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Gradient-enhanced TOCSY experiments with improved sensitivity and solvent suppression

D. Bruce Fulton, Richard Hrabal and Feng Ni*

Biotechnology Research Institute and The Montreal Joint Centre for Structural Biology, National Research Council Canada, 6100 Royalmount Avenue, Montreal, PQ, Canada H4P 2R2

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Summary

Gradient-enhanced versions of the homonuclear TOCSY experiment are described, with solvent suppression and sensitivity superior to that of a conventional TOCSY experiment. The pulse sequences are constructed by appending a WATERGATE module to a z-filtered TOCSY experiment. Pulsed-field gradients and appropriately phased selective rf pulses are used to maintain precise control of the water magnetization vector. Problems associated with radiation damping and spin-locking of the water magnetization are thus alleviated. The water magnetization is returned to equilibrium prior to each acquisition, which improves water suppression and minimizes signal losses due to saturation transfer.

It is now a common practice to achieve solvent suppression in 2D homonuclear NMR experiments by appending a WATERGATE (Piotto et al., 1992) sequence at the end of the pulse sequence. For example, this approach has been used successfully for NOESY (Piotto et al., 1992) and DQF-COSY (Trimble and Bernstein, 1994) experiments. The use of WATERGATE has two principal advantages over presaturation. First, there is no loss of information at the water frequency along F1, since selective suppression of signals occurs after the mixing period. Second, there is less attenuation of protein signals due to saturation transfer and spin diffusion.

The WATERGATE procedure is very effective at dephasing transverse magnetization at the water frequency, but has no effect upon magnetization aligned with the z-axis. If water magnetization is aligned with the negative z-axis during the WATERGATE period, transverse water magnetization will build up in the acquisition period via radiation damping. This situation can arise in 2D experiments due to the t_1-dependent phase cycling necessary for quadrature detection in the indirectly detected dimensions (Bax et al., 1987; Stonehouse et al., 1994b). The water magnetization vector enters the mixing period along the positive z-axis, in the transverse plane, or along the negative z-axis, and continually cycles among these states as the t_1 value is incremented. The behavior of the water magnetization may be further complicated by radiation damping during the evolution period. In the case where a component along the negative z-axis survives after the WATERGATE sequence, the receiver may be overloaded. Even in less severe cases, an imbalance in the water suppression efficiency over the TPPI cycle causes a t_1 modulation of the baseline, giving rise to antidiagonal spectral artifacts. This type of modulation is not encountered in the NOESY-WATERGATE experiment if the mixing time is longer than about 100 ms. Radiation damping during the mixing period restores the water magnetization to equilibrium for each t_1 increment (Stonehouse et al., 1994a), and ensures that it will be transverse during the WATERGATE period. In the case of MQF-COSY, the majority of water magnetization is rejected by the multiple-quantum filter (Hurd, 1990) prior to the WATERGATE. However, in a conventional TOCSY experiment (Braunschweiler and Ernst, 1983; Davis and Bax, 1985) there is no provision to ensure that the water magnetization will be well behaved upon entering a subsequent WATERGATE module.

It is very important to consider radiation damping effects (Bloembergen and Pound, 1954; Abragam, 1961) in the design and implementation of NMR experiments

*To whom correspondence should be addressed.

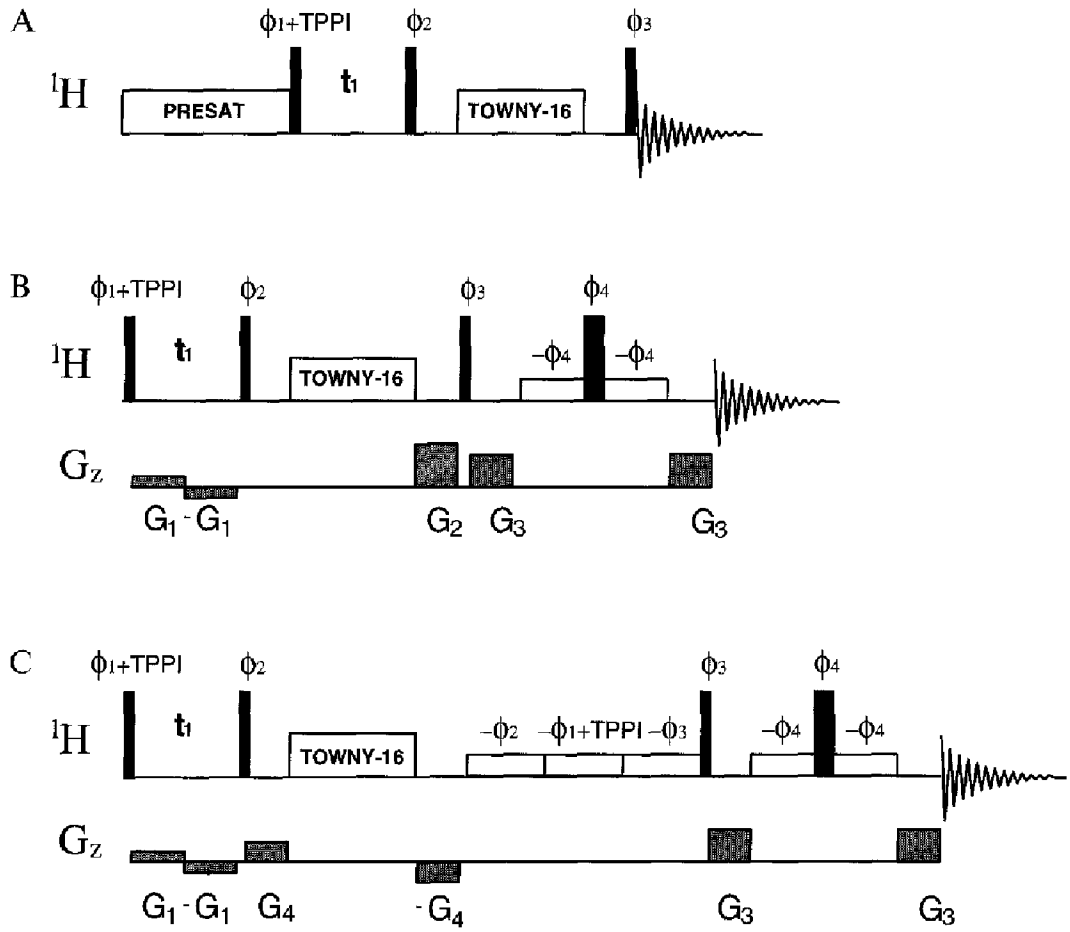

Fig. 1. TOCSY pulse sequences used in this work. (A) Conventional z-filtered TOCSY with presaturation (Rance, 1987); (B) z-filtered TOCSY with WATERGATE; and (C) flip-back TOCSY with WATERGATE (with water magnetization restored to equilibrium prior to acquisition). Narrow and wide dark rectangles indicate nonselective 90° and 180° pulses, respectively. Water-selective 90° pulses are indicated by open rectangles. The transmitter is on-resonance at the water frequency. In all sequences TOWNY-16 (Kadkhodaei et al., 1993) was used for isotropic mixing to minimize NOE artifacts, although any mixing scheme compatible with z-filtered TOCSY could be used. Selective rf pulses are low-power (ca. 100 Hz) rectangular pulses calibrated on water to give a 90° effective flip angle. The phase cycle for all three sequences was as follows: $\phi_1 = 4(x),4(y),4(x),4(y),4(-x),4(-y),4(-x),4(-y)$, $\phi_2 = 4(x),4(y),4(-x),4(-y)$, $\phi_3 = x,y,-x,-y$, $\phi_4 = x,y,-x,-y,-x,-y,x,y$, $\phi_{Receiver} = 4(x,y,-x,-y),4(-x,-y,x,y)$. Rectangular gradient pulses were used with the following strengths: $G_1 = 0.25$ G cm^{-1}, $G_2 = 15$ G cm^{-1}, $G_3 = 13$ G cm^{-1} and $G_4 = 1.3$ G cm^{-1}. Gradient pulses G_2, G_3 and G_4 are 1 ms long, and are followed by a 100 μs recovery delay. Gradient pulses G_1 are each of duration $t_1/2$, i.e., their duration is incremented during the 2D experiment (Skleňár, 1995), and no recovery delays were used.

for aqueous samples, particularly at high magnetic fields and when using high-Q probeheads (Gueron et al., 1991). Radiation damping causes transverse water magnetization to return to the z-axis at a much faster rate than its intrinsic longitudinal relaxation rate. This can cause two classes of problems in NMR experiments. First, when radiation damping occurs during the evolution period of a 2D experiment, an undesirable broadening of water cross peaks results along F1 (Otting, 1994). Second, a pulse sequence may have a decidedly different effect on the water magnetization than on the magnetization of dilute spins, because considerable radiation damping can occur during delays or during selective pulses with durations of a few milliseconds (Warren et al., 1989). Thus, the effective flip angle of a selective rf pulse is dependent upon the state of the water magnetization vector prior to the pulse (e.g., at equilibrium, inverted, or transverse). Furthermore, radiation damping effects may vary considerably at different stages of an rf phase cycle (Stonehouse et al., 1994a). Magnetization that has been spatially phase-encoded has no net transverse magnetic moment and is therefore not subject to radiation damping. Thus, B_0 field gradients (Kay et al., 1994; Skleňár, 1995) or B_1 field gradients (Otting, 1994) may be used to eliminate or delay the onset of radiation damping. Alternatively, modifications to probehead electronics have been reported that permit the control of radiation damping (Anklin et al., 1995; Broekaert and Jeener, 1995).

The above considerations can be addressed by combining WATERGATE with z-filtering for the TOCSY experiment (Rance, 1987). The pulse sequence for a conventional z-filtered TOCSY experiment is depicted in Fig. 1A. Note that the coherence of interest is stored as z-magnetization between the second and third rf pulses, and

will be unaffected by field gradient pulses during this interval. Figure 1B shows a gradient-enhanced version of the z-filtered TOCSY experiment. The first notable element of the sequence is a pair of gradient pulses (G_1) during the evolution period to prevent radiation damping (Skleňár, 1995). These gradient pulses must be very weak (0.25 G cm^{-1}) to avoid signal losses due to molecular diffusion and to allow the usual delays for eddy current recovery to be omitted. Homospoil pulse G_2 dephases any transverse magnetization present following the spin-lock period. This serves three purposes. First, no z-component of the water magnetization will be generated by the read-out pulse. Second, radiation damping is halted, which facilitates the adjustment of the WATERGATE parameters for optimal water suppression. Third, the gradient pulse enhances the function of the z-filter to purge phase anomalies from the TOCSY cross peaks.

An additional sensitivity enhancement can be achieved by restoring the water magnetization to the positive z-axis prior to acquisition, particularly for protein and peptide samples at neutral pH (Grzesiek and Bax, 1993; Stonehouse et al., 1994b). If the water magnetization remains partially dephased continuously through an experiment, sensitivity is attenuated in a manner similar to saturation

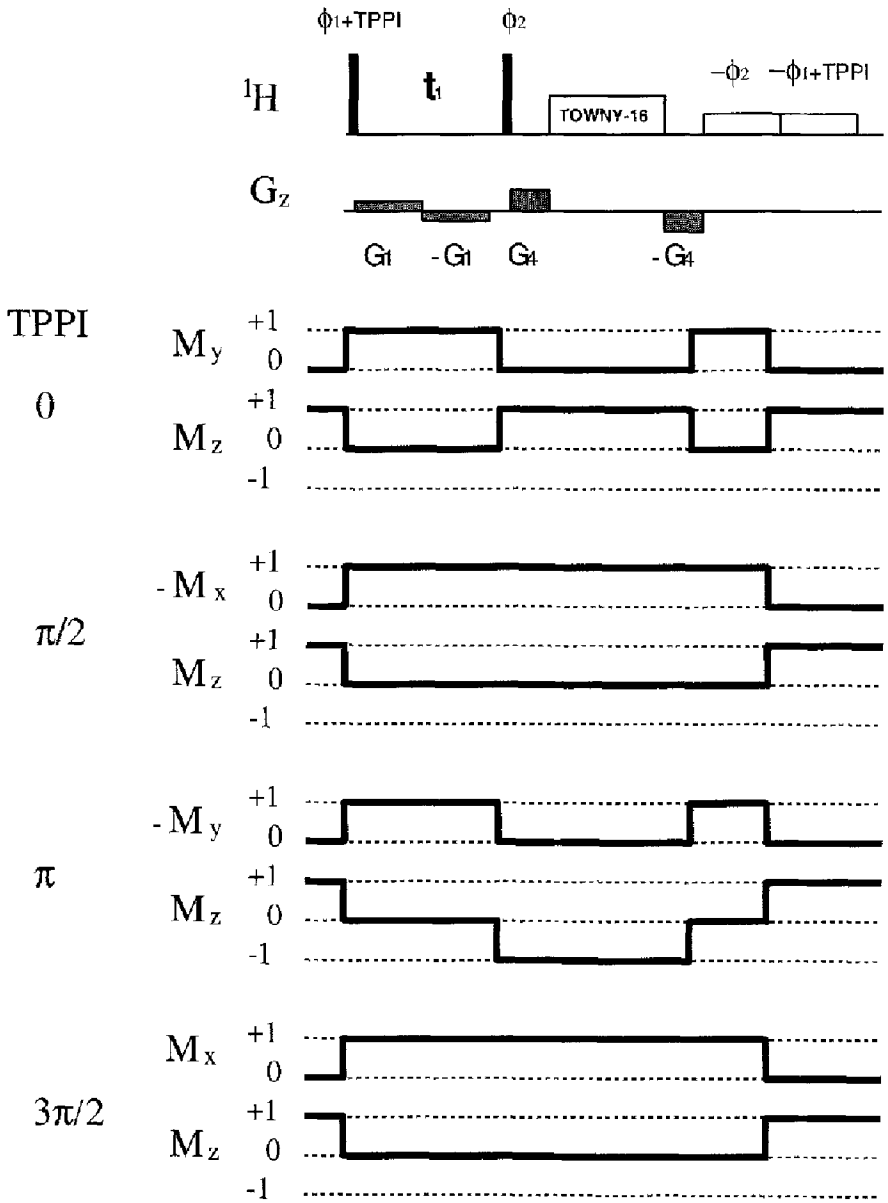

Fig. 2. Schematic diagram of the trajectory of the water magnetization during the flip-back TOCSY pulse sequence depicted in Fig. 1C. Trajectory diagrams are shown for the first step of phase cycles ϕ_1 and ϕ_2 (see Fig. 1) for the four steps of the TPPI cycle. The corresponding diagrams for the remaining steps of ϕ_1 and ϕ_2 would be identical, except for permutation of M_x, M_y, $-M_x$ and $-M_y$. Only the first half of the sequence is shown; in each case the water magnetization is maintained along the positive z-direction for the remainder of the sequence. These trajectories are valid only if radiation damping is suppressed during the evolution and isotropic mixing periods, which is accomplished by the use of pulsed-field gradients (Skleňár, 1995).

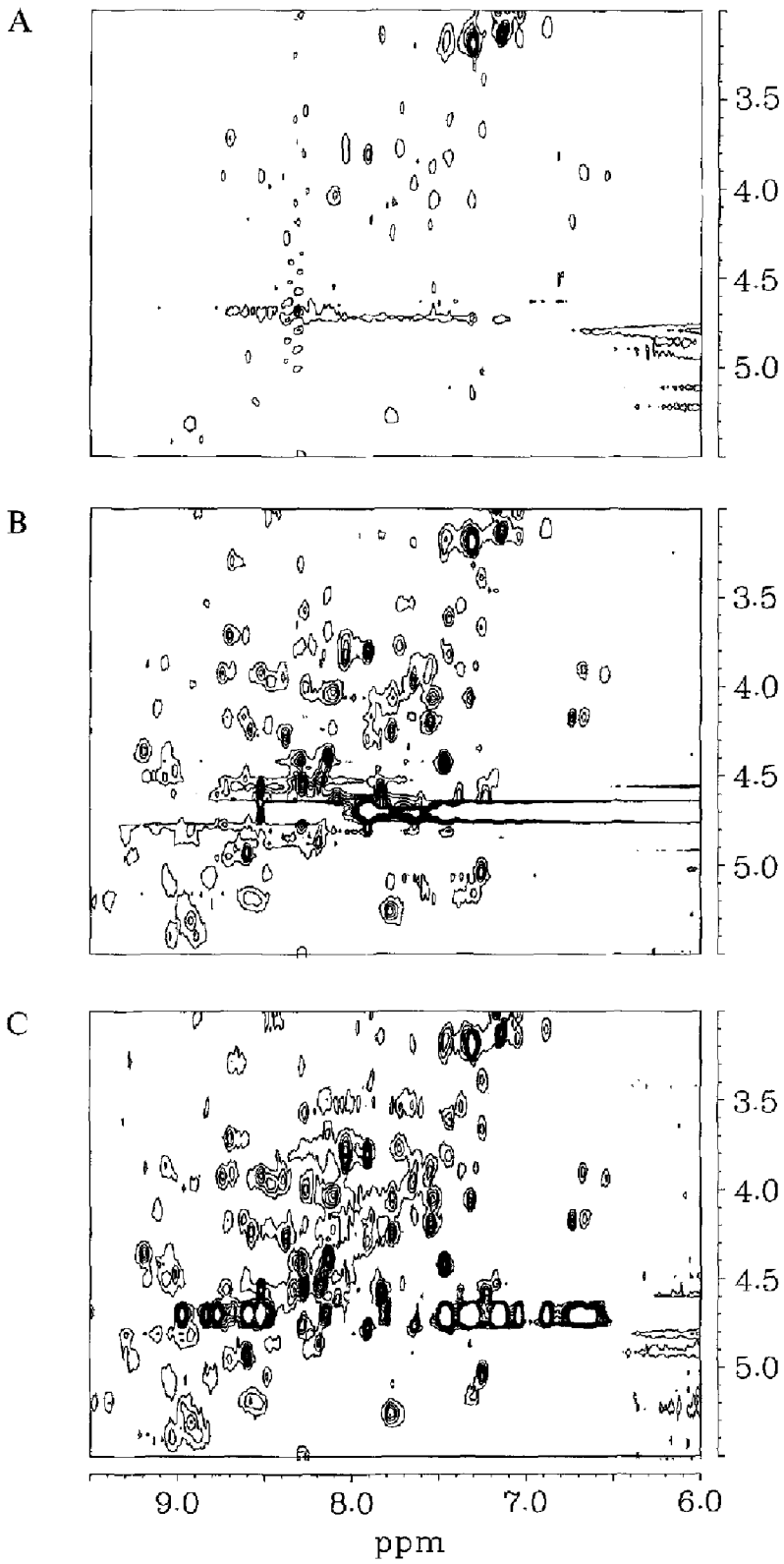

Fig. 3. Fingerprint regions of 2D TOCSY spectra of a 0.7 mM sample of angiogenin, a 13 kD protein, in 90% H_2O/10% D_2O at pH 7.0 and 25 °C. (A) TOCSY with presaturation spectrum (see Fig. 1A), obtained using an 80 Hz presaturation field (attenuation of 64 dB on our Bruker AMX-500 console) during the relaxation delay. (B) Basic TOCSY-WATERGATE spectrum (see Fig. 1B). (C) Flip-back TOCSY-WATERGATE spectrum (see Fig. 1C). For all spectra a relaxation delay of 1.2 s was used. The spin-lock power during isotropic mixing was 6.25 kHz, with a mixing time of 73.4 ms. Selective rf pulses had a duration of 2.5 ms. Acquisition parameters for each 2D experiment were: 2048 complex points in t_2, 400 increments in t_1, 64 scans per t_1 increment, 10.5 h total experiment time. Each spectrum was processed and plotted identically. The raw data were multiplied in both dimensions by a sine window function, phase shifted by 30°. The data were zero-filled and transformed to give 2048 × 1024 real points. No baseline correction or post-acquisition solvent suppression was performed.

transfer, unless very long relaxation delays are employed (Li and Montelione, 1993). Recently, sensitivity enhancement has been demonstrated in the homonuclear NOESY experiment (Fulton et al., 1995; Lippens et al., 1995) by use of a selective flip-back pulse just prior to the read-out pulse. However, since radiation damping is essentially quenched during spin-locking (Warren et al., 1989), this simple strategy will not work for the TOCSY experiment.

Figure 1C depicts a flip-back TOCSY-WATERGATE pulse sequence that uses field gradients and selective rf pulses to maintain control of the water magnetization over all stages of the phase and TPPI cycles. The trajectories of the water magnetization vector for the four steps of the TPPI cycle (Marion and Wüthrich, 1983) are shown schematically in Fig. 2. Note that the problems and solutions presented here are generally applicable when alternate methods of quadrature detection are used (States et al., 1982; Marion et al., 1989). Suppression of radiation damping during evolution (accomplished by gradient pulses G_1) is crucial for this sequence, because the behavior of the water magnetization must be reproducible throughout the 2D experiment. The isotropic mixing sequence is flanked by a pair of gradient pulses G_4 of opposite sign. Their function is to refocus only magnetization that has been subjected to a net flip angle of exactly 0° during isotropic mixing, in a manner similar to that used for 180° decoupling pulses (Bax and Pochapsky, 1992). Of particular importance, the bipolar gradients G_4 remove artifacts arising from flip-angle errors of the water magnetization orthogonal to the spin-lock axis, caused by radiation damping effects (Warren et al., 1989). Once again, the gradient strength G_4 must be low to avoid loss of magnetization by molecular diffusion. A pair of selective flip-back pulses then rotate the refocused water magnetization to the positive z-axis. The two pulses are phased so that the water magnetization vector is rotated by a net flip angle of 0°, 90° or 180°, appropriate for each step of the phase and TPPI cycles (see Fig. 2). The z-filter period ends with a read-out pulse, preceded by a selective flip-back pulse of opposite phase to maintain the water magnetization along the z-axis.

A potential pitfall of sequences 1B and 1C is the possibility of cross peaks developing via longitudinal cross-relaxation, because of the additional delay time relative to a conventional z-filtered TOCSY. In practice, the additional time amounts to 5–8 ms, during which cross-relaxation effects are generally negligible.

Experimental results confirm the improved sensitivity and solvent suppression of sequences 1B and 1C. All NMR spectra were recorded at 500 MHz on a Bruker AMX500 spectrometer equipped with a z-gradient accessory and a 5 mm inverse broadband probehead with actively shielded z-gradient coils. The efficiency of recovery of the water magnetization in the flip-back experiment was measured by inserting a nonselective 90° pulse at the end of the WATERGATE module, and comparing the integral of the water peak to that obtained in a simple one-pulse experiment. The efficiency of recovery was found to be at least 80% for all values of the TPPI cycle for $t_1 = 10$ μs, and at least 60% for $t_1 = 50$ ms. A set of 1D spectra corresponding to the first FID of a TOCSY were recorded as a function of relaxation delay for an aqueous sample of a 16-residue peptide at pH 7.0, to compare the sensitivities of sequences 1A, 1B and 1C. At least a 20% increase in signal intensity was observed in the WATERGATE spectra relative to spectra obtained by presaturation with an 80 Hz rf field. The sensitivity increase for the signals of labile protons was up to 50%. In the flip-back version of the experiment, maximum signal intensity was reached at a relaxation delay of 2 s, versus up to 5 s for the conventional WATERGATE experiment. Thus, there will be an increase in sensitivity per unit time when a short relaxation delay is used.

Figure 3 shows 2D TOCSY spectra of a 0.7 mM solution at pH 7.0 of angiogenin, a 13 kD protein for which both exchange and spin-diffusion effects are severe. Figure 3A, recorded using TOCSY with an 80 Hz presaturation field (with the sequence of Fig. 1A) shows considerable bleaching of cross peaks near the water frequency in F1. The corresponding spectrum obtained using TOCSY with WATERGATE (the sequence of Fig. 1B) is shown in Fig. 3B. The spectrum is cleaner and cross peaks are visible at the water frequency, although there is a residual water streak. The flip-back TOCSY with WATERGATE (the sequence of Fig. 1C) spectrum is shown in Fig. 3C. There is a further improvement in water suppression, and an increase in the intensity of many cross peaks. The line widths of the water exchange peaks along F1 are narrow, and cross peaks involving α-protons very close to the water frequency are resolved. There is also an absence of antidiagonal artifacts in the spectra in Figs. 3B and 3C.

In conclusion, we have presented two TOCSY-WATERGATE pulse sequences. Problems associated with radiation damping and spin-locking of water have been solved by use of pulsed-field gradients and selective rf pulses. Both sequences have no selective excitation elements until after the isotropic mixing period, so there is a minimal loss of information along the F1 dimension. The first sequence, shown in Fig. 1B, works by selectively dephasing the water magnetization prior to acquisition. This sequence is straightforward, robust and gives superior water suppression and sensitivity compared to TOCSY with presaturation. Further gains in both sensitivity and water suppression efficiency were obtained in the water flip-back version of the experiment, shown in Fig. 1C, particularly for dilute and high-molecular-weight protein samples at neutral pH. The inclusion of water flip-back introduces no constraint on the phase cycling of the z-filtered TOCSY (Rance, 1987), and no restriction on the mode used for quadrature detection in F1, an im-

provement over the previous schemes for acquiring TOCSY spectra of aqueous samples (Bax et al., 1987). Therefore, sequence 1C can be easily incorporated as an element of more complex experiments. For example, the modification described by Cavanagh and Rance (1990) could be implemented to achieve a further increase in sensitivity. It should also be noted that alternative methods of selective excitation can be used. For example, we routinely use a version of sequence 1B employing the 3-9-19 pulse train (Skleňár et al., 1993) for selective inversion during WATERGATE, with excellent results.

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Note added in proof

Lippens and co-workers [*J. Magn. Reson.* (1996) **B111**, 168–170] described in a recent paper a similar approach to minimize water saturation in homonuclear TOCSY experiments. The pulse sequences developed in the current work have better control of the water magnetization, hence achieve further reduction of radiation damping effects.

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