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JE-TROSY: combined *J*- and TROSY-spectroscopy for the measurement of one-bond couplings in macromolecules

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Abstract

With the application of RDCs in high-resolution NMR studies of macromolecules, there has been an interest in the development of accurate, sensitive methods for measuring ^{15}N–^{1}H and ^{13}C–^{1}H one-bond coupling constants. Most methods for determining these couplings are based on the measurement of the displacement between cross-peak components in *J*-coupled spectra. However, for large macromolecules and macromolecular complexes, these methods are often unreliable since differential relaxation can significantly broaden one of the multiplet components (i.e., the anti-TROSY component) and thereby make accurate determination of its position difficult. To overcome this problem, a *J*-evolved transverse relaxation optimized (JE-TROSY) method is presented for the determination of one-bond couplings that involves *J*-evolution of the sharpest cross-peak multiplet component selected in a TROSY experiment. Couplings are measured from the displacement of the TROSY component in the additional *J*-evolution dimension relative to a zero frequency origin. The JE-TROSY method is demonstrated on uniformly labeled ^{15}N,^{13}C-labeled RNA and peptide samples, as well as with an RNA–protein complex, in which the protein is uniformly ^{15}N,^{13}C-labeled. In all cases, resolved, sensitive spectra are obtained from which heteronuclear one-bond *J*-couplings could be accurately and easily measured.
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Keywords: TROSY; *J*-spectroscopy; Scalar couplings; Residual dipolar couplings; RNA; Protein

1. Introduction

The use of RDCs in high-resolution NMR studies of macromolecular structure and dynamics [1,2] has brought about an interest in methods for rapid and accurate determination of heteronuclear [3–15] and homonuclear [16–24] coupling constants. Most methods designed for the determination of large heteronuclear one-bond *J*-couplings are based on the measurement of the displacement between cross-peak components in *J*-coupled spectra [2,7,25,26]. The application of such methods to larger macromolecules and macromolecular complexes, however, is often unreliable because differential relaxation significantly broadens and subsequently decreases the resolution of one of the two components of the cross-peak doublet (i.e., the anti-TROSY component) needed for accurate measurements [8,27]. The effect of cross-correlated relaxation on the accuracy with which one-bond heteronuclear coupling constants can be determined using *J*-coupled spectra for proteins with short transverse relaxation times is well documented [27]. The observation of doublets in *J*-coupled spectra also leads to a greater potential for cross-peak overlap in these experiments. However, this problem can be mitigated through the use of multiplet selective experiments [4,28–30]. For larger macromolecules and macromolecular complexes, *J*-modulated based methods [31,32] can provide an alternative approach for the accurate measurement of coupling constants. Here, a *J*-evolved heteronuclear transverse relaxation optimized (JE-TROSY) method (Fig. 1) is presented for the measurement of ^{15}N–^{1}H and ^{13}C–^{1}H one-bond couplings that circumvents the potential problems of differential line broadening and overlap in *J*-coupled spectra.

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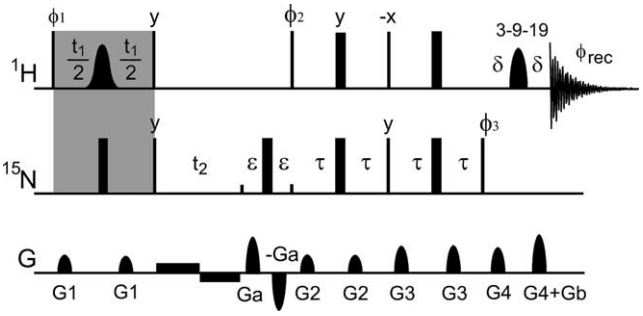

Fig. 1. An example of the JE-approach is shown by incorporating a *J*-evolution into the initial INEPT transfer step of an SG-TROSY-sequence [40]. Gradients were applied as published with $g1 = g2 = g3 = 1.5$ G/cm, $g4 = 3.0$ G/cm, Ga = 15 G/cm, and Gb = 3.05 G/cm for the P-type signal and Ga inverted for the N-type signal of the echo/anti-echo acquisition. Using a Bruker Avance 600 MHz spectrometer, phases for the P-type signals for the most slowly relaxing cross-peak component were $\phi_2 = y, \phi_3 = x$, and $\phi_2 = -y, \phi_3 = -x$ for the N-type signal, while the first proton pulse was phase-alternated together with the receiver phase $\phi_1 = \phi_{rec} = x, -x$. The gradient during the *J*-evolution period can be applied by starting with the dwell time of the *J*-spectrum as the first time increment, as described in the text. The first 180° proton pulse (drawn as a Gaussian shape) can be applied either as a hard or selective pulse. Application of a selective pulse effectively decouples ^{1}H–^{1}H couplings between amide and aliphatic protons.

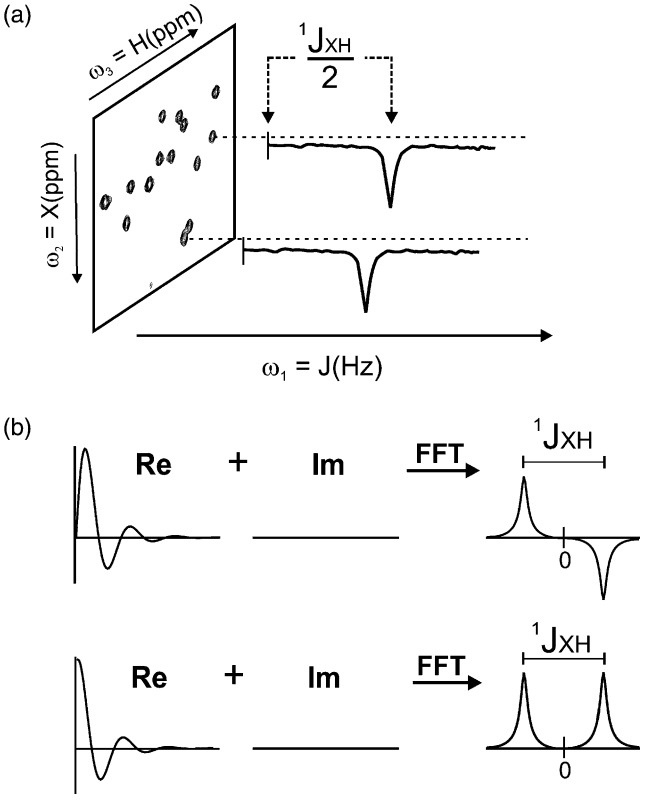

Fig. 2. (a) Schematic representation of the JE-TROSY experiment. Using an incremented spin-echo delay instead of a fixed INEPT period, the TROSY selected multiplet component can be evolved to generate a *J*-spectrum (shown representatively as one-dimensional traces projected from the 2D TROSY cross peaks). (b) Coupling constants can be measured easily from the separation between positive and negative components generated through a complex Fourier transform of the data. Data collected as a sine-modulated real signal are combined with a zeroed imaginary part and complex Fourier transformed to yield a mirror image, antiphase cross-peak pattern centered on the zero frequency. Data collected as a cosine-modulated signal and processed in a similar fashion result in a mirror image, in-phase pattern. Both patterns can be produced with the JE-TROSY approach using a single recorded experiment. The generation of in-phase/antiphase spectra allows application of Keeler–Titman type methods to measured one-bond *J*-couplings even if the linewidth exceeds $J/2$.

The JE-TROSY experiment combines the sensitivity and resolution of TROSY correlation [33,34] with the measurement of coupling constants through a time incremented spin-echo delay that generates a *J*-spectrum [35–38]. The method employs *J*-evolution of the sharpest cross-peak multiplet component selected in a TROSY experiment in a third dimension so that heteronuclear one-bond couplings can be determined from the frequency displacement of the TROSY component in this dimension relative to a zero frequency origin (Fig. 2). The JE-TROSY experiment is demonstrated on uniformly labeled ^{15}N,^{13}C-labeled RNA and peptide samples, as well as with an RNA–protein complex sample in which the protein is uniformly ^{15}N,^{13}C-labeled.

2. Theory

In the JE-TROSY experiment (Fig. 1), an INEPT spin-echo delay (highlighted by a shaded box) is incremented to produce a *J*-spectrum [35–38] in an independent dimension. During the spin-echo evolution period, scalar and residual dipolar couplings evolve while chemical shift and artifacts like B1-field inhomogeneities are refocused. In the experiment, time incrementation of the INEPT spin-echo delay results in the evolution of real data which can be transformed using a discrete version of the real Fourier transform. The real Fourier transform of the time-domain *J*-spectrum produces a frequency domain *J*-spectrum with a single line at half the frequency of the coupling constant (Fig. 2a). Alternatively, the data can be processed using a complex Fourier transform by adding a zeroed imaginary part to the real data. The complex Fourier transform of the sum of the acquired real data and an artificially zeroed imaginary part produces an antiphase spectrum that can be phased to pure absorption. Spectra processed according to this method can be used to measure coupling constants directly from the displacement between the two peaks (Fig. 2b). The antiphase nature of the signal is a consequence of using a zero delay for the first point in the *J*-evolution. If the first point is set to $1/2J_{HX}$, the *J*-evolution starts with maximum signal and the processed spectrum results in an inphase pattern. In cases where the linewidth is comparable to half the $^1J_{HX}$ coupling

constant, spectra can be acquired as described in the experimental section with the first increment set to $\sim 1/2J_{max}$ such that the resulting data can be processed as inphase signals and then with the first point set to zero to obtain antiphase signals. The two resulting spectra can then be used to determine the coupling constants by methods originally developed by Keeler and Titman [39].

The JE-TROSY experiments implemented in this study have been adopted from previously published TROSY pulse schemes [33,40]. Fig. 1 shows an example of a *J*-evolution incorporated in the initial INEPT transfer step of the SG-TROSY-sequence [40]. Apart from incrementation of the first INEPT spin-echo period to generate the *J*-spectrum, only slight modifications have been made to the sequences to suppress artifacts in the *J*-dimension that can result from proton exchange and/or imperfect pulses and lead to zero-frequency signals. The zero-frequency signal can be suppressed simply through an additional phase cycling of the first proton pulse (ϕ_1 in Fig. 1) that is synchronized with the receiver phase. Pulse-field gradients can also be applied during the *J*-evolution period to suppress artifacts. However, since it is desirable to start with an initial spin-echo delay of zero to achieve clean phase behavior, no delay time is available for the application of pulsed-field gradients during the first acquisition point. This problem can be circumvented by setting the first increment to the dwell time of the *J*-evolution and then manually adding a zero as the first point during post-acquisition processing of the data since an INEPT transfer step of zero duration should not give any detectable signal. In addition to providing the possibility for applying gradients during all t_1 increments, this approach also reduces the overall experimental time by eliminating the need to acquire the first 2D plane of the 3D experiment.

The increment for the spin-echo delay and the number of points for the *J*-spectrum should be chosen such that the spectral width is at least twice as large as the observed splitting. In most cases the increment can be chosen as $1/2J_{max}$, with J_{max} being the largest one-bond coupling expected in the spectrum. In cases where the cross-peak linewidth is in the order of or larger than the $1/2J_{max}$ it might be necessary to reduce the time increment and acquire a sweep width in the *J*-spectrum that is larger than twice the largest splitting to avoid folding artifacts. The optimal number of time increments in the *J*-spectrum also depends in part on the signal-to-noise ratio of the experiment. In principle, the acquisition of more time increments will lead to longer evolution times and therefore better resolved *J*-spectra. In practice, relaxation of magnetization in the transverse plane will strongly reduce the detected signal for longer t_1 evolution periods. The total number of points collected for the *J*-spectrum is therefore a compromise between the inherent signal-to-noise ratio of the sample and the desired resolution. To determine the optimal number of t_1 increments, a series of one-dimensional (1D) experiments can first be recorded with increased spin-echo delays to determine the maximum evolution time that still displays a detectable signal.

3. Experimental

The accuracy of the JE-TROSY experiment was first demonstrated through the measurement of $^1J_{NH}$ coupling constants associated with imino resonances in the uniformly ^{15}N,^{13}C-labeled 16mer RNA oligonucleotide CopT16 (5′-GCACUUUGGCGAGUGC-3′). In this application, measured $^1J_{NH}$ coupling constants (Fig. 3c) were found to be in excellent agreement with couplings determined from a ^{15}N-coupled 1D proton spectrum (Fig. 3b). In all cases, measured $^1J_{NH}$ coupling constants were within the error for the given signal-to-noise ratio of the experiments, which were estimated to be 0.3 Hz for the 1D and 0.4 Hz for the JE-TROSY using the peak picking routine of nmrPipe [41]. Application of the JE-TROSY for the measurement of $^1J_{NH}$ coupling constants associated with amide resonances was also demonstrated using the ^{15}N,^{13}C-labeled cyclic pentapeptide, D-Pro-Ala-Ala-Ala-Ala (PA_4), shown in Fig. 3d. Fig. 3e shows the trace of the directly zero-filled and processed spectrum with an inphase pattern. In Fig. 3f, the same zero-filled data were circular left shifted by one point prior to Fourier transform to produce the expected antiphase pattern. From each of the resulting *J*-spectra the $^1J_{NH}$ coupling constant of 90.2 Hz, as well as the $^3J_{HNH\alpha}$ coupling of 9.7 Hz, can be directly measured from the peak splitting.

In the JE-TROSY experiment, measurement of one-bond couplings while proton magnetization is transverse results in the simultaneous evolution of ^1H–^1H couplings (Fig. 3e and f). The evolution of these additional couplings is usually undesirable since they not only result in line broadening but can also lead to inaccurate coupling constant determination due to cross-peak distortion from differential relaxation of the subcomponents [3,8,27]. In situations where the coupled proton is sufficiently separated in chemical shift, as is the case for protein amide and nucleic acid imino protons, a selective 180° proton pulse can be used in the spin-echo of the INEPT step to effectively decouple most ^1H–^1H couplings. This is shown for the peptide PA_4 in Fig. 3g and h for the inphase and antiphase processed versions of the *J*-spectra.

The $^3J_{HNH\alpha}$ coupling is clearly decoupled in these spectra, as is any line distortion that resulted from the differential relaxation due to the H_α spin. In addition, the period of the proton selective pulse, during which *J*-couplings can partially evolve, appears to be of minor

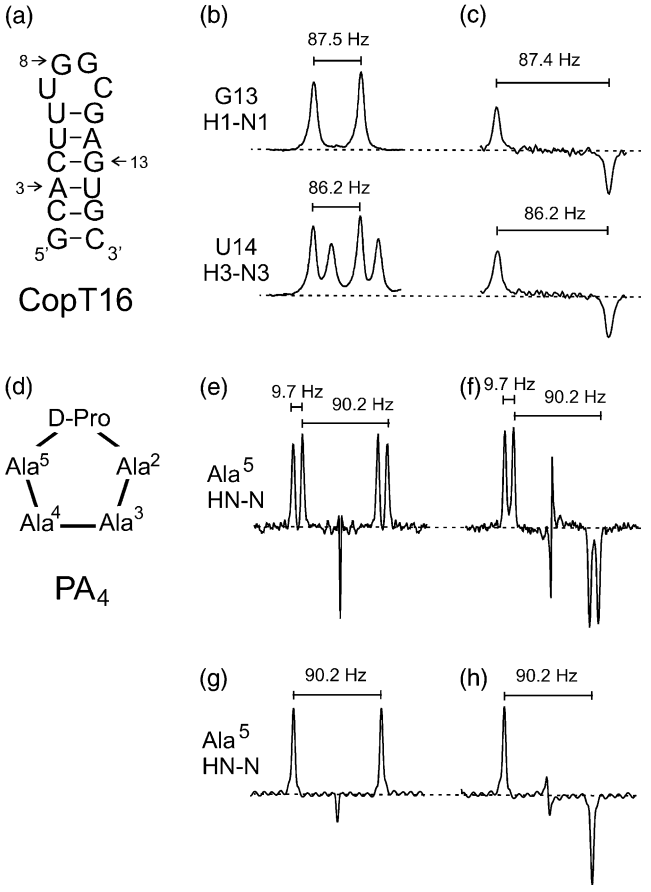

Fig. 3. (a) Sequence and secondary structure of the CopT16 RNA hairpin. Comparison of the coupling constants measured from the ^{15}N-coupled proton acquisition dimension of a 1D jump–return-spin-echo experiment (b) and traces from a 2D JE-TROSY (c) applied to the CopT16 sample using a Bruker Avance 500 MHz spectrometer. For the JE-TROSY experiment as shown in (c), no t_2-evolution was applied since the imino spectrum was already resolved. For the J-evolution the sweep width was set to 200 Hz corresponding to a t_1-time increment of 5.0 ms starting with zero transfer time. The 64 real increments were complex Fourier transformed after zeroing the added imaginary part as shown in Fig. 2b, leading to the 1D traces shown. (d) Schematic of the cyclic pentapeptide, PA_4. Two versions of the pulse sequence of Fig. 1 were applied to the ^{15}N,^{13}C-labeled cyclic pentapeptide PA_4 using a Bruker Avance 600 MHz spectrometer and processed in different ways to demonstrate the two acquisition and processing techniques described in the text. Using a hard proton pulse during the spin-echo and a dwell time of 5.55 ms, which is approximately $1/2J_{NH}$, the inphase pattern (e) is obtained and addition of zeroes as the first J-increment results in an antiphase pattern (f). The spectra recorded with a selective G3 pulse of 1.5 ms applied on the amide protons during the spin-echo and identical processing parameters to (e) and (f) are shown in (g) and (h). The observed splitting due to the $^3J_{HNH\alpha}$ coupling in (e) and (f) is clearly decoupled in (g) and (h). Sixty-four time increments were recorded for the J-spectra and zero-filled to 512 points prior to Fourier transform. The addition of the first zero points to the J-time increments was achieved with a circular shift of the zero-filled data by a single point. Measured coupling constants are indicated above the traces.

consequence to the accuracy of the coupling measurement. This is shown for the coupling constants measured in PA_4 (Fig. 3g and h) which are identical within

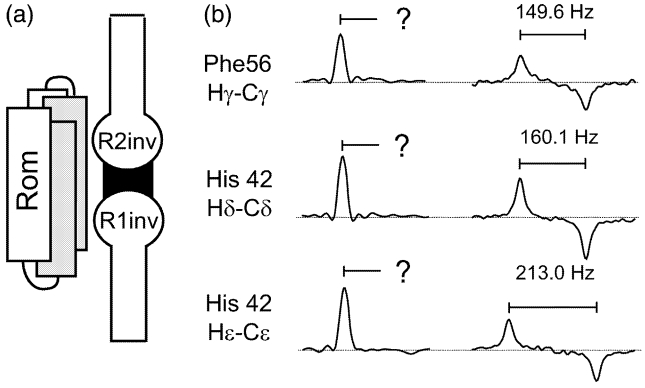

Fig. 4. (a) Schematic of the trimolecular RNA–protein complex formed between the ColE1 Rom protein and the R1inv (21mer) and R2inv (19mer) RNA hairpins. (b) Traces through the t_1-coupled ^{13}C-CT-HSQC with preliminary assignments acquired for ^{15}N,^{13}C-labeled Rom in complex with R1inv and R2inv show only the downfield-multiplet component with the second part of the multiplet broadened away due to differential relaxation. With the addition of a J-evolved dimension to the coupled ^{13}C-CT-HSQC, the coupling constants could be readily measured as shown in the corresponding traces of the J-spectra above. For the J-dimension, 32 real increments were collected with a sweep width of 400 Hz. A complex FT was applied to the data after addition of a zeroed imaginary part.

0.1 Hz when compared to the coupling constants measured from the 1D spectrum of resolved amide signals.

To demonstrate the use of the JE-TROSY approach for measuring $^1J_{CH}$ couplings in a relatively large macromolecular complex, the method was applied to an RNA–protein complex involving two RNA hairpins, so-called R1inv and R2inv, and a ^{15}N,^{13}C-labeled protein, Rom, with an overall molecular weight of 28.6 kDa for the complex (Fig. 4a). In a t_1-coupled ^{13}C-CT-HSQC [42] applied to this RNA–protein complex, only the downfield TROSY multiplet component was observed for most of the aromatic protein signals due to the effect of differential relaxation and the long constant time period of $2T = 14$ ms. This made the accurate determination of the one-bond $^1J_{CH}$ coupling constant impossible from the spectrum. To determine these couplings, the ^{13}C-CT-HSQC was expanded into a 3D experiment by incrementing the first INEPT spin-echo period to generate a J-spectrum. Using the JE-TROSY approach, $^1J_{CH}$ coupling constants were readily measured in the J-dimension (Fig. 4b). In this example of ^{1}H–^{13}C correlation of aromatic resonances, use of the $H_x \rightarrow H_yC_z$ coherence transfer period for the J-spectrum provides the most favorable relaxation behavior [4] since the faster relaxing carbon magnetization is kept along the Z-axis.

The measurement of the coupling constants using the JE-TROSY approach can be very accurate as shown by comparison with coupling constants derived from resolved 1D data. With increasing molecular size, however, the signal will relax significantly faster and fewer time increments in the J-dimension can be recorded.

Nonetheless, since the linewidth also increases simultaneously, the peak center in the *J*-spectrum of the JE-TROSY experiment should still be well defined and the accuracy of the coupling measurement remain predominately determined by the signal-to-noise ratio of the acquired data. As presented, both ^{15}N–^{1}H and ^{13}C–^{1}H versions of the JE-TROSY experiment are limited by the relaxation of the proton resonances. Nonetheless, for the systems investigated in this study, including the 28.6 kDa protein–RNA complex, proton relaxation was not a limiting factor.

4. Discussion

The determination of one-bond ^{15}N–^{1}H and ^{13}C–^{1}H couplings in larger macromolecular systems from *J*-coupled spectra is often unreliable due to the significant decrease in the resolution of one of the two components of the cross-peak doublet needed for accurate measurements [27]. The JE-TROSY method presented here for the measurement of one-bond couplings in the presence of significant differential line broadening, involves the direct measurement of the couplings through *J*-evolution of the TROSY multiplet component in an independent dimension. Since the JE-TROSY method is most closely related to *J*-modulated approaches, its advantages and disadvantages are made in comparison to these methods.

In *J*-modulated experiments, additional homonuclear or heteronuclear couplings can lead to serious damping effects and even sign reversal at higher-order zero crossings that might cause misinterpretations of the experimental data. These additional passive couplings, however, can be directly observed and readily decoupled in most cases using the JE-TROSY approach, thus allowing accurate measurement of the one-bond coupling constants even though transverse proton magnetization also evolves during the *J*-evolution period. Moreover, in comparison to *J*-modulated experiments where a number of 2D spectra need to be recorded close to a zero crossing with low signal-to-noise ratios, the JE-TROSY has an inherent signal-to-noise ratio advantage since increments with maximum transfer are included in the processing. A disadvantage of the JE-TROSY approach is the additional measurement time needed as a result of the introduction of an additional frequency dimension. This measurement time, however, is comparable to the time required for the acquisition of the series of experiments required by the original *J*-modulated method. Another limitation is the linewidth in the *J*-spectrum, which does not correspond to the TROSY linewidth but to the linewidth of the decoupled signal.

In comparison with JE-TROSY, *J*-scaled TROSY methods [27] have an advantage in terms of measurement time since *J*-scaling occurs in the same dimension as the heteronuclear chemical shift evolution. However, these methods also require a mixture of TROSY and anti-TROSY multiplet components to generate the frequency displaced component and are therefore limited by the resolution of decoupled cross-peak linewidth in the heteronuclear dimension. In addition, acquisition of an additional decoupled or TROSY reference spectrum to quantify the frequency displacement of the multiplet component is required.

For the JE-TROSY method, accurate measurement using the *J*-spectrum is possible so long as the natural linewidth of the signal of interest is less than half the measured coupling constant. For large one-bond couplings, proton linewidths would have to approach 45 and 75 Hz for $^{1}J_{NH}$ and $^{1}J_{CH}$ couplings, respectively, before this limitation is encountered. In addition, coupling constants can be determined from cross peaks with slightly larger linewidths if extraction methods, like the Keeler–Titman approach, are used. However, if there is a large variation in coupling constant magnitude, a single initial delay $\sim 1/2J_{max}$ will not produce pure in-phase spectra for all signals and thus introduce larger errors. Although the error of the coupling constant measurement increases for linewidths exceeding $J/2$, it can still be estimated using calculated linewidths [43] that the ^{15}N–^{1}H JE-TROSY method is applicable to macromolecules up to a molecular weight of ~ 100 kDa if no additional relaxation mechanisms, like broadening due to chemical exchange, are present.

In addition to its advantage in systems that experience significant differential line broadening, the JE-TROSY approach also allows measurement of one-bond couplings in cases where overlap of multiplets exists. In smaller macromolecules, a case might be considered where the α- (β-) components of two neighboring cross peaks overlap while no overlap is observed for the β- (α-) components. With methods that rely on the displacement of α and β components for measurement of a coupling constant, no accurate measurement is possible. In contrast, the *J*-spectroscopy approach, applied with either a β- or α- spin state selectivity [28,29,33], could be used to select the non-overlapping multiplet components and make possible the detection of the coupling constant.

In summary, the JE-TROSY method is presented for the measurement of one-bond couplings in large macromolecular systems at high magnetic field strengths. As long as the linewidth is less than roughly half the measured coupling, no significant errors are expected in the measurement of coupling constants using this method and for slightly larger linewidths accurate coupling constants should still be measurable using a Keeler–Titman approach. Since only ^{1}H spin operators are in the x,y-plane during the *J*-evolution, the presented experiments are expected to show the greatest sensitivity enhancements in systems where the proton magnetization relaxes more slowly than the directly coupled heteronucleus.

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