Full Correlations across Broad NMR Spectra by Two-Field Total Correlation Spectroscopy

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Abstract: Total correlation spectroscopy (TOCSY) is a key experiment to assign nuclear magnetic resonance (NMR) spectra of complex molecules. Carbon-13 TOCSY experiments are essential to assign signals of protein side chains. However, the performance of carbon-13 TOCSY deteriorates at high magnetic fields since the necessarily limited radiofrequency irradiation fails to cover the broad range of carbon-13 frequencies. Here, we introduce a new concept to overcome the limitations of TOCSY using two-field NMR spectroscopy. In two-field TOCSY experiments, chemical shifts are labelled at high field but isotropic mixing is performed at a much lower magnetic field, where the frequency range of the spectrum is drastically reduced. We obtain complete correlations between all carbon-13 nuclei belonging to amino acids across the entire spectrum: aromatic, aliphatic and carboxylic. Two-field TOCSY should be a robust and general approach for the assignment of uniformly carbon-13 labelled molecules in high-field and ultra-high field NMR spectrometers beyond 1000 MHz.

Introduction

High magnetic fields have greatly contributed to the success of nuclear magnetic resonance (NMR), in particular to characterize molecules of ever increasing complexity. Very high magnetic fields offer spectacular resolution and sensitivity but also come at a price, since the performance of some important experiments may dramatically deteriorate when the static field is increased. This is the case for total correlation spectroscopy (TOCSY) [1] of nuclei that feature a wide range of chemical shifts such as carbon-13. TOCSY spectra reveal networks of nuclei that belong to a common coupling network: amino acids in proteins, nucleotides in nucleic acids, monosaccharide units in polysaccharides, or constituents of complex mixtures [2-5]. Carbon-13 TOCSY experiments are essential for the assignment of signals from protein side chains, a crucial step for structural studies of proteins by NMR [6]. In current liquid-state probes, typical radio-frequency (*rf*) fields have amplitudes $\omega_1(^{13}C)/(2\pi)$ = 10 to 15 kHz that are insufficient to cover the broad range of carbon-13 resonance frequencies (about 40 kHz at B_0 = 18.8 T) during the TOCSY mixing time. It has been shown that by pushing actively cooled cryogenic probes beyond their specifications [7] one can achieve efficient coherence transfer across wide carbon-13 spectra at moderate static fields (up to 700 MHz) [8]. To cope with higher static fields, salty solutions, samples containing paramagnetic shift reagents or nuclei such as fluorine-19, the *rf* amplitudes will have to be Increased further, which may not be possible (arching, sample heating). Thus, protein side chain assignment strategies are best carried out in moderate magnetic fields, usually below 800 MHz, as a compromise between resolution (which is best at the highest fields) and efficiency of TOCSY mixing (which is better at lower fields). Could we possibly access optimal TOCSY mixing while benefiting from the resolution of the highest magnetic fields? Here, we demonstrate a universal solution to this problem by using different magnetic fields for chemical shift labelling and detection on the one hand, and for TOCSY mixing on the other. This can be achieved with a two-field NMR spectrometer [9, 10].

Total coherence transfer, the basic idea underlying TOCSY, is based on so-called isotropic mixing [1]. Many irradiation schemes have been proposed, all of which strive to reduce the average Hamiltonian to the strong coupling limit by eliminating the chemical shifts while preserving the scalar couplings. Under isotropic mixing, the magnetization is not only transferred between directly coupled nuclei but throughout the entire network of coupled spins. Suitable pulse sequences have been optimized to cover large ranges of frequencies with minimal *rf* amplitudes. Supercycles of *rf* pulses such as DIPSI [11, 12] and FLOPSY [13] where developed about thirty years ago. The steady increase of static magnetic fields has led to further improvements [14] that use adiabatic pulses [15, 16], in some cases designed using optimal control [17, 18]. Yet, with current high-resolution probe designs, one cannot apply continuous *rf* pulses with high amplitudes during relatively long intervals (typical *rf* amplitudes of $\omega_1(^{13}C)/(2\pi)$ = 10 kHz can be sustained up to 20 ms). As a result, the magnetization cannot be transferred efficiently between all types of carbon nuclei. Therefore, several TOCSY experiments are usually performed for different types of carbon-13 nuclei, by focusing first on the aliphatic region, then on the aromatic region, and finally on the carboxyl region. With few exceptions [17], correlations between these regions cannot be observed, which makes the assignment of aromatic side chains particularly difficult. At the highest available magnetic fields, even mixing within the aliphatic region alone becomes challenging.

We have recently introduced a two-field NMR spectrometer [9, 10] that combines two magnetic centres at greatly different fields. Spin systems can be manipulated by applying *rf* pulses at two different magnetic fields. Detection is performed at high field. In systems were chemical exchange leads to line-broadening, we have shown that our two-field spectrometer allowed us to recover signals that were broadened beyond detection at high fields [9]. Here, we exploit the dramatically reduced spectral width for carbon-13 nuclei at low fields to obtain efficient isotropic mixing across the entire carbon-13 spectrum. This conceptual approach opens the way for a new class of experiments for resonance assignment in carbon-13 labelled molecules.

Results and Discussion

The pulse sequence of the 2F-TOCSY experiment starts with an interval where the carbon-13 polarization is enhanced by proton irradiation to induce a nuclear Overhauser enhancement (Fig. 1). The carbon-13 magnetization is labelled according to the chemical shifts during an evolution interval t_1, and stored along the z-axis. The sample is then transferred to the low-field position where a FLOPSY-16 isotropic mixing sequence can be applied during the TOCSY mixing period (Fig. 1a). The sample is subsequently transferred back to high field where the carbon-13 magnetization is detected. This experiment was compared with (i) a standard high-field TOCSY experiment where a FLOPSY-16 sequence is applied at high field (Fig. 1b), and (ii) with a two-field experiment without applying any isotropic mixing sequence at low field (Fig. 1c). In the latter case, isotropic mixing occurs naturally as a result of the scalar couplings that become comparable to or stronger than the chemical shift differences at low field. Since the limited sensitivity of our prototype makes experiments on protein samples challenging, the two-field TOCSY experiment was tested on a mixture of two carbon-13 enriched amino acids. Phenylalanine was chosen as a typical amino acid with an aromatic sidechain, and leucine because of its long aliphatic sidechain, where the entire ^{13}C spectrum spans 155 ppm between the carboxyl and methyl groups, which amounts to only 540 Hz at B_0^{LF} = 0.33 T, but to as much as 23 kHz at B_0^{HF} = 14.1 T, far more than the RF field strength ($\gamma B_1^{HF}/(2\pi)$ = 5.5 kHz) that is available at high field on our system. At low field, even weak radiofrequency pulses ($\gamma B_1^{LF}/(2\pi)$ = 1.5 kHz in our prototype) are more than enough to cover the entire range of frequencies efficiently, from carboxyl to methyl resonances. Indeed, all possible correlations between all carbon-13 signals within each amino acid molecule were observed (Fig. 2a). Close inspection of the two-dimensional 2F-TOCSY spectrum shows cross peaks between aliphatic and aromatic carbon-13 nuclei, between aliphatic and carboxylic carbon-13 nuclei, and between aromatic and carboxylic carbon-13 nuclei (Fig. 2b). The observation of such cross-peaks is challenging in high-field TOCSY experiments. Note that there is no price to pay in terms of resolution, since the evolution under the chemical shifts occurs at high field in both evolution and detection intervals. The only disadvantage is the loss of sensitivity due to

longitudinal $T_1(^{13}C)$ relaxation during the shuttling of the sample (230 ms total for up and down transfers) and the use of a small sample volume in our prototype. The sensitivity of the current system is about one order of magnitude lower than a typical 600 MHz spectrometer equipped with a room-temperature probe.

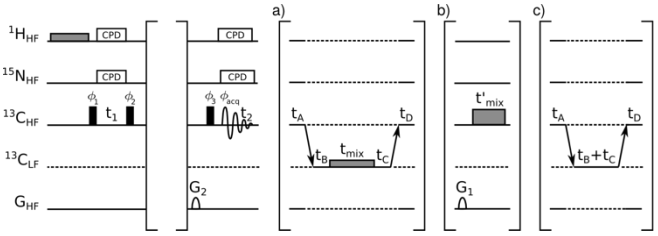

Figure 1. Pulse sequences employed for TOCSY experiments. The subscripts HF and LF designate channels at high field and low field, respectively. Non-selective $\pi/2$ pulses are denoted by filled rectangles. The grey rectangular box on the $^1H_{HF}$ channel depicts a 1.3 s WALTZ-16 composite-pulse irradiation [19] with an amplitude $\gamma B_1^{HF}/(2\pi)$ = 714 Hz to allow the Overhauser effect to build up after a delay of 1 s between subsequent scans. WALTZ-16 composite pulse decoupling [20] with $\gamma B_1^{HF}/(2\pi)$ = 1667 Hz is used for $^1H_{HF}$ and GARP-4 [27] with $\gamma B_1^{HF}/(2\pi)$ = 1.04 kHz for $^{15}N_{HF}$ (rectangular boxes labelled CPD.) Three TOCSY experiments can be implemented: (a) with FLOPSY-16 [17] isotropic mixing (grey rectangle) with $\gamma B_1^{LF}/(2\pi)$ = 1.5 kHz during t_{mix} = 31.28 ms. (b) high-field TOCSY experiment with FLOPSY16 [13] with $\gamma B_1^{LF}/(2\pi)$ = 5.5 kHz applied at high field during t_{mix} = 16.96 ms. (c) Two-field TOCSY experiment without any *rf*-pulses at low field. The carbon-13 carrier frequency was placed at 100 ppm at both high and low fields. The phases cycles were φ_1 = {x, -x}, φ_2 = {4x, 4(-x)}, φ_3 = {2x, 2(-x)}, and φ_{acq} = {x, -x, - x, x, -x, x, x, -x}. Pulsed field gradients have smoothed rectangular shapes, with maximum amplitudes G_1 = 20 G/cm and G_2 = 17 G/cm, and durations of 0.5 ms. Sign discrimination in the indirect dimension is achieved by an alternation of φ_1 in the States-TPPI manner [21]. The spectra were acquired with N_1 = 230 t_1 time increments in the indirect dimension. The sample reached the low-field position in 120 ms and the return to the high-field position took 110 ms. Delays required because of hardware limitations prior to the sample transfer were t_A = 25 ms and t_C = 30 ms, respectively. The delays t_B = 10 ms and t_D = 300 ms allow possible vibrations to be attenuated.

By contrast, a TOCSY experiment performed at high field (B_0^{HF} = 14.1 T) when the carrier frequency is positioned in the centre of the carbon-13 spectrum (100 ppm) leads to a spectrum where many cross-peaks are missing (Fig. 3a) despite the stronger *rf* amplitude ($\gamma B_1^{HF}/(2\pi)$ = 5.5 kHz > $\gamma B_1^{LF}/(2\pi)$ = 1.5 kHz). Only the correlation peaks between aromatic carbon-13 nuclei were clearly detected, which is not surprising since their resonance frequencies were close to the carrier frequency during isotropic mixing. Only a few weak dispersive peaks that barely exceed the noise level show correlations between carboxylic and alpha carbon-13 nuclei (see supporting information). At low magnetic fields, strong couplings are commonly found in carbon-13 spin systems. In the limit where the chemical shift differences are much smaller than the scalar coupling constants, the Hamiltonian is identical to the average Hamiltonian operator obtained during ideal isotropic mixing. In particular, we expect to observe strong couplings within the aliphatic region: the δ_1 and γ carbon-13 nuclei in leucine have a chemical shift difference of only 2.2 ppm (7.7 Hz at 0.33 T) while the scalar coupling constant is about $^1J_{CC}$ = 35 Hz. Likewise, in the aromatic ring of phenylalanine the maximum chemical shift difference is 7.3 ppm (25.7 Hz at 0.33 T), leading to a network of strongly coupled nuclei. Indeed, a two-field correlation experiment without any *rf* irradiation at low field leads to cross-peaks within the aliphatic region of both amino acids as well as within the aromatic region of phenylalanine (Fig. 3b). On the other hand, the chemical shift difference between directly bound α and β carbon-13 nuclei of phenylalanine is 19.6 ppm (69 Hz at 0.33 T), so that mixing due to strong coupling is expected to be less efficient in the absence of *rf* irradiation. More generally, no cross peaks could be detected between aromatic and aliphatic, aliphatic and carboxylic, or aromatic and carboxyl carbon-13 nuclei since the scalar couplings $^1J_{CC}$ are small compared to the chemical shift differences at B_0^{LF} = 0.33 T, so that the weak coupling regime applies. Although it would be technically feasible to reach lower magnetic fields to obtain isotropic mixing without *rf* irradiation, this may not be desirable for all spin systems. First, the longitudinal relaxation rates $1/T_1(^{13}C)$ may be large at very low fields, in particular in macromolecules such as proteins [22-25]. Second, on the way to very low fields, one would necessarily be exposed to level anti-crossings (LACs) [26-29], which could result in coherent effects that would be difficult to control.
The two-field TOCSY experiment introduced here provides by far the best correlations throughout networks of scalar-coupled carbon-13 nuclei over the entire range of chemical shifts typical for biomolecules, which are often uniformly labelled with carbon-13. We observed peaks that correlate carbon-13 nuclei such as the δ_2 and carboxyl carbon-13 nuclei of leucine that are separated by as many as four bonds and have chemical shifts that are separated by as much as 154.7 ppm. In phenylalanine, cross-peaks could be readily observed between nuclei such as the ζ and carboxyl carbon-13 nuclei that are separated by no less than six bonds. In the latter case, the transfer requires efficient mixing between aromatic and aliphatic carbon-13 nuclei, as well as between aliphatic and carboxylic carbon-13 nuclei. The assignment of aromatic side chain resonances is usually a challenge in proteins [30]. The two-field TOCSY methodology should allow to overcome this hurdle. Our approach opens the way to the development of a host of two-field TOCSY-based NMR experiments.

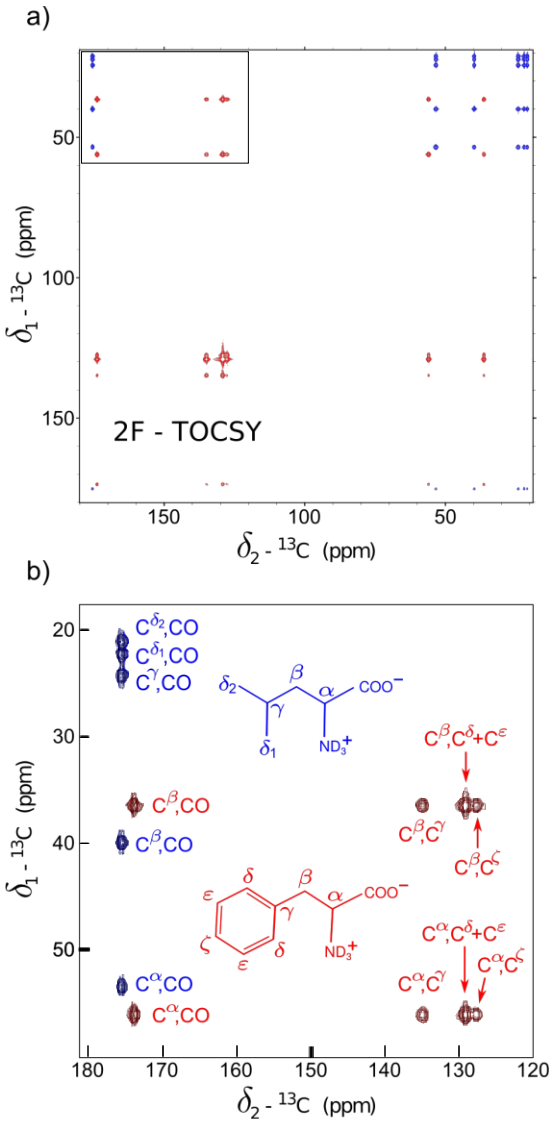

Figure 2. (a) Full two-field TOCSY spectrum of a mixture of 0.1 M leucine and phenylalanine in D_2O and (b) the region identified by a rectangle in (a) showing correlations between aliphatic (21-56 ppm), aromatic (128–135 ppm), and carboxylic (174–176 ppm) peaks. The peaks corresponding to leucine (blue) and phenylalanine (red) are distinguished by colours for clarity. All multiplets due to the homonuclear carbon-carbon couplings were masked in both dimensions to simplify the spectrum by multiplying the signals by an exponential function (line-broadening 40 Hz). All possible correlations were detected.

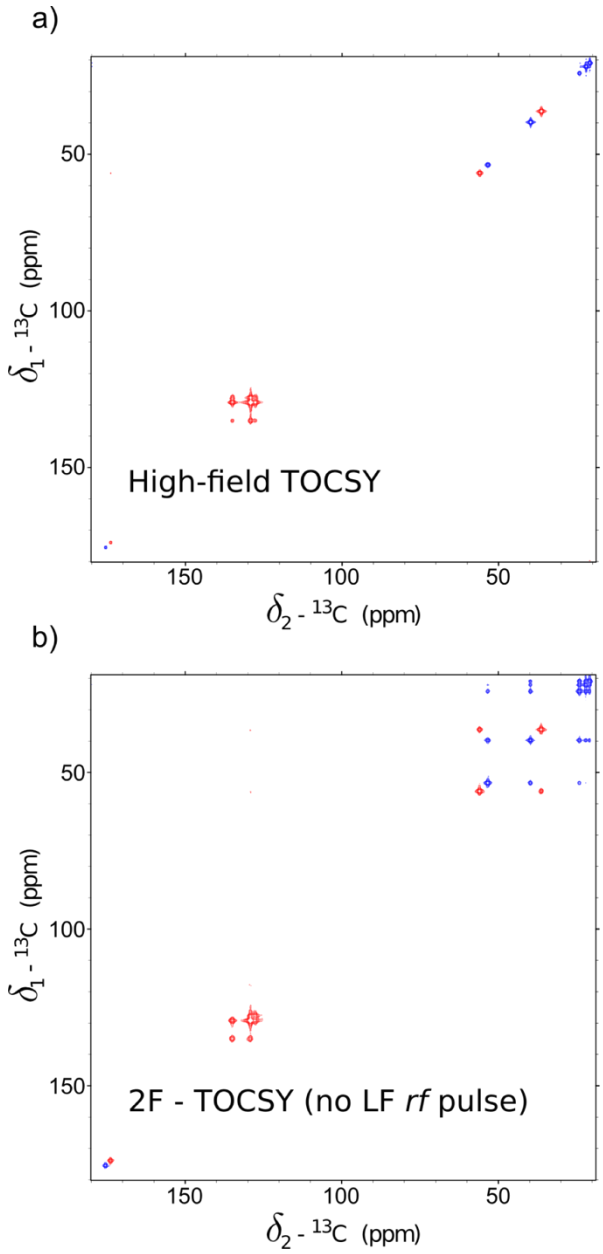

Figure 3. (a) Conventional TOCSY spectrum of the same sample as in Fig. 2 obtained at high field B_0^{HF} = 14.1 T. (b) Two-field TOCSY experiment acquired without any rf irradiation at low field. The peaks corresponding to the leucine (blue) and phenylalanine (red) molecules are distinguished by colours for clarity. Only cross-peaks close to the *rf* carrier frequency can be observed in (a). Only cross-peaks corresponding to spin networks that are strongly coupled at 0.33 T are detected in (b).

Conclusions

We have presented a two-field TOCSY experiment that is designed for a two-field NMR spectrometer [9, 10]. This experiment greatly improves isotropic mixing at low fields since the range of resonance frequencies of carbon-13 nuclei is dramatically reduced, so that the Hamiltonian is switched from the weak- to the strong-coupling limit. To achieve a similar efficiency of isotropic mixing at high fields, one would have to apply *rf* amplitudes that are at least comparable to the range of the chemical shifts $\gamma B_1^{HF}/(2\pi)$ > 60 kHz for 200 ppm at 28.2 T (1.2 GHz for protons), which does not appear to be realistic with current probe technology. High resolution can be preserved in both direct and indirect dimensions in 2F-TOCSY experiments. Application to a mixture of two amino acids shows that correlations between all types of carbon-13 nuclei can be observed: aromatic, aliphatic, and carboxylic. By performing isotropic mixing and chemical shift labelling at different fields, the two-field TOCSY approach should be particularly suited for running TOCSY experiments at fields beyond 20 T. Two-field TOCSY can be incorporated into many known experiments and applied to other spin-1/2 nuclei such as fluorine-19. As the intrinsic sensitivity of two-field NMR spectrometers will increase, this approach is expected to give rise to the development of new methods for the resonance assignment of uniformly carbon-labelled (bio)molecules.

Experimental Section

All experiments were applied to a mixture of uniformly ^{13}C and ^{15}N labelled leucine and phenylalanine (Cortecnet) both at 0.1 M in D_2O at 25 ºC on a Bruker Avance III HD spectrometer (B_0^{HF} = 14.1 T) augmented by a series of accessories to perform 2F-NMR experiments with a second magnetic centre (B_0^{LF} = 0.33 T) with residual field inhomogeneity of *ca*. 10 ppm over a height of 2 cm [9, 10]. The sample (100 µL) can be moved between the two magnetic centres separated by 49 cm in *ca.* 110 ms by a pneumatic shuttle [23]. Each magnetic centre is equipped with a triple-resonance probe tuned to the resonance frequencies of nitrogen-15, carbon-13 and protons, plus a deuterium channel at 14.1 T for field-frequency locking. The FLOPSY-16 isotropic mixing [13] has been used to induce TOCSY transfer at both B_0^{LF} = 0.33 T and B_0^{HF} = 14.1 T (Figure 1). The spectra were processed using NMRpipe [31] and analysed using SPARKY [32].

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Keywords: Nuclear magnetic resonance (NMR) • Total correlation spectroscopy (TOCSY) • Two-field NMR (2F-NMR) • Two-field TOCSY (2F-TOCSY)

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