Field gradient techniques in NMR spectroscopy

Lewis E Kay

The University of Toronto, Toronto, Canada

In recent years NMR spectroscopy has emerged as a powerful technique for studying the structure and dynamics of biomolecules in solution. The development of shielded pulsed-field gradient coils for high-resolution NMR spectroscopy has led to significant improvements in a large number of experiments which are used to provide such information. Experiments enhanced with pulsed-field gradients have fewer artifacts, suffer far less from problems of solvent suppression and have reduced phase cycles relative to their non-gradient counterparts. The whole array of NMR experiments available for macromolecular structure determination is likely to benefit substantially from the incorporation of gradient pulses.

Current Opinion in Structural Biology 1995, 5:674–681

Introduction

The past several years have witnessed a tremendous growth in the application of NMR spectroscopy to a wide array of problems of biological interest. This growth is the result of a number of important methodological and instrumental improvements. On the methodology side, the development of multi-dimensional NMR in concert with triple-resonance techniques has significantly reduced the molecular mass restrictions previously imposed on structural studies by NMR [1]. From an instrumental perspective, increased field strength and improved radio-frequency electronics have had a significant impact. However, an equally important breakthrough has been the recent development of pulsed-field gradient technology and the availability of such instrumentation from all leading commercial NMR manufacturers.

Although pulsed-field gradients were introduced into NMR imaging more than 20 years ago [2], a number of technical problems had to be overcome before such gradients could be successfully incorporated into high-resolution NMR studies. Specifically, the major technical problem stems from the fact that the application of a pulsed-field gradient in the static field B_0 interferes with the receiver and the lock systems of the spectrometer. This interference introduces a significant dead time before data acquisition or the application of a subsequent radio-frequency pulse. The development of probes with actively shielded gradient coils has drastically reduced recovery times, permitting the use of short and intense static gradients.

Before the development of gradient technology, artifacts in NMR experiments were eliminated by phase cycling, i.e. by repeating the experiment several times with different phases for pulses and/or the receiver. This process requires excellent spectrometer stability, often for a period of several days. Even with perfect stability, data acquisition times in practice often limit the extent of phase cycling, leading to incomplete suppression of artifacts. In contrast, the use of pulsed-field gradients permits the suppression of artifacts on a per scan basis, thereby reducing the demand for long-term spectrometer stability [3,4]. This is particularly important, for example, in heteronuclear applications involving unlabeled compounds [5•].

The usefulness of pulsed-field gradients in high-resolution NMR spectroscopy can be understood by noting that the application of a gradient pulse imparts a phase shift to transverse magnetization, the amplitude of which is proportional to the position of the resonating nuclei in the magnetic field, the gyromagnetic ratio of the spins affected by the gradient, and the magnitude and duration

Abbreviations

BPTI—bovine pancreatic trypsin inhibitor; **CBCA(CO)NH**—correlation of the $^{13}C^{\beta}$,$^{13}C^{\alpha}$ chemical shifts of residue (i–1) with the ^{15}N and NH chemical shifts of residue i; **2D**—two-dimensional; **3D**—three-dimensional; **4D**—four-dimensional; **H(CA)NNH**—correlation of the $^{1}H^{\alpha}$ chemical shift of residue i with the ^{15}N and NH chemical shifts of residues i and (i+1); **HCCH-TOCSY**—proton-carbon-carbon-proton correlation using carbon total correlated spectroscopy; **HMQC**—heteronuclear multiple quantum coherence; **HNCACB**—correlation of the $^{13}C^{\beta}$,$^{13}C^{\alpha}$ chemical shifts of residues (i–1) and i with the ^{15}N and NH chemical shift of residue i; **H(N)CAHA**—correlation of the $^{13}C^{\alpha}$ and $^{1}H^{\alpha}$ chemical shifts of residues (i–1) and i with the NH chemical shift of residue i; **HOHAHA**—homonuclear Hartmann-Hahn spectroscopy; **HSQC**—heteronuclear single quantum coherence; **NOE**—nuclear Overhauser enhancement; **NOESY**—NOE spectroscopy; **ROE**—rotating frame Overhauser enhancement.

of the gradient pulse. We can express this mathematically as:

$$\Delta\phi = \gamma_i z G \tau_G$$

where $\Delta\phi$ is the phase change of transverse magnetization arising from spins located a distance z from the center of the gradient coil, γ_i is the gyromagnetic ratio of the spins, G is the strength of the gradient pulse and τ_G its duration.

Note that in this expression it is assumed that the gradient pulse creates a magnetic field that varies linearly along the Z-axis, which is the most common case. Because the signal intensity is the sum of magnetization from all regions of the sample, a sufficiently strong gradient pulse will completely attenuate the signal. A clear discussion of these results has been provided by van Zijl and co-workers [6] and recently in an excellent review by Keeler *et al.* [7•].

Pathway rejection versus pathway selection

Gradients can eliminate undesired signals in NMR spectra in at least two distinct ways. The first approach is based on the concept of pathway rejection. In an NMR experiment the signal of interest follows a distinct magnetization-transfer pathway, while would-be artifacts originate from other pathways. During the course of a pulse scheme it is likely at some point that the magnetization of interest lies along the Z-axis while the remaining signals are in the transverse plane. In its most simplistic form, the application of a pulsed-field gradient at this point preserves the signals along Z and dephases transverse magnetization. As long as this magnetization is not refocussed by subsequent gradient pulses, potential artifacts originating from such signals are eliminated.

One of the earliest uses of such an approach involved the application of a homospoil pulse during the mixing period of a nuclear Overhauser enhancement spectroscopy (NOESY) experiment. More recently, Bax and Pochapsky [8] have described in detail how gradients can be employed to reject unwanted pathways and eliminate artifacts caused by pulse imperfections.

An alternative strategy involves the use of gradients to actively select for the magnetization pathway of interest. In this case, application of the first gradient-selection pulse dephases all of the magnetization in the transverse plane, including the desired signal, and a subsequent gradient pulse refocuses only the magnetization of interest. A simple application of coherence pathway selection is provided in the early work of Bax *et al.* [9], in which gradients were used to select particular orders of multiple-quantum coherence.

More recent applications include coherence transfer pathway selection in multi-dimensional heteronuclear spectroscopy, in which the first selection gradient pulse is applied when the desired magnetization resides on the heteronucleus, and the final gradient selection occurring before detection, when proton magnetization is present. In this case, the amplitudes and/or durations of these gradient pulses must be adjusted accordingly to reflect the differences in gyromagnetic ratios of the heteronuclear and proton spins.

Initial examples of the use of gradients to select for coherence-transfer pathways provided spectra featuring cross peaks with mixed mode lineshapes; for biological applications this is particularly undesirable because resolution is critical. Subsequently, a number of groups have demonstrated the feasibility of obtaining pure absorptive spectra by either time-domain or frequency-domain manipulation of data sets obtained by alternating the relative signs of the coherence-transfer selection gradients [10–12].

A major limitation of the pathway selection approach described above is that only one of the two pathways which normally contribute to the observed signal can be selected. As a result, sensitivity is reduced by a factor of $\sqrt{2}$ at least, depending on the positioning of the selection gradients in the pulse scheme. Fortunately, the non-gradient enhanced-sensitivity heteronuclear pulse schemes developed by Rance and coworkers [13] are easily modified to incorporate gradients [14,15,16•]. In the absence of relaxation and pulse imperfections these enhanced-sensitivity pulsed-field gradient experiments are a full factor of 2 more sensitive than gradient-based experiments with coherence-transfer pathway selection that do not make use of the enhanced-sensitivity approach, and a factor of $\sqrt{2}$ more sensitive than non-gradient pure-absorption experiments.

Unfortunately, these sensitivity gains are only possible for the detection of spin systems where the heteronucleus is coupled to a single proton. Whereas experiments to assign the backbone protons of labeled proteins which are based on the detection of the NH proton fall into this category, other important classes of experiments require detection of magnetization from methylene and methyl groups. Griesinger and coworkers [17•] have recently shown that, with a small number of modifications, it is possible to use the same gradient-based selection approach to observe cross peaks from CH_2 and CH_3 groups, albeit without the sensitivity gain of $\sqrt{2}$ that accompanies cross-peak intensities from CH spin systems. The reader should note that it is not possible to maximize the sensitivities of signals originating from CH, CH_2 and CH_3 groups simultaneously using this approach; compromise delays must be chosen when signals from methyl, methylene and methine groups are to be observed.

Solvent suppression

One of the key advantages associated with the use of pulsed-field gradients is the suppression of the intense solvent signal in samples dissolved in H_2O. This is of particular importance in biological applications where the concentration of solvent protons relative to individual solute protons is in the order of 10^5. The use of gradients for coherence-transfer pathway selection suppresses the solvent signal *de facto* because the solvent protons are unable to traverse the selected pathway. In cases where coherence-transfer selection is not employed, a popular alternative strategy of solvent suppression, termed WATERGATE, and developed by Sklenar and coworkers [18], can be used. In this method, a selective ^1H refocusing pulse which excites the region of interest, but not the H_2O signal, is applied at the end of the pulse sequence. The insertion of gradients on opposite sides of the proton pulse ensures that the water signal is completely dephased while the signal of interest is unaffected. This approach is extremely robust and easy to use.

Recently, Hwang and Shaka [19] have developed a more general approach for solvent suppression. In their pulse scheme, denoted by $A=G_1\text{-}S\text{-}G_1\text{-}G_2\text{-}S\text{-}G_2$, where S is any pulse sequence, and G_1 and G_2 are gradients satisfying the condition that the application of G_2 does not refocus magnetization dephased by G_1, transverse magnetization is returned to its original position by the action of A. However, the amplitude of the signal is attenuated by a factor, P^2, where P is the probability that a spin is flipped by S ($P^2=0$ corresponds to the elimination of signal). The authors demonstrate water-suppression levels of >30 000-fold for

$$S = (soft - 180_x) - (hard - 180_{-x})$$

where the soft 180_x pulse is water-selective.

Although gradient approaches that dephase water result in excellent solvent suppression, they should be avoided if at all possible. Magnetization exchange between dephased water and labile solute protons, such as NH or OH protons in proteins, for example, decreases the intensity of the labile protons. Furthermore, saturation transfer can result in a significant decrease of the ^1H spectral envelope [20]. The deleterious effects of water saturation and dephasing have long been known and the importance of minimizing water saturation has recently been articulated in an excellent paper by Grzesiek and Bax [21]. These workers point out that once the water protons become saturated (dephased) during an experiment, they will remain in this state because the repetition rate between scans is much more rapid than the longitudinal relaxation rate of the H_2O ^1H spins. Consequently, it is important that gradient pulses that act as 'homospoils' be applied only when the water magnetization is stored along the Z-axis and that water magnetization be returned to the +Z-axis before detection. Significant improvements in the signal-to-noise ratio of spectra that include such 'water flip back methods' have been reported [21,22•,23•]. The incorporation of this methodology into enhanced-sensitivity experiments that make use of gradients for coherence transfer selection has also recently been described [22•,23•].

Utility of pulsed-field gradients in high-resolution studies of protein structure

Backbone and side-chain resonance assignment

A particularly powerful approach for the backbone assignment of proteins making use of ^{15}N,^{13}C and ^1H multi-dimensional spectroscopy has been developed over the past few years [1]. Two of the most useful experiments include the HNCACB [24] and the CBCA(CO)NH [25], which provide correlations linking $^{13}C\alpha/^{13}C\beta$, ^{15}N and NH chemical shifts. Because both of these experiments detect magnetization arising from a single backbone NH proton attached to a heteroatom, it is straightforward to incorporate the enhanced sensitivity pulsed-field gradient methodology described above [16•]. Moreover, the large number of delays in the triple-resonance experiments required for magnetization transfer between coupled nuclei allows the insertion of coherence-transfer selection gradients into these pulse schemes with only a minimal increase in the duration of the experiments. An enhanced-sensitivity pulsed-field gradient version of the HNCO experiment has been reported where the benefits of the 'water flip back approach' for a cellulose-binding-domain fragment at pH 7.0 were clearly established [23•].

Recently Mariani *et al.* [26] reported a time-shared experiment where H(N)CAHA and H(CA)NNH schemes were recorded simultaneously. In this case, gradients were used to eliminate magnetization from spurious pathways. Further, Sattler *et al.* [27] have developed a clever way of using gradients to select for both (^{15}N,NH) and (^{13}C,CH) correlations in a simultaneous ^{15}N,NH and ^{13}C,CH HSQC experiment. Excellent water suppression is demonstrated in these experiments, although the benefits of enhanced sensitivity for the carbon experiment are significantly less than for the nitrogen version because of the use of compromise delays necessary to maximize the sensitivity from CH, CH_2 and CH_3 groups, pulse imperfections arising from the large dispersion of carbon chemical shifts, and evolution attributable to carbon–carbon scalar couplings during the delays in the pulse scheme.

The use of gradients to suppress so efficiently the solvent signal has led to the development of gradient-based experiments for side-chain assignment that allow recording of spectra in H_2O. An INEPT-based version of the HCCH-TOCSY with gradients used to remove

artifacts and eliminate the water signal has been reported [28], and more recently Wang and Zuiderweg [29••] have developed a heteronuclear-cross polarization version of the experiment with gradients used for pathway rejection that provides high-quality spectra with outstanding water suppression. Sattler *et al.* [30••] have reported elegant HCCH-TOCSY experiments which make use of two enhanced-sensitivity elements and in which coherence transfer selection is achieved via pulsed-field gradients. The ability to assign completely a protein based on spectra recorded on a single H_2O sample is significant because ambiguities associated with comparing spectra obtained on different samples possibly prepared under different conditions or showing isotope shifts (H_2O versus D_2O) can be avoided.

Gradient-enhanced nuclear Overhauser enhancement (NOE) experiments

NMR-based structures are obtained by exploiting the 1/r6 distance dependence of the NOE intensity on the distance (r) between two proximal spins. Because of the central importance of these experiments in the structure determination process it is not surprising that considerable efforts have been directed towards their improvement through the design of experiments which incorporate pulsed-field gradients. Recently, enhanced-sensitivity pulsed-field gradient ^{15}N-edited NOESY experiments have been developed [31,32]. These experiments utilize radiation damping as an active element to ensure that the water magnetization is returned to the +Z axis before detection in a manner independent of the phases of the radio-frequency pulses. ^{13}C-Edited NOESY-HSQC experiments have been reported in which water suppression is achieved through the use of intense homospoil gradient pulses applied when the magnetization of interest lies along the Z axis and the water magnetization is in the transverse plane [33,34]. The recording of NOE experiments on samples dissolved in H_2O allows the simultaneous observation of NOEs directed to nitrogen-bound and carbon-bound protons. Pascal *et al.* [35•] have demonstrated a simultaneously $^{15}N,^{13}C$-edited three-dimensional (3D) NOESY-HSQC experiment which records the chemical shifts of the two heteroatoms coupled to the protons giving rise to an NOE.

The incorporation of field gradients in four-dimensional (4D) NOE experiments has had a particularly significant impact on spectral quality. Muhandiram *et al.* [36] have developed a 4D $^{15}N,^{13}C$ NOESY experiment with gradients used to select for the coherence-transfer pathway traversing nitrogen using the enhanced-sensitivity approach described previously. The use of gradients in this way has led to a reduction by a factor of 2 in the number of phase-cycling steps, allowing measuring time to be allotted to improving resolution, and to an increase in sensitivity of ~40%. Significant improvements in the original 4D ^{13}C–^{13}C NOESY experiments were made by Vuister and colleagues [37] in their development of gradient-based HMQC-NOESY-HMQC and HMQC-NOESY-HSQC pulse schemes with excellent levels of artifact suppression. Very recently, Farmer and Mueller [38] have demonstrated a 4D simultaneous $^{15}N,^{13}C$ and $^{15}N,^{15}N$ gradient-enhanced NOE experiment and have provided a very clear description of the usefulness of gradients in the elimination of artifacts.

Gradient-based experiments for the study of molecular complexes

With recent significant improvements in NMR methodology, the technique has become increasingly attractive for the study of molecular complexes. The approach most often taken is one in which one class of molecules (protein) in the complex is labeled with $^{15}N,^{13}C$ while the other molecular class (DNA/RNA, drug, peptide) is unlabeled. The $^{15}N,^{13}C$-labeled component can be readily studied using the large array of triple-resonance multi-dimensional experiments that now exists in the literature. A substantial body of experiments has also been developed for the assignment of the unlabeled ligand and for obtaining NOE constraints connecting the ligand with the labeled molecule.

Several significant improvements in these experiments have been made through the use of pulsed-field gradients. In a recent publication, Bax and co-workers [39•] have described an isotope-filtered two-dimensional (2D) HOHAHA experiment for providing correlations within spin systems of the unlabeled ligand. Signals from protons bound to ^{13}C spins are dephased during the HOHAHA period and pulsed-field gradients are employed to minimize artifacts arising from spurious magnetization-transfer pathways.

Building on earlier work by Folkers *et al.* [40], Boelens and colleagues [41] have described 2D time-shared $^{15}N,^{13}C$ double half-filtered experiments in which it is possible to record: (a) NOEs between protons within either the labeled or unlabeled component exclusively, or (b) inter-monomer NOEs between protons across the molecular interface. Gradients are employed to minimize the phase cycle and to aid in the elimination of artifacts. It is very straightforward to extend the 2D NMR experiments which provide inter-molecular NOE contacts to a third dimension in cases where spectral overlap precludes the use of 2D spectroscopy.

Lee *et al.* [42] have recently developed a 3D ^{13}C F_1-edited, F_3-filtered HMQC NOESY experiment for assigning intermolecular NOEs between isotope-labeled and -unlabeled components of a complex. The experiment makes use of pulsed-field gradients to dephase magnetization arising from protons one-bond coupled to ^{13}C spins during the purging portion of the sequence; this reduces significantly an extensive phase cycle that would otherwise be necessary.

Application of pulsed-field gradient spectroscopy in the study of protein dynamics

Developments in heteronuclear NMR methods have found important applications in areas outside protein structure determination. Heteronuclear spectroscopy combined with uniform labeling of the protein of interest with either ^{15}N or ^{13}C permits the study of the dynamics at virtually every position in the molecule. Early versions of ^{15}N relaxation experiments relied on saturation or dephasing to suppress the signal from the water peak. Saturation transfer from the water to labile spins on the protein via either the NOE or chemical exchange can lead to errors in measured relaxation rates or ^{15}N–^{1}H NOE values, and subsequently to errors in the extracted motional parameters [21,43].

The use of pulsed-field gradients in combination with water flip back methods results in significant improvements in the accuracy of measured relaxation rates. Recently, pulse schemes that incorporate coherence transfer selection with enhanced sensitivity as well as water-selective pulses to minimize water dephasing/saturation have been proposed [44•,45•] for the measurement of ^{15}N T_1, T_2 relaxation times and the ^{15}N–^{1}H heteronuclear NOE. Tolman and Prestegard [46] have described a gradient-based experiment which permits simultaneous measurement of (a) the heteronuclear (X) transverse relaxation rate and (b) cross correlation between the heteronuclear ^{1}H-X dipolar and the X spin chemical shift anisotropy interactions.

The relaxation experiments described above provide a measure of molecular motions with correlation times less than the overall molecular correlation time although the T_2 relaxation time is also sensitive to dynamics in the microsecond to millisecond range.

Slower process can be monitored via hydrogen-exchange spectroscopy, and elegant NOE and rotating frame Overhauser enhancement (ROE) experiments have been developed by Grzesiek and Bax [47] for obtaining quantitative measures of NH hydrogen-exchange rates with water so long as the exchange rates are greater than $\sim 0.2\ s^{-1}$.

Pulsed field gradients for the measurement of diffusion

An important criterion to establish at the start of any structure determination is the aggregation state of the molecule in question. Very recently, Altieri *et al.* [48••] have developed pulsed-field gradient NMR experiments that are suitable for measuring diffusion constants of macromolecules in aqueous solution. The methods have been applied to determine the aggregation states of a number of different protein systems, including a dimer of molecular mass 38 kDa. Pulsed-field gradient diffusion experiments have also been used to estimate an upper bound for the lifetime of bound water molecules in the protein bovine pancreatic trypsin inhibitor (BPTI). The time dependence of NOE cross peaks between the bound waters and protons of the protein is obtained using a modified 2D homonuclear NOESY experiment containing self-compensating pulsed-field gradients [49] applied before and after the mixing time. A fit of the intensity of the cross peaks as a function of increasing gradient strength allows the extraction of the residence lifetime of the bound waters. Using this novel approach, Dotch *et al.* [50••] obtain a maximum lifetime of ~1 ms for internal water molecules in BPTI.

Three-axis versus one-axis gradients

A recent paper by Warren and co-workers [51] has demonstrated intermolecular multiple-quantum coherences between bulk water and solute. This surprising result can be explained by disregarding the high-temperature approximation normally used in NMR and by including long-range dipolar interactions between spins. Magic-angle gradients can dramatically reduce this effect. For this reason, the use of magic-angle coherence-selection gradients in double quantum filtered COSY (DQF COSY) experiments offers far superior water-suppression performance compared with other gradient combinations [52]. Nevertheless, improved performance of a three-axis system in relation to a one-axis coil in the context of other experiments has not been clearly established so far. It is clear, however, that in experiments that employ more than a single gradient it is easier to ensure that successive gradient pulses do not refocus magnetization dephased by earlier gradients using a three-axis system.

NMR spectroscopy with B_1 gradients

The use of B_0 gradients for coherence transfer selection and artifact suppression has become well established in the past several years; it is also possible to use B_1 field gradients for the same purpose, although the technology has until now been much less popular. That the application of B_1 gradient pulses does not perturb the spectrometer and that very negligible rise and fall times are associated with the gradient pulses makes their use very attractive.

One of the most popular applications of B_1 field gradients is in heteronuclear NMR, where the inherent B_1 field inhomogeneity supplied by a standard probe is used to dephase signals arising from proton spins which are not coupled to the heteroatom of interest [53]. More sophisticated uses of the technology include employing B_1 gradients to induce coherence transfers in single-scan NOESY [54] and correlation spectroscopy

[55] experiments and in heteronuclear pulse schemes [56].

Otting [57•] has proposed a clever application of B_1 gradients for improving the resolution and sensitivity in NOE and ROE experiments. The application of spin lock pulses of equal duration on opposite sides of the t_1 evolution period serves to defocus magnetization (including water) before the evolution period and refocus it afterwards. This leads to the suppression of radiation damping during t_1 and subsequently improves the quality of NOE/ROE cross peaks connecting water and protein protons.

Only a few applications of B_1 gradients in macromolecular NMR are known at present, but this is perhaps not surprising, considering the early stages in development of this technology. This area may well expand rapidly during the next few years.

Conclusions

The development of pulsed-field gradient NMR experiments represents an important advance over the NMR methods of a few years ago. Gradients provide for a significant reduction in the artifact content of spectra, aid in the suppression of the intense solvent peak in H_2O experiments, and reduce the requirements for phase cycling. Moreover, gradient-based experiments open up the possibility of studying interactions between water and solute in a far more quantitative fashion than would otherwise be possible. It is likely that pulsed-field gradients will be used in every high-resolution NMR laboratory within the not too distant future.

Note added in proof

Recently Dingley *et al.* [58••] have developed pulsed field gradient methods for the measurement of translation diffusion constants of molecules. The methodology presented is similar to results published by Byrd and co-workers [48••].

Acknowledgements

LE Kay thanks all of his NMR colleagues in Toronto for stimulating discussions.

This paper is dedicated to Professor CM Kay on the occasion of his retirement from the Biochemistry Department, University of Alberta, Edmonton, Alberta, Canada.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Bax A, Grzesiek S: **Methodological advances in protein NMR.** *Accounts Chem Res* 1993, **26**:131–138.

2. Lauterbur PC: **Image formation by induced local interactions: examples employing nuclear magnetic resonance.** *Nature* 1973, **242**:190–191.

3. Hurd RE, John BK: **Three-dimensional gradient-enhanced relay-edited proton spectroscopy. GREP-HMQC-COSY.** *J Magn Resonance* 1991, **92**:658–668.

4. Vuister GW, Boelens R, Kaptein R, Burgering M, Van Zijl PCM: **Gradient-enhanced 3D NOESY-HMQC spectroscopy.** *J Biomol NMR* 1992, **2**:301–305.

5. • Weigelt J, Otting G: **^{1}H-Detected INEPT-INADEQUATE at natural ^{13}C abundance.** *J Magn Resonance [A]* 1995, **113**:128–130.
Coherence transfer selection gradients are employed to select for ^{13}C–^{13}C double-quantum coherence, allowing > 10 000-fold suppression of signals originating from ^{12}C-bound protons.

6. Von Kienlin M, Moonen CTW, Van der Toorn A, Van Zijl PCM: **Rapid recording of solvent-suppressed 2D COSY spectra with inherent quadrature detection using pulsed field gradients.** *J Magn Resonance* 1991, **93**:423–429.

7. • Keeler J, Clowes RT, Davis AL, Laue E: **Pulsed field gradients: theory and practice.** In *Methods in Enzymology, Nuclear Magnetic Resonance, Part C, Vol 239.* Edited by James TL and Oppenheimer NJ. New York: Academic Press; 1994:145–207.
An excellent and current review of the theory and applications of pulsed-field gradients in high-resolution NMR.

8. Bax A, Pochapsky S: **Optimized recording of heteronuclear multidimensional NMR spectra using pulsed field gradients.** *J Magn Resonance* 1992, **99**:638–643.

9. Bax A, De Jong DE, Mehlkopf AF, Smidt J: **Separation of the different orders of NMR multiple-quantum transitions by the use of pulsed field gradients.** *Chem Phys Lett* 1980, **69**:567–570.

10. Davis AL, Keeler J, Laue ED, Moskau D: **Experiments for recording pure-absorption heteronuclear correlation spectra using pulsed field gradients.** *J Magn Resonance* 1992, **98**:207–216.

11. Tolman JR, Chung J, Prestegard JH: **Pure-phase heteronuclear multiple-quantum spectroscopy using field gradient selection.** *J Magn Resonance* 1992, **98**:462–467.

12. Boyd J, Soffe N, John B, Plant D, Hurd R: **The generation of phase-sensitive 2D ^{15}N–^{1}H spectra using gradient pulses for coherence-transfer-pathway-selection.** *J Magn Resonance* 1992, **98**:660–664.

13. Palmer AG, Cavanagh J, Wright PE, Rance M: **Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectroscopy.** *J Magn Resonance* 1991, **93**:151–170.

14. Kay LE, Keifer P, Saarinen T: **Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity.** *J Am Chem Soc* 1992, **114**:10663–10665.

15. Schleucher J, Sattler M, Griesinger C: **Coherence selection by gradients without signal attenuation: application to the three-dimensional HNCO experiment.** *Angew Chem Int Ed Engl* 1993, **32**:1489–1491.

16. • Muhandiram DR, Kay LE: **Gradient-enhanced triple-resonance three-dimensional NMR experiments with improved sensitivity.** *J Magn Resonance [B]* 1994, **103**:203–216.
This paper demonstrates both experimentally and theoretically the relative sensitivities associated with a number of non-gradient and gradient-based HNCO pulse schemes. Sensitivity gains associated with the enhanced sensitivity pulsed-field gradient approach for proteins as large as 173 residues relative to unenhanced methods are established.

17. • Schleucher J, Schwendinger M, Sattler M, Schmidt P, Schedletzky O, Glaser SJ, Sorensen OW, Griesinger C: **A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients.** *J Biomol NMR* 1994, **4**:301–306.
Building on the results of previous studies [14,15], a general scheme for enhancement in gradient-selected heteronuclear experiments is presented. The $\sqrt{2}$ sensitivity gains that are possible for CH and NH groups can not be realized with NH_2, CH_2 or CH_3 groups.

18. Piotto M, Saudek V, Sklenar V: **Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions.** *J Biomol NMR* 1992, **2**:661–665.

19. Hwang TL, Shaka AJ: **Water suppression that works. Excitation sculpting using arbitrary waveforms and pulsed field gradients.** *J Magn Resonance [A]* 1995, **112**:275–279.

20. Li YC, Montelione GT: **Solvent saturation-transfer effects in pulsed-field-gradient heteronuclear single-quantum-coherence (PFG-HSQC) spectra of polypeptides and proteins.** *J Magn Resonance [B]* 1993, **101**:315–319.

21. Grzesiek S, Bax A: **The importance of not saturating H_2O in protein NMR. Application to sensitivity enhancement and NOE measurements.** *J Am Chem Soc* 1993, **115**:12593–12594.

22. • Stonehouse J, Shaw GL, Keeler J, Laue ED: **Minimizing sensitivity losses in gradient-selected ^{15}N–^{1}H HSQC spectra of proteins.** *J Magn Resonance [A]* 1994, **107**:178–184.
This paper articulates the importance of minimizing saturation of H_2O in HSQC spectra. Modifications of a number of different HSQC schemes that minimize water dephasing and saturation and result in improved sensitivity are presented.

23. • Kay LE, Xu GY, Yamazaki T: **Enhanced-sensitivity triple-resonance spectroscopy with minimal H_2O saturation.** *J Magn Resonance [A]* 1994, **109**:129–133.
An enhanced-sensitivity HNCO pulse sequence is modified to minimize dephasing of water by: (a) the insertion of a single water-selective pulse, (b) the careful choice of phases of all ^{1}H radio-frequency pulses and (c) by insuring that when ^{1}H decoupling occurs in the pulse sequence the water magnetization is locked along the decoupling field. Improvements in sensitivity are demonstrated for a protein sample at pH 7. This builds on the important results presented in [21].

24. Wittekind M, Mueller L: **HNCACB, a high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with alpha- and beta-carbon resonances in proteins.** *J Magn Resonance [B]* 1993, **101**:201–205.

25. Grzesiek S, Bax A: **Correlating backbone amide and sidechain resonances in larger proteins by multiple relayed triple resonance NMR.** *J Am Chem Soc* 1992, **114**:6291–6293.

26. Mariani M, Tessari M, Boelens R, Vis H, Kaptein R: **Assignment of the protein backbone from a single 3D, ^{15}N, ^{13}C, time-shared HXYH experiment.** *J Magn Resonance [B]* 1994, **104**:294–297.

27. Sattler M, Maurer M, Schleucher J, Griesinger C: **A simultaneous ^{15}N,^{1}H- and ^{13}C,^{1}H-HSQC with sensitivity enhancement and a heteronuclear gradient echo.** *J Biomol NMR* 1995, **5**:97–102.

28. Kay LE, Xu GY, Singer A, Muhandiram DR, Forman-Kay JD: **A gradient-enhanced HCCH-TOCSY experiment for recording sidechain ^{1}H and ^{13}C correlations in H_2O samples of proteins.** *J Magn Resonance [B]* 1993, **101**:333–337.

29. •• Wang H, Zuiderweg ERP: **HCCH-TOCSY spectroscopy of ^{13}C-labeled proteins in H_2O using heteronuclear cross-polarization and pulsed-field gradients.** *J Biomol NMR* 1995, **5**:207–211.
A heteronuclear cross-polarization experiment for protein samples dissolved in H_2O with outstanding water suppression. Pulsed field gradients aid in solvent suppression and in the elimination of artifacts.

30. •• Sattler M, Schwendinger, MG, Schleucher J, Griesinger C: **Novel strategies for sensitivity enhancement in heteronuclear multidimensional NMR experiments employing pulsed field gradients.** *J Biomol NMR* 1995, **6**:11–22.
Experiments that include two sensitivity-enhancement elements are described. In the absence of relaxation and pulse imperfections sensitivity gains of a factor of 2, relative to unenhanced sequences, can be realized.

31. Zhang O, Kay LE, Olivier JP, Forman-Kay, JD: **Backbone ^{1}H and ^{15}N resonance assignments of the N-terminal SH3 domain of drk in folded and unfolded states using enhanced-sensitivity pulsed field gradient NMR techniques.** *J Biomol NMR* 1994, **4**:845–858.

32. Jahnke W, Baur M, Gemmecker G, Kessler H: **Improved accuracy of NMR structures by a modified NOESY-HSQC experiment.** *J Magn Resonance [B]* 1995, **106**:86–88.

33. Majumdar A, Zuiderweg ERP: **Improved ^{13}C-resolved HSQC-NOESY spectra in H_2O, using pulsed field gradients.** *J Magn Resonance [B]* 1993, **102**:242–244.

34. Muhandiram DR, Farrow N, Xu GY, Smallcombe SH and Kay LE: **A gradient ^{13}C NOESY-HSQC experiment for recording NOESY spectra of ^{13}C-labeled proteins dissolved in H_2O.** *J Magn Resonance [B]* 1993, **102**:317–321.

35. • Pascal SM, Muhandiram DR, Yamazaki T, Forman-Kay JD, Kay LE: **Simultaneous acquisition of ^{15}N- and ^{13}C-edited NOE spectra of proteins dissolved in H_2O.** *J Magn Resonance [B]* 1994, **103**:197–201.
Simultaneous ^{15}N- and ^{13}C-edited NOESY spectra are recorded on water samples, allowing, in principle, the observation of symmetry-related cross peaks for each NOE peak in the spectrum.

36. Muhandiram DR, Xu GY, Kay LE: **An enhanced-sensitivity pure absorption gradient 4D ^{15}N,^{13}C-edited NOESY experiment.** *J Biomol NMR* 1993, **3**:463–470.

37. Vuister GW, Clore GM, Gronenborn AM, Powers R, Garrett DS, Tschudin R, Bax A: **Increased resolution and improved spectral quality in four-dimensional ^{13}C/^{13}C-separated HMQC-NOESY-HMQC spectra using pulsed field gradients.** *J Magn Resonance [B]* 1993, **101**:210–213.

38. Farmer BT, Mueller L: **Simultaneous acquisition of [^{13}C,^{15}N]- and [^{15}N,^{15}N]-separated 4D gradient-enhanced NOESY spectra in proteins.** *J Biomol NMR* 1994, **4**:673–687.

39. • Bax A, Grzesiek S, Gronenborn AM, Clore GM: **Isotope-filtered 2D HOHAHA spectroscopy of a peptide–protein complex using heteronuclear Hartmann-Hahn dephasing.** *J Magn Resonance [A]* 1994, **106**:269–273.
Combination of homonuclear TOCSY transfer between protons in the unlabeled ligand with heteronuclear dephasing of protons from the labeled molecule avoids an increased number of delays in the experiment.

40. Folkers PJM, Folmer RHA, Konings RNH, Hilbers CW: **Overcoming the ambiguity problem encountered in the analysis of nuclear Overhauser magnetic resonance spectra of symmetric dimer proteins.** *J Am Chem Soc* 1993, **115**:3798–3799.

41. Burgering M, Boelens R, Kaptein R: **Observation of intersubunit NOEs in a dimeric P22 Mnt repressor mutant by a time-shared [^{15}N,^{13}C] double half-filter technique.** *J Biomol NMR* 1993, **3**:709–714.

42. Lee W, Revington MJ, Arrowsmith CH, Kay LE: **A pulsed-field gradient isotope-filtered 3D ^{13}C HMQC-NOESY experiment for extracting intermolecular NOE contacts in molecular complexes.** *FEBS Lett* 1994, **350**:87–90.

43. Li YC, Montelione GT: **Overcoming solvent saturation-transfer artifacts in protein NMR at neutral pH. Application of pulsed field gradients in measurements of ^{1}H–^{15}N Overhauser effects.** *J Magn Resonance [B]* 1994, **105**:45–51.

44. • Farrow NA, Muhandiram DR, Singer AU, Pascal SM, Kay CM, Gish G, Shoelson SE, Pawson T, Forman-Kay JD, Kay LE: **Backbone dynamics of a free and a phosphopeptide-complexed Src homology 2 domain studied by ^{15}N NMR relaxation.** *Biochemistry* 1994, **33**:5984–6003.
This report presents enhanced-sensitivity pulse sequences for the measurement of ^{15}N T_1,T_2 and the steady-state ^{1}H–^{15}N NOE with gradients to select for the transfer pathway traversing nitrogen and which minimize saturation and dephasing of water (as described in [21]).

45. • Dayie KT, Wagner G: **Relaxation-rate measurements of ^{15}N–^{1}H groups with pulsed-field gradients and preservation of coherence pathways.** *J Magn Resonance [A]* 1994, **111**:121–126.
Enhanced-sensitivity pulse schemes for the measurement of ^{15}N T_1,T_2, the steady-state ^{1}H-^{15}N NOE, heteronuclear longitudinal two-spin order, antiphase heteronuclear two-spin coherence, amide-proton in-phase co-

herence and amide-proton longitudinal magnetization are reported. Water flip-back methods are also included in the pulse sequences.

46. Tolman JR, Prestegard JH: **Simultaneous collection of two transverse ^{15}N relaxation pathways in isotopically labeled proteins.** *J Magn Resonance [B]* 1995, **106**:97–100.

47. Grzesiek S, Bax A: **Measurement of amide proton exchange rates and NOEs with water in $^{13}C/^{15}N$-enriched calcineurin B.** *J Biomol NMR* 1993, **3**:627–638.

48. •• Altieri AS, Hinton DP, Byrd RA: **Association of biomolecular systems via PFG-NMR diffusion measurements.** *J Am Chem Soc* 1995, **117**:7566–7567.
A pulsed-field gradient method is described for the measurement of translational diffusion constants, from which the aggregation state of biomolecules can be measured. Unlike many other methods, this approach allows measurements to be made on the sample under conditions that are identical to those used for structural studies.

49. Wider G, Dotsch V, Wüthrich K: **Self-compensating pulsed magnetic field gradients for short recovery times.** *J Magn Resonance [A]* 1994, **108**:255–258.

50. •• Dotsch V, Wider G: **Exchange rates of internal water molecules in proteins measured using pulsed field gradients.** *J Am Chem Soc* 1995, in press.
Gradient-based NOESY is used to establish that the lifetime of bound waters in the protein BPTI is < 1 ms.

51. Warren WS, Richter W, Andreotti AH, Farmer BT Jr: **Generation of impossible cross-peaks between bulk water and biomolecules in solution NMR.** *Science* 1993, **262**:2005–2009.

52. Van Zijl PCM, Johnson MO, Mori S, Hurd RE: **Magic-angle-gradient double-quantum-filtered COSY.** *J Magn Resonance [A]* 1995, **113**:265–270.

53. Messerle BA, Wider G, Otting G, Weber C, Wüthrich K: **Solvent suppression using a spin lock in 2D and 3D NMR spectroscopy with H_2O solutions.** *J Magn Resonance* 1989, **85**:608–613.

54. Eads CD: **Single-scan, phase-sensitive NOESY using radio-frequency-field inhomogeneity for coherence selection.** *J Magn Resonance [A]* 1994, **107**:109–112.

55. Mutzenhardt P, Brondeau J, Canet D: **COSY experiments and solvent suppression with B_1 gradients.** *J Magn Resonance [A]* 1994, **108**:110–115.

56. Mass WE, Cory DG: **Heteronuclear correlation spectroscopy with RF gradients.** *J Magn Resonance [A]* 1995, **112**:229–236.

57. • Otting G: **Improved resolution and sensitivity in NOE and ROE experiments with water by the use of B_1 gradients.** *J Magn Resonance [B]* 1994, **103**:288–291.
Improvements in sensitivity and resolution of cross peaks to water in NOE and ROE experiments can be obtained with the use of B_1 gradients applied on opposite sides of the t_1 evolution period. The application of such pulses eliminates radiation damping during the t_1 period.

58. •• Dingley AJ, Mackay JP, Chapman BE, Morris MB, Kuchel PW, Hambly BD, King GF: **Measuring protein self-association using pulsed-field-gradient NMR spectroscopy: application to myosin light chain 2.** *J Biomol NMR* 1995, in press.
This paper discusses the development of pulsed field gradient methods for the measurement of translation diffusion constants of molecules.

LE Kay, Department of Medical Genetics, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8.