any denatured material which may have been produced during the final steps of sample preparation. The clear supernatant may then be removed with a pipette or syringe and transferred to the NMR tube. Using a vial with a conical bottom minimizes the amount of solution that may be lost, which should not be more than 10-20 μl. Where the contaminants remain in suspension or the pellet is easily disturbed, the solution must be filtered. All filtering apparatus should be washed through with the NMR buffer and blown dry with a stream of dry, inert gas prior to use. This also serves to remove fibrous materials from the filters. In all cases the outflow from the filter can be directed straight into the NMR tube.

A plug of medical cotton wool or glass wool in the neck of a Pasteur pipette serves as a simple filter, although it is quite difficult to get the last drops of solution out and so the retentate is relatively large. Commercial glass fibre filters are available (e.g. Whatman GF/D) which overcome this problem. The best filters are those commonly used for HPLC samples. A 0.45 μm filter fitted on the end of a syringe has the advantages of high efficiency, a compact size leading to easy handling, and a negligible retention volume.

4.4 Degassing the sample-removal of dissolved oxygen

Oxygen has a dual deleterious effect on samples of biological macromolecules for NMR analysis, in that it is both a paramagnetic species and an oxidizing agent. The former property leads to the broadening of the lines of the NMR spectrum, whilst the latter can cause chemical damage to the macromolecule. The linewidths of resonances of most macromolecules are quite large in the first place, and so the paramagnetic broadening effect of the concentrations of oxygen found in buffer solutions in equilibrium with air is proportionally small and is usually ignored. This is not the case for oligosaccharides, however, which have much sharper lines, and for high resolution spectra of these compounds, appropriate precautions should be taken. On the other hand, oxidation is a particular problem for some protein samples, where cysteine and methionine residues are especially susceptible. Free thiol groups may be protected from oxidation by the addition of a small amount of DTE or DTT.

Freeze thaw cycles are one way to degas the sample. Given the difficulty of freezing a sample within the NMR tube, it is best to perform this in a small vial, such as those used for cryo-storage. After the requisite number of cycles, the sample can be transferred to the NMR tube, taking care to minimize the possibility of reintroducing air into the solution at that time. For maximum oxygen removal, the whole process can be performed in an inert atmosphere in a dry box. If the final step in the sample preparation process is a lyophilization, then it will probably be sufficient to redissolve the biopolymer in degassed sample buffer.

Vacuum inert gas cycles will also degas the sample. The sample contained in the NMR tube can be alternately evacuated and then exposed to inert gas, such as dry argon, from which the residual oxygen has been removed by passage over a BTS catalyst. The tube should be tilted during these operations, to maximize the surface area of the sample and facilitate gas exchange.

Long NMR tubes are available from all the manufacturers for sealing. Whilst attached to the valve and under an inert atmosphere, the tube can be tipped-off with a small flame. This may only be necessary for the most sensitive samples, since a snug-fitting plastic NMR tube cap routinely provides a reasonably gas-tight fit.

5. Sample parameters

The buffer constituents are a vital component in maximizing the information that can be obtained by NMR about a particular biopolymer sample. Correct choice of pH, ionic strength, buffer type, concentration, and temperature can make a very real difference to the quality of the NMR spectrum, particularly for protein samples. The signal-to-noise ratio of the spectrum can be increased by increasing the concentration of the biological macromolecule. However, aggregation of the macromolecule must also be minimized at the same time, so that production of a clear solution is not the sole criterion that must be met. The only way to tell whether the sample is going to provide a good NMR spectrum is to run that spectrum. The effect of variation of the sample parameters on both the intensity and the resolution of the NMR spectrum then provides information as to the optimal conditions to be used. A short time spent running simple ^{1}H NMR spectra under varying sample conditions will enable the best quality multi-dimensional spectra to be obtained in the most efficient manner. It is important to realize that the best conditions worked out for, say, an enzyme assay, are not necessarily the best conditions under which to run the NMR spectrum. Given the significant effect which the sample conditions can have on resonance position, variation of these conditions (particularly pH and temperature) can also help to overcome overlap problems within the spectrum. The various components of the buffer are treated separately below, but it should be emphasized that changing one parameter may allow the spectrum to be further improved by the subsequent variation of one or more of the others.

5.1 Definition of pH in $^{2}H_2O$ - the isotope effect

For a sample in 100% $^{2}H_2O$, the pH reading on the pH meter is 0.4 units lower than the true p^{2}H, due to an isotope effect on the glass electrode. The meter reading is normally reported uncorrected and designated pH*. If a sample made up in 90% H2O/10% $^{2}H_2O$ has a measured pH of 7.0, then the same sample in 100% $^{2}H_2O$ will have a measured pH* of 6.6. This is an important point to remember if direct comparisons are to be made between the NMR spectra of H_2O and $^{2}H_2O$ solutions of the same biopolymer under otherwise identical sample conditions. A better estimate of the true p^{2}H is obtained by adding 0.4 units to the meter reading or by using the standard buffers calibrated for $^{2}H_2O$ for which recipes are available. The reason this is not commonly done, and the pH* value is used, is that there is also a deuterium isotope effect on the ionization equilibrium of the ionizable groups in the sample. For instance, most acids are between three to five-fold weaker in $^{2}H_2O$ than they are in H_2O, corresponding to a rising of their *pKs* by 0.5-0.7 units. This then tends to offset the error obtained from not correcting the pH

meter reading to p^{2}H. Since, when comparing data in H_2O and ^2H_2O solution, one is usually more concerned that the ionizable groups of the molecule of interest should have the same charge state than that the free proton/deuteron concentrations should be the same, the use of the simple meter reading may be more useful. Care should however be taken to report this as pH*, not pH.

5.2 pH adjustment

For NMR tubes larger than 5 mm diameter, the adjustment of the pH of the sample is as simple as for any other solution in the laboratory. A long, narrow bore, combination pH electrode (such as that available from Aldrich) is inserted into the NMR tube. Small amounts of acid or base, as appropriate, are then added by micropipette or syringe. The solution is stirred throughout by a small magnetic stirrer bar (always remembering to remove this before returning the tube to the spectrometer magnet!). However, it is extremely difficult to adjust the pH of a sample conveniently whilst it is in a 5 mm NMR tube. Efficient mixing is a serious problem, and this can lead to local extremes of pH, which may be highly deleterious to the sample, causing precipitation in some cases. Although very thin pH electrodes are available, they are expensive and delicate and probably not suitable for routine use in most laboratories. pH electrodes which just fit inside the NMR tube (around 3.5 mm diameter), and are therefore more robust, tend to smear the sample all over the inside of the NMR tube during use. The simplest answer to these problems is to remove the sample from the NMR tube for pH adjustment, as detailed in *Protocol* 2.

Protocol 2. Adjustment of pH in a 500 µl NMR sample

Materials

- a long glass Pasteur pipette or syringe fitted with a long needle (preferably platinum)
- an Eppendorf tube or similar, with a screw cap and tapered bottom
- a vortex mixer
- a bench ultracentrifuge (microfuge or equivalent)
- a pH meter fitted with a pH electrode of about 4 mm diameter
- solutions of acid and base of various concentrations (ten-fold serial dilutions of stock preferably)

1. Using the pipette or syringe transfer the sample from the NMR tube to the vial. To prevent unnecessary loss of the sample, put the tip of the pipette just under the surface and move it down smoothly as the solution is drawn up.
2. Cap the vial and spin the sample to the bottom of the vial by a few seconds centrifugation.
3. Measure the pH of the sample. On removal of the pH electrode from the sample, do not wash down the electrode; the short amount of time that the electrode is left dry should not harm it.
4. Holding the vial at a 45° angle, place a few mic rolitres of the pH adjustment solution at the top of the vial, above the surface of the sample solution. This will be held in place by surface tension. Cap the vial and vortex the main bulk of the solution through the droplet of acid or base. This will ensure swift and thorough mixing.
5. Spin the sample for a few seconds in the centrifuge.
6. Repeat steps 3-5 as necessary. At the point where the measured pH is close to that required,

use lower concentration acid or base for the adjustment. If the pH change is slow, use higher concentration. By this means, the amount of added solution will be a few microlitres each time, and the sample dilution can be kept to a minimum.

7. Spin the sample for 5-10 min to pellet any dust, fibres, or denatured material.
8. Remove the sample from the vial in an analogous manner to step 1, being careful not to disturb any sediment and transfer it back to the NMR tube. Place the tip of the pipette a few millimetres above the bottom of the NMR tube and slowly deliver the solution, moving the tip up smoothly to keep it just above the liquid surface. Try to keep the neck of the pipette away from the sides of the NMR tube. This will minimize the amount of fluid lost on to the outside of the pipette and up the walls of the NMR tube.

In situations where the sample being adjusted is in ^2H_2O, a few additional precautions need to be taken to prevent proton contamination. After referencing the pH electrode as normal (in H_2O buffers), rinse it off thoroughly with ^2H_2O and then soak it in a vial containing ^2H_2O. Before inserting the electrode in the sample, dry it off with a lint-free tissue. NaO^2H and ^2HCl solutions in ^2H_2O are commercially available or can be easily prepared by lyophilization of the protonated solutions and redissolving them in ^2H_2O, and a series of varying concentrations of these should be made by diluting the stocks with ^2H_2O. These solutions can be stored, tightly stoppered, until required. For NMR samples containing a small amount of water in the first place, following the protocol in the open laboratory leads to a small, but not unacceptable, increase in the size of the water resonance in the ^1H NMR spectrum. Where the intrinsic humidity is high or where absolute exclusion of water is necessary, the NMR tube can be transferred straight from the magnet to a dry box for subsequent handling.

5.3 Choice of buffer

The most convenient buffers for ^1H NMR are those containing no nonexchangeable protons. Since we commonly require to have the buffer concentration at 10-50 mM to maintain the pH, any protons on the buffer are likely to give rise to ^1H NMR signals many times larger than those from the macromolecule of interest. These will obscure some regions of the spectrum and may give rise to a dynamic range problem. Some simple inorganic buffers can be exchanged very readily by simple lyophilization and redissolution in ^2H_2O, whilst others (particularly acetate and Tris) can now be purchased fairly cheaply with deuterium at non-labile positions. The most commonly used NMR buffers are shown in *Table* 2. If a buffer other than these is absolutely required, then it may be available in deuterated form from one of the isotope suppliers, though at a price. For NMR of nuclei other than protons, the choice of buffer is much less restricted. ^{13}C and ^{15}N NMR would normally be performed on isotopically labelled macromolecules, so that the effective concentrations from the natural abundance heavy isotope (<1%) of the buffer would be at most approximately equal to that of the atom(s) of interest and would be unlikely to provide a dynamic range problem. There are no common fluorinated buffers that might interfere in ^{19}F NMR. ^{31}P NMR should not generally be attempted in phosphate buffer.

Inorganic buffers may appear to be the best for ^1H NMR work, but certain points should be borne in mind. Phosphate, cacodylate, borate, and bicarbonate can all interact with certain enzymes and their substrates. Borate can complex with saccharides and with the ribose moiety

of oligonucleotides. Phosphate will, at least to some extent, inhibit kinases, dehydrogenases, and other enzymes which require phosphate esters as substrates. Bicarbonate requires a closed system, as it is in equilibrium with CO_2 gas. Cacodylate has found extensive use in ^{1}H NMR studies of oligonucleotides, despite its toxicity and the presence of non-exchangeable methyl protons, since it will buffer around neutrality and does not catalyse proton exchange in the way that phosphate does, making the observation of the base imino protons possible.

Table 2. Buffers for ^{1}H NMR of biological samples.

Buffer	pK_a	Useful pH range	Notes
Acetate	4.76	3.7-5.6	a, b
Cacodylate	6.27	5.0-7.4	c
Phosphate	7.20 (2.15, 12.33)	5.8-8.0	
Tris	8.06	7.1-8.9	a, d
Borate	9.24 (12.74, 13.80)	8.0-10.2	
Glycine	2.35, 9.78	2.2-3.6, 8.6-10.6	a
(Bi)carbonate	6.35, 10.33	6.0-8.0, 9.2-10.8	e, e
Succinate	4.21, 5.64	3.5-6.0	a

a Readily available in deuterated form.

b Neutral species volatile to lyophuilization.

c Poisonous, and has methyl proton resonances at 3.8 ppm.

d The pH of Tris solutions is very temperature sensitive.

e Requires closed system.

5.4 Ionic strength effects

Variation of the concentration of salts in the solution has far reaching consequences for the solubility of a particular biological macromolecule and therefore for the intensity of the NMR signal from it. The salt concentration also has bearing on the state of aggregation and therefore on the spectral resolution.

As can be seen, the solubility of proteins in solutions of very low ionic strength is itself low, and this solubility steadily increases as the salt concentration is increased. This is due to the increase in the dielectric constant of the solution, which helps to shield the charges on individual protein molecules from each other. In this part of the solubility curve, there is little difference between one salt and another. As the salt concentration increases it starts to compete with the protein for the solvent molecules. Water is excluded from the immediate vicinity of the protein and it 'salts-out', as will be familiar to anyone who has ever done an ammonium sulfate precipitation. In this part of the solubility curve, the nature of the salt is very important. The effect is more dependent on the anion than the cation, and generally, multi-charged anions are least efficient at solubilizing proteins. But the effect is dependent on the size and charge distribution on the ion and on the protein, so the best salt for maximizing the solubility of each particular protein must be determined afresh. The Hofmeister series orders ions by their ability to 'salt-out' proteins, and may be used in this case as an approximate indication of their solubilizing power. However, care should be taken in using this, as ions at the left-hand side of each series may solubilize proteins at the expense of their native structure, (e.g. thiocyanate anion). Sodium chloride represents a good starting point for a systematic search for the correct salt to use in the

NMR sample buffer.

The Hofmeister series
Anions: SCN^- > $N03^-$ > Cl^- > citrate > acetate> phosphate> SO_4^{2-}
Cations: Ca^{2+} > Mg^{2+} > Na^+ = K^+ > NH_4^+ > $(CH_3)_4N^+$

Within the NMR experiment, variation in both ionic strength and ionic type can be used to maximize the solubility of the protein, in order to maximize the NMR signal. This is important, as the concentrations of protein necessary for NMR analysis (~1 mM) are close to the maximum solubilities that can be achieved for a large number of proteins. On the other hand, high salt concentrations above 100 mM reduce the efficiency of the NMR probes leading to longer pulses a decreased sensitivity. The cryogenic probes are especially sensitive to these effects. Slight variations in the sample conditions may make significant differences to the attainable solubility. In practical terms, one way of doing this is described in *Protocol* 3.

Protocol 3. Optimizing the NMR spectrum by varying the ionic strength

1. Make up the protein solution under some particular conditions of pH with an ionic strength of, say 50 mM (for some proteins, a lower ionic strength will be possible). The concentration of the protein should be the maximum possible that still produces a clear solution. This will vary from protein to protein.
2. Run a 1D ^{1}H NMR spectrum.
3. Add to the solution a small amount of a highly concentrated buffered salt solution (5 M NaCI is convenient) to raise the ionic strength by 50 mM.
4. Rerun the 1D ^{1}H NMR spectrum and compare with the one run previously. Look for the sharpening of signals within the spectrum as evidence for decreased aggregation.
5. Continue adding small aliquots of the salt solution until there is no further evidence for improvement in the spectrum or until the spectrum starts to deteriorate. This can be taken as evidence for the beginning of aggregation and salting-out.
6. Make up a fresh solution of protein at the newly established optimal ionic strength and at the maximum concentration possible to obtain a clear solution.
7. Run a 1D ^{1}H NMR spectrum and compare with the one run at the same ionic strength but at lower protein concentration. The sensitivity will be greater, and if the resolution is comparable, then this solution can be used for subsequent NMR experiments. If the resolution is poorer, then the solution should be diluted with buffer until the resolution is acceptable.
8. For maximum resolution, and where protein stocks allow, the ion type can also be varied. Try repeating the above steps with a different salt.
9. If a specific component is required for enzyme function, e.g. Ca^{2+} is often an essential ion, try adding aliquots of a concentrated solution of this, and comparing NMR spectra as before.

pH and temperature can also be varied in concert with ionic strength; their effects are described elsewhere. High ionic strengths (≥ 500 mM) do have some deleterious effects on the NMR experiment. Samples of high ionic strength require longer 90° pulses than those of lower ionic strength, and if these pulses become excessively long this may compromise a wide range

of NMR experiments. In addition, the high dielectric that is produced leads to heating when a radiofrequency pulse is applied, and the temperature gradients that are set up within the sample decrease the resolution of the spectrum; this can be a serious problem if solvent presaturation or long isotropic mixing pulses are being used.

For oligonucleotides, the effect of ionic strength is not primarily on the macromolecular solubility, but on its conformation. The small oligonucleotides studied by NMR so far have tended to be inverted repeat sequences for reasons of spectral simplicity. At low ionic strength, these sequences can form hairpins, and in order to be sure of generating duplexes in solution, ionic strengths of 100-200 mM should be used. Mg^{2+} is an almost universal component of oligonucleotide sample buffers.

NMR studies of oligosaccharides are generally carried out in water. The effect of varying the ionic strength is negligible.

5.5 Organic solvents

The addition of organic solvents to solutions of biological macromolecules can increase solubility, decrease aggregation, and improve the resolution of the NMR spectrum, particularly for protein samples. Exactly which solvent and what concentration to use can be determined in a similar manner to that described for ionic strength in *Protocol* 3, that is: systematic variation of the solvent concentration by the addition of small aliquots whilst monitoring the NMR spectrum. The problem with the addition of organic solvent, which also applies to a lesser extent to high ionic strengths, is the perennial one of whether the data obtained is relevant to more physiological conditions. Generally, concentrations of organic solvent less than 10% are used and other techniques are available to determine whether a gross change in protein structure is occurring under these conditions (e.g. enzyme assay, CD spectroscopy). Organic solvents can bind directly to the protein via hydrophobic interactions, which at high concentrations will turn the protein inside out, and they can decrease the bulk dielectric, enhancing inter- and intramolecular electrostatic interactions. Alcohols, dioxane, acetone, and DMSO can all help to stabilize a protein structure under very specific conditions. At 10℃, 0-20% ethanol will stabilize ribonuclease, whilst at 50℃ it has a concentration dependent denaturing effect. Addition of non-aqueous solvents will elevate the *pK* of carboxylates and other neutral acids, but has little or no effect on amines, imidazoles, and other cationic acids.

The addition of organic solvents also permits solution NMR at temperatures below 0°C. Methanol and DMSO have been used as co-solvents to extend the fluid range as low as -50℃, for investigation of thermally unstable molecules or where reaction intermediates can be 'frozen out'. The viscosity of the solutions at these temperatures seriously affects resolution, however. This problem essentially rules out the use of other cryo-solvents, such as ethylene glycol or glycerol, for NMR, and indeed presents difficulties in the use of glycerol, a common stabilizer, even at temperatures greater than 0°C.

Spectra of oligosaccharides are often run in neat DMSO, allowing identification of alcohol and amine proton resonances in ^{1}H NMR. These are necessary for primary sequence determination and for obtaining information on intramolecular H-bonding.

5.6 Detergents

Whilst chaotropic agents are useful for the solubilization of proteins, this is at the expense of

native structure, and there is, therefore, nothing to be gained in the addition of such compounds to cytosolic or extracellular proteins. Structural and functional studies on membrane bound proteins can however benefit from the use of detergents, since in these cases detergents can stabilize the native conformation. NMR studies of peptides and proteins incorporated into SOS micelles are thus possible. Both deuterated SDS and dodecylphosphocholine are commercially available for studies involving ^{1}H NMR.

5.7 Temperature

Whilst temperature is not strictly a sample preparation parameter, it is important to consider it in the same light as pH, ionic strength, and additives; the optimization of conditions for the NMR experiment requires variation of all the possible parameters. In general, the higher the temperature within the probe of the spectrometer, the better is the resolution of the NMR spectrum, as a consequence of the decrease in the correlation time. This assumes, of course, that the sample is still stable at the elevated temperature over the time period of the experiment. The increase in resolution achieved may not be sufficient to justify the risk of losing the entire sample, since the thermal unfolding of some proteins is effectively irreversible, due to aggregation of the unfolded protein, at the concentrations used for NMR. For protein NMR, temperatures somewhat greater than room temperature, normally in the range 25 - 45° C, have become standard for structure determination work. Individual overlaps within a spectrum can often be resolved by slight changes in temperature. The differential temperature dependence of the amide proton chemical shifts within a protein have been used to estimate the extent of hydrogen bonding at each amide, although the exchange rate is more commonly used for this purpose. A series of 10 spectra, run at slightly different temperatures, in a manner similar to that described in *Protocol* 3, will allow one to determine the optimum temperature for each particular sample.

If, as is often the case, exchange processes are taking place in the sample such as conformational equilibria, ionization equilibria, or ligand binding the appearance of the spectrum may be particularly sensitive to temperature.

5.8 Chemical shift references

Chemical shift referencing can be either external or internal, direct or indirect. External references are contained in a capillary within the NMR tube and do not contact the sample directly. The presence of an external capillary makes it much more difficult to attain good field homogeneity and resolution, and an external reference does not properly compensate for susceptibility changes in the sample solution. External referencing should be avoided if at all possible. A direct internal reference is dissolved in the sample buffer and should therefore be biochemically inert. This reference will correct properly for susceptibility effects, but its resonance may be affected by changes in the sample conditions. Indirect reference can be made by running a spectrum of the reference compound on its own, subsequent chemical shift measurements on the sample of interest then being made relative to the calibrated resonance(s) of the reference solution. In this case it is not always possible to duplicate the sample conditions for the reference solution, which may affect the exact chemical shift measured. After indirect referencing, it may be possible to use a strong, invariant line within the spectrum of the biopolymer itself for subsequent referencing-but one will often not know *a priori* which line(s) will

be invariant.

For ^{1}H NMR, a number of different references may be used *(Table* 3). The residual water peak is often used and assigned a chemical shift of 4.76 ppm at 25°C with the temperature dependence approximately 0.01 ppm/℃.

Comparison of chemical shifts for resonances assigned under different conditions of pH, ionic strength, and temperature can be extremely difficult, especially if the data has been obtained in different laboratories. This is further complicated by the fact that the chemical shift given in the literature for the reference compound is often not consistent with the sample conditions actually used, and differs from the value that would have been obtained had it been measured relative to a second reference.

To alleviate the situation, IUPAC recommends that a unified chemical shift scale for all nuclides be based on the proton resonance of TMS as the primary reference (4). The chemical shifts of other nuclei are then referenced indirectly using ratios given in Table 3. For aqueous solutions, DSS is used rather that TMS because of its water solubility.

Table 3. Relative frequencies (Ξ) of nuclei of biomolecular interest to be used for indirect referencing.[a]

Nucleus	Secondary reference sample	Ξ /MHz
^{1}H		100.000000
^{2}H	DSS (internal)	15.3506088
^{13}C	DSS (internal)	25.1449530
^{15}N	Liquid NH_3 (external)	10.1329118
^{31}P	trimethylphosphate	40.4808636

[a] These Ξ values were derived originally (as indicated in the table) from the ratio of the signal frequency of a secondary reference to that of internal DSS in D2O as the primary reference.

6. Preventing sample contamination

Standard laboratory practice should prevent most contamination by unwanted materials. Disposable gloves should be worn throughout, as fingertip contamination is otherwise very common. This is manifested by the presence of lactate, observable as a sharp doublet around 1.4 ppm and a quartet around 4 ppm in the ^{1}H NMR spectrum. There are some additional pre-cautions that should be taken, particularly concerning the elimination of paramagnetic species and the prevention of microbial growth.

6.1 Paramagnetic contamination

The effect of the presence of metal ions such as Cu(II), Mn(II), Cr(III), high spin Fe(III), and low spin Co(II) is to broaden beyond detection the resonances of many nuclei in the neighbourhood of these paramagnetic centres. This can be a particular problem for proteins that bind divalent cations such as Ca^{2+} and Mg^{2+}, where the paramagnetic ion can substitute, and for phosphorylated proteins, or proteins with phosphate containing coenzymes, which can bind cations such as Mn^{2+} at the phosphate anion. The best way of dealing with this problem is not to introduce metal ions into the solution in the first place.

- avoid contact with metal, including so-called stainless steel
- where practicable, substitute non-contaminating materials - use FPLC instead of HPLC, platinum needles, PTFE coated spatulas
- do not use chromic acid to clean apparatus
- treat all glass and plastic ware with 0.1 M HCI and 1 mM EDTA prior to use, rinsing thoroughly with deionized water
- use commercially available 'low heavy metal' grades of inorganic salts throughout
- treat all buffer solutions with a metal chelating agent such as Chelex prior to use

The sample itself can be treated, prior to transfer into the NMR tube. Chelex is best for the removal of metal ions since its non-specific absorption is low. It is highly effective at neutral pH and in the absence of Mg^{2+} and Ca^{2+}, which compete for the chelation sites. Passage through a column of Chelex will lead to dilution of the sample and some slight loss of material, but it is just as effective to add a few particles of Chelex to the sample solution, stir gently for a few minutes and then decant, filter, or centrifuge.

Low concentrations of soluble chelating agents can be added to the sample during the NMR experiment. 5-10 μM EDTA will usually be sufficient. Fully deuterated EDTA is not commercially available, but at these concentrations its proton resonances should not interfere in ^{1}H NMR. If deemed necessary, the α-CH2 protons can be exchanged for deuterium by boiling the Mg^{2+} salt of EDTA in $^{2}H_2O$.

6.2 Microbial contamination

A concentrated solution of a biological macromolecule at high temperature and around neutral pH represents an ideal growth medium for algae, bacteria, and fungi. As well as consuming the compound of interest, the excretion of proteases and nucleases from the microbes will cause long-term damage. Algal growth can be eliminated by keeping the NMR sample out of the light whilst it is not in the magnet. Extremes of pH and temperature will retard microbial growth, but these are not generally useful conditions for NMR studies. Solutions containing greater than 80% $^{2}H_2O$ act as effective antimicrobial agents and this is possibly the simplest way of preventing microbial contamination. For water solutions, azide and fluoride at less than 50 μM are effective. However, azide is volatile at pH values less than 7 and fluoride can not be used in the presence of metal ions. The antibiotic chloramphenicol is highly effective at 10-50 μM, is chemically inert, and can be used with metal ions, but it does have non-exchangeable proton resonances which may interfere in ^{1}H NMR. Metal chelators (EDTA, EGTA) and rigorous exclusion of metal ions (Chelex) will also suppress microbial growth. The best anti-microbial agent to be used will depend on the exact nature of the sample involved.

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