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Spinning-rate encoded chemical shift correlations from rotational resonance solid-state NMR experiments

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Abstract

Structural measurements in magic-angle-spinning (MAS) solid-state NMR rely heavily on ^{13}C - ^{13}C distance measurements. Broadbanded recoupling methods are used to generate many cross-peaks, but have complex polarization transfer mechanisms that limit the precision of distance constraints and can suffer from weak intensities for distant peaks due to relaxation, the broad distribution of polarization, as well as dipolar truncation. Frequency-selective methods that feature narrow-banded recoupling can reduce these effects. Indeed, rotational resonance (R^2) experiments have found application in many different biological systems, where they have afforded improved precision and accuracy. Unfortunately, a highly selective transfer mechanism also leads to few cross-peaks in the resulting spectra, which complicates the extraction of multiple constraints. R^2 -width (R^2W) measurements that scan a range of MAS rates to probe the R^2 matching conditions of one or more sites can improve precision, and also permit multiple simultaneous distance measurements. Unfortunately, multidimensional R^2W can be very time-consuming. Here, we present an approach that facilitates the acquisition of 2D-like spectra based on a series of 1D R^2W experiments, by taking advantage of the chemical shift information encoded in the MAS rates where matching occurs. This yields a more time-efficient experiment with many of the benefits of more conventional multidimensional R^2W measurements. The obtained spectra reveal long-distance ^{13}C - ^{13}C cross-peaks resulting from R^2 -mediated polarization transfer. This experiment also enables the efficient setup and targeted implementation of traditional R^2 or R^2W experiments. Analogous applications may extend to other variable-MAS and frequency-selective solid-state NMR experiments.

Introduction

Structural measurements on immobilized proteins by magic angle spinning solid-state NMR (MAS ssNMR) often center on distance measurements, in particular ^{13}C - ^{13}C contacts enabled by a variety of dipolar recoupling methods [1, 2]. Generally one relies heavily on large numbers of approximate distances obtained via broad-banded transfer mechanisms that simultaneously recouple as many interactions as possible. Such methods tend to feature a magnetization transfer pathway that mixes direct transfers and relayed transfers, thus complicating data analysis and limiting the precision of obtained distances. Furthermore,

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these methods often are affected by dipolar truncation, which also limits the distance range that can be observed [3]. One approach to overcome these challenges is to employ band- or frequency-selective experiments that simplify the transfer pathway, improve the reliability and precision of distance constraints, and avoid or reduce dipolar truncation concerns. A subset of, though not all [4–11], practical approaches for band- or frequency-selective ^{13}C - ^{13}C transfer directly rely on rotational resonance (RR, or R^2) recoupling. Here, magnetization transfer occurs specifically as an integer multiple of the MAS frequency matches the frequency difference of the recoupled nuclei, with both the extent of transfer and the MAS-rate-selectivity being dependent on the internuclear distance. Thus, at any particular MAS rate, only a few specific ^{13}C sites are being recoupled, thereby minimizing dipolar truncation and providing a well-defined transfer mechanism. R^2 experiments have been used to measure ^{13}C - ^{13}C distances in various biological systems, ranging from membrane-embedded proteins to amyloid fibrils [12–22]. As it can be very narrow-banded, it is particularly powerful in cases where a highly targeted approach is taken, for instance due to site-specific labeling, to detect selected interactions within a protein or its substrate, or to map out specific interactions within a protein's hydrophobic core [12, 16, 21].

The narrow-bandedness is such that each particular cross peak is only maximally observed at one particular MAS rate. This works well if one targets only one specific internuclear distance, and avoids interference from other contacts. Then, one can measure the cross-peak build-up profile at the 'optimal' MAS rate, as a function of R^2 mixing time, and extract the distance based on this profile. An alternative approach is to determine the off-matching 'width' of the R^2 condition, as it is also dependent on the distance. Such R^2 -width (R^2W) experiments typically use a single mixing time, but vary the spinning rate, in a constant-time approach that further improves the ability to obtain precise distance constraints [23]. One consequence of this changing MAS rate is that different cross-peaks (i.e. internuclear contacts) are being matched throughout the experiment, permitting one to measure multiple distances in one set of experiments. This approach has been applied to a number of different peptides and proteins, often by acquiring an individual 2D spectrum at each of the explored spinning rates [17, 20–22, 24, 25]. Since the matching conditions of most interest (i.e. over long ^{13}C - ^{13}C distances) are also relatively narrow, MAS rate increments have to be quite small (unless the matching condition is purposely 'broadened' [17, 25]). Whilst the narrow-bandedness of the experiment is key to obtain reliable distance measurements, running series of 2D experiments at many different MAS rates gets very time-consuming. This means that it is particularly crucial to elucidate the optimal MAS rates where useful recoupling of sites of structural value (via R^2) occurs, before one commits to the time-consuming pseudo-3D experiment. Long-mixing conventional ^{13}C - ^{13}C experiments (e.g. using proton-driven spin diffusion at lower MAS rates [21], or third-spin-assisted recoupling (TSAR) under faster spinning [26, 27]) can be used to qualitatively identify potential long-distance contacts, but do not necessarily reflect a recoupling mechanism that is directly comparable to the spin physics active during the R^2 experiment (i.e., involving non-relayed transfer pathways).

Inspired by a desire to improve this setup process and help address the time-consuming nature of R^2 -based experiments, we have devised a variable-MAS pseudo-2D experiment that uses constant-time R^2 recoupling as usual in R^2W experiments, but is re-processed into a single reconstructed spectrum that resembles a conventional ^{13}C - ^{13}C 2D. This approach takes advantage of the fact that the MAS rate at which optimal recoupling occurs actually encodes the frequencies of the recoupled nuclei. We show how customized processing of systematically acquired 1D data can be employed to generate 2D ^{13}C - ^{13}C spectral reconstructions that are easily analyzed. The resulting spectrum contains multiple cross-peaks, yet still reflects the well-defined narrow-banded R^2 -based recoupling mechanism and thus features long distance ^{13}C - ^{13}C cross-peaks. The data are acquired in a small fraction of the time it would take to obtain the corresponding series of variable MAS 2D spectra that

contain the analogous cross-peaks and much of the same information. This experiment can serve as an efficient tool to complement and guide more conventional multidimensional R^2W experiments (see e.g. [2, 21]). In addition, these types of reconstructions should be useful in their own right, and also be applicable in combination with other narrow-banded MAS-dependent recoupling methods.

Methods

Sample Preparation

Isotopically $U\text{-}^{13}C$, ^{15}N -labeled N-acetyl-Val-Leu (N-Ac-VL) was obtained from New England Peptide (Gardner, MA), diluted with unlabeled peptide (at a 1:9 molar ratio), and recrystallized as previously described [28, 29]. The T2Q mutant of the model protein GB1 (the 1 immunoglobulin-binding domain of streptococcal protein G) was expressed in *Escherichia coli*, grown on ^{15}N -labeled ammonium chloride and $1,3\text{-}^{13}C$ -labeled glycerol, after which microcrystals were prepared from the isotopically labeled protein, following protocols from previous reports [21, 30, 31]. The obtained crystalline N-Ac-VL and GB1 were each packed into 3.2mm MAS rotors from Bruker Biospin (Billerica, MA).

NMR Experiments

All experiments were performed in the Department of Structural Biology's NMR facility, on a wide-bore 600MHz (1H frequency) spectrometer from Bruker Biospin (Billerica, MA), using a 3.2mm triple-channel HCN Efree MAS probe from Bruker. MAS spinning rates were controlled via a Bruker MAS control unit (MAS II model), and were stable to within less than ± 4 Hz. The temperature was controlled via pre-cooled nitrogen gas at a temperature of $5^\circ C$ (for GB1) or $0^\circ C$ (for N-Ac-VL). The pulse sequence was implemented analogous to previous reports [24, 32], using a selective z-filter initiated by a SNOB excitation pulse [33] to select the CO resonances in N-Ac-VL or the Tyr C resonances in GB1 [21]. High power two-pulse phase modulation (TPPM) decoupling was applied during the 40ms fixed R^2 mixing time, at ~ 99 kHz for the GB1 sample or ~ 83 kHz for N-Ac-VL [34]. The DARR and R^2W comparison experiments (Fig. 4) were done immediately after each other, using 83kHz TPPM decoupling during acquisition, while cooling with $5^\circ C$ gas. The DARR spectra were obtained at a MAS rate of 13kHz. In these R^2W measurements at each MAS rate multiple 1D spectra at different mixing times were acquired, with ~ 90 kHz TPPM 1H decoupling during the R^2 mixing.

Data Processing

The NMR data were processed and reconstructed using customized scripts that control different software utilities within the NMRPipe software package [35]. The reconstruction process is completely based on built-in NMRPipe algorithms. Detailed notes on the reconstruction approach, specific to NMRPipe, are provided in the Supplementary Data that are made available online. The scripts designed to facilitate the processing are available upon request, or downloadable via the internet (<http://www.structbio.pitt.edu/webusers/pvdwel/software>). The reconstructed spectral regions were analyzed, plotted and integrated using the NMRPipe, CCPNMR Analysis, and Sparky software suites [36–38]. Assignments were facilitated by reference to previous work [21, 30] and reference distances were determined based on the respective X-ray crystallographic structures [21, 29, 39].

Results

Acquisition and spectral reconstruction

To demonstrate the protocol and describe its implementation, we initially applied it to the model compound N-Ac-VL, which has a known structure [29]. Isotopically dilute N-Ac-VL

has also served as a reference compound in various related studies [23–25, 32]. We applied a selective z-filter to eliminate all signals except those from the carbonyl groups (ref. Fig 1c). A 40ms longitudinal mixing time, under high power TPPM decoupling, then permits R^2 -mediated polarization transfer for appropriate MAS rates. Under these high-power decoupling conditions, only at or near specific MAS rates is the R^2 condition matched and transfer occurs. Away from these conditions, the 40ms mixing does not produce any cross-peaks (e.g. Fig. 1c), whilst at matched MAS rates efficient and highly selective transfer occurs. The choice of mixing time here reflects our desire to emphasize the longest-distance peaks and ensure a narrow matching condition (which is narrower at longer mixing times [24]), while avoiding the application of unnecessarily long high-power ^1H decoupling to the probe and sample.

To monitor the MAS-dependent transfer and occurrence of cross-peaks we recorded a systematic array of 1D spectra, at MAS rate intervals of 15 Hz. Fig. 1e shows the resulting 1Ds as a pseudo-2D spectrum, with the MAS rate on the y-axis. There are a number of observations that are worth making. First, the systematic shift of aliphatic cross-peaks as a function of MAS rate can be clearly observed, with any particular cross-peak only occurring over a narrow MAS rate range. As previously noted, the width in this dimension is dependent on the decoupling applied during the longitudinal mixing period [25]. In the presence of high-power decoupling, as used here, this width is narrow as long as the ^{13}C - ^{13}C distance is relatively long (i.e., those that are of structural interest [40]). For short distances, in particular one-bond contacts, the R^2 condition is much broader, and the R^2 matching leads to significant peak distortions. This is visible in Fig. 1e for the CO-C cross-peaks near a 9kHz MAS rate.

As we examine in more detail below, the combined MAS rate and observed peak position (i.e. the aliphatic ^{13}C chemical shift) of a particular cross-peak actually encode the frequency of the corresponding ‘source nucleus’ (in this case a carbonyl group). This implies that, in principle, the patterns of observed cross-peaks in 1D data should suffice to deduce the same information contained in typical 2D spectra, without the need to acquire series of 2D spectra across numerous MAS rates. We examine our implementation for a practical automation of this process on the basis of the data shown in Figure 1 and 2. For this process to work best, the linewidth in the MAS-rate dimension needs to be relatively narrow, to minimize peak overlap. Thus one-bond (CO-C) cross-peaks should be avoided, but there is little structural information of value in those cross-peaks anyway [40]. Here, we focus on the CH_2/CH_3 region (Fig. 2a) of the pseudo-2D shown in Fig. 1e. As noted above, the cross-peaks occur when the MAS rate R matches half the frequency difference between the ‘source’ carbon C1 (C_1) and the observed carbon C2 (C_2): $2 \times \omega_R = \Delta\omega = \omega_{C_2} - \omega_{C_1}$. Thus, whenever a transfer optimum occurs in these 1D spectra, this condition must be matched and one should be able to extract the CO frequency based on: $\omega_{CO} = 2\omega_R - \omega_{obs}$ (where ω_{obs} is the frequency of the observed peak in the 1D spectrum). Although this in principle allows identification of the matched nuclear pairs, doing so manually is time-consuming, in particular for uniformly or extensively labeled proteins.

Fortunately, this analysis can be emulated by an automated process that centers around the application of a shearing operation to the spectrum. During shearing, each vertical slice in the spectrum is vertically shifted to an extent that depends linearly on the X-axis position of the slice: $\Delta\omega_{shear} = C_{shear} \times \omega_{obs}$. After shearing the new Y-axis frequency ($\omega_{Y,sheared}$) is given by $\omega_{Y,sheared} = \omega_{Y,orig} + C_{shear} \times \omega_{obs}$, which conveniently parallels the equation correlating the R^2 -matched C1 and C2 ^{13}C frequencies (see above). Thus, when $\omega_{Y,orig} = 2\omega_R$ and $C_{shear} = -1$, the shearing equation becomes $\omega_{Y,sheared} = 2\omega_R - \omega_{obs} = 2\omega_{CO}$. In other words, Y-axis frequencies are automatically translated into the chemical shift of the ‘source’ carbon (here the COs), as is the case in conventional 2D spectra.

Initially, in Fig. 1e, γ_{orig} is set to the MAS rate of each to the 1D spectra in the pseudo-2D. To attain the $\gamma_{\text{orig}} = 2 \text{ R}$ prerequisite, we simply scale the y-axis by the R^2 matching coefficient (here $n=2$), as shown in Fig. 2a. This then sets the stage for the shearing operation itself, using $C_{\text{shear}} = -1$, which transforms the Y-axis into the appropriate frequency range such that $\gamma_{\text{sheared}} = \text{CO}$. This whole process is conveniently done via built-in NMRPipe functions, and its outcome is shown in Fig. 2b. Some care has to be taken in the automating and executing this shearing process due to its algorithmic reliance (in the NMRPipe code) on spectral points, rather than frequencies (see the Supplemental Data for more details).

The resulting 2D spectrum is shown in Fig. 2c, with the Y-axis expressed in ^{13}C chemical shift equivalents (in ppm). Indeed, as expected the cross-peaks now occur at the frequencies of the CO polarization sources. Note that the conversion of MAS frequency to the appropriate nuclear frequencies is fully predictable and can thus be automated, without need for manual intervention or re-calibration. Furthermore, the implementation of the shearing and processing code is designed to be as general as possible, and accommodates other R^2 matching conditions and arbitrary MAS rate increments.

Application to microcrystalline GB1

To further evaluate the utility of this approach, we also applied it to microcrystals of the model protein GB1. In particular we explored the same aromatic-methyl contacts in its hydrophobic core that were previously studied through R^2W experiments based on a series of 2D spectra, which took over a week of acquisition time [21]. Here, we used an analogous experimental setup as used for the N-Ac-VL sample, except that for these experiments the selective z-filter was configured to select the Tyr C sites. A series of 1D spectra were obtained at MAS rates ranging from ~10 to 11 kHz. In this MAS regime, the $n=2 \text{ R}^2$ matching condition is met for transfer from the different Tyr C s to a range of aliphatic carbons (predominantly methyl groups), mapping out much of the hydrophobic core (Fig. 3e). Processing the resulting data using the same processing scripts used above for N-Ac-VL, we obtained the 2D panel shown in Fig. 3d, reminiscent of a conventional 2D ^{13}C - ^{13}C spectrum but with each apparent cross-peak being the result of very narrow-banded R^2 -mediated polarization transfer. Assignments were identified based on our earlier R^2W experiments on this system and reference crystal structures [21, 39]. As before, our current R^2W data reveal a number of long distance cross-peaks between the Tyr side chains and methyl-containing side chains (up to ~7 Å [39]). This includes the same contacts probed by R^2W before [21]. However, as a direct consequence of the substantial time-savings produced by recording 1D rather than 2D data at each MAS rate, we now were able to scan an increased range of MAS rates and observe additional ^{13}C - ^{13}C pairs.

Distance dependence

In the R^2W data obtained in Fig. 2 and Fig. 3, we observe ^{13}C - ^{13}C distances up to ~7 Å, consistent with previous applications of R^2W to these two model systems [21, 24]. As noted, in such experiments both the peak intensity and the width of the matching condition are sensitive to the internuclear distance [23]. To compare these distance dependencies to the distance-dependent cross-peak buildup seen in DARR spectra, we performed several DARR 2D acquisitions with different mixing times and a 1D-based R^2W experiment in which we varied both the MAS rate and mixing time. These measurements were performed on the same sample and under identical conditions to allow the most direct comparison. First, we compared the peak volumes of corresponding cross-peaks seen in both experiments, normalized to the corresponding zero-mixing CO source peak volume (Fig. 4 and Table S1). With a sufficiently long mixing time, both approaches provide transfer over long ^{13}C - ^{13}C distances (up to a distance of ~6.5 Å [29]). However, there are two notable differences. First,

the individual peak volumes as a fraction of the source peak volume are significantly higher for the R^2 -based transfers. Second, the correlation between cross-peak volume and distance is found to be more systematic in the R^2 -based data, consistent with the cleaner, non-relayed, non-truncated polarization transfer mechanism. Fig. 4c also shows that in the R^2W data, it is not only the peak volume, but also the matching condition width (in terms of MAS rate frequencies where matching occurs) that correlates to the distance. Although this correlation is less sensitive to distances longer than $\sim 4\text{\AA}$ [23, 24], in combination, these dependencies allow for the extraction of ^{13}C - ^{13}C distances with good precision and accuracy. These observations are not necessarily surprising, as they are consistent with earlier analyses on this model system, and the underlying data used for the reconstruction approach are the same as employed in previous work [23, 24]. For more in-depth and quantitative discussion of distance-measurements and data analysis (including effects of CSA orientations and dynamics) we refer to those reports. Briefly, depending on the specific conditions, a detailed quantitative analysis may need to account for the influence and orientation of the CSA and relaxation, but also for variations among crystal structures, localized dynamics, and isotopic labeling patterns [21].

Discussion

R^2 experiments permit one to extract precise ^{13}C - ^{13}C distance measurements and have found productive applications in numerous biological contexts. However, as MAS ssNMR is increasingly applied to extensively or uniformly labeled samples, there has been a growing reliance on larger numbers of less precise measurements rather than the use of R^2 and related frequency-selective approaches. One concern for narrow-banded experiments is that they can be rather time-consuming when applied to measure multiple distances, as a side effect of their site-selectivity. Approaches to increase the efficiency with which these experiments can be executed are therefore desirable [17, 25]. Realizing that the MAS-rates at which the recoupling occurs encode frequency information on the recoupled nuclei, we here described a reconstruction method relying purely on 1D data obtained under narrow-banded R^2 recoupling conditions.

We have shown application of this approach to two model samples that have been previously used to demonstrate and test canonical R^2W experiments. In detail we described its application to the commonly used N-Ac-VL peptide sample (Fig. 1 & Fig. 2). We acquired a systematic set of 1D data over a range of MAS rates, and developed automated scripts that parse the data and (without Fourier transformation of the indirect dimension) use the MAS rate frequencies to extract the chemical shift resonances of the recoupled nuclei. Indeed, we successfully used these scripts to generate a pseudo-2D spectral reconstruction that facilitates the convenient identification of the recoupled nuclei (Fig. 2). These data can simply be treated as a 'normal' 2D spectrum for assignments and data analysis, avoiding the need for any manual calculations. Of course, in contrast to conventional spectra obtained by Fourier transformation of the indirect dimension, the line-width in the indirect dimension actually reflects the width of the R^2 condition. As shown in Fig. 4, both this resonance width and the peak intensities correlate to the internuclear distance [23, 24]. Our approach proved similarly effective with 1D R^2W data acquired on the microcrystalline protein GB1 (Fig. 3). In both test cases, cross-peaks were observed for ^{13}C pairs up to $6\text{--}7\text{\AA}$ apart in the respective X-ray structures. The observance of slightly longer distances in GB1 probably indicate that the maximum distance seen in the VL case is not limited by the spin dynamics, but rather by the size of the peptide model system. We reiterate that the underlying R^2W data obtained here are analogous to data previously reported [21, 24].

Although our aim here is to describe the reconstruction method, rather than repeat the quantitative analysis of these data, it is worth examining the usage of these kinds of

experiments. For the most precise data analysis, one needs to perform peak fitting as a function of MAS rate, with proper deconvolution of overlapping peaks being crucial for the optimum extraction of distances (see e.g. [21]). The approach delineated here is substantially faster than the acquisition of corresponding 2D data, but it may increase concerns of peak overlap. For longer distances, which are of most structural interest[40], the high selectivity of the recoupling process (under the high-power decoupling and longer mixing times applied here) helps to reduce cross-peak overlap. In those cases where the overlap is still significant, and unequivocal assignments are difficult, one may gain additional resolution by incorporation of additional magnetization transfer steps into the pulse sequence design [15, 17]. Furthermore, for proper calibration of the extent of transfer, at least some reference data with no longitudinal mixing are typically required. The use of 1D data makes the timely acquisition of additional (zero and non-zero) mixing times much more feasible (e.g. the data in Fig. 4a). Thus, our approach should preserve the beneficial distance-dependence of R^2W measurements, while making them more practical in their time constraints. This in turn facilitates more extensive variations in both MAS rates and mixing times. Since there is no need to decouple during the (missing) t_1 evolution, a reliance on 1D data may also permit longer R^2 mixing times (under high-power decoupling), without increasing the maximum power input into the probe.

A key consideration that drove us to investigate these experiments was the time requirement of traditional multidimensional R^2W experiments, which has limited their application despite the demonstrated precision, accuracy and ability to probe long distances. In 3D R^2W experiments, an entire 2D spectrum is acquired at each explored MAS rate. In practice, this implies multiple hours of acquisition time for each of these datasets, leading to a multiday experiment for any appreciable range of MAS rates. For instance, this meant ~5 hours per MAS rate in an earlier study [21] on a crystalline GB1 sample at high field (750MHz 1H frequency). In contrast, the R^2W data discussed here were acquired in 21 and 30 hours, for the N-Ac-VL and GB1 samples, respectively. Exploring these same MAS rates via 2D spectra would require more than a week of spectrometer time, without a corresponding gain in information. Clearly, unless other time-saving measures are implemented (e.g. via broadening of the R^2 condition), such experiments necessitate a well-thought-out setup and experimental design. We believe that the methods delineated here will help facilitate this in a practical and time-effective manner.

Features of the reconstructed spectra

Although the reconstructions are reminiscent of ‘normal’ 2D spectra, it is important to point out some key differences. First, as indicated above, the peak width in the indirect dimension reflects the width of the recoupling condition. This provides distance information used in the quantitative analysis of R^2W data (Fig. 4). However, it can also make for peak-shapes that may be harder to integrate using typical NMR software. Second, in contrast to normal 2Ds we obtained only one set of cross peaks reflecting transfer from CO/Tyr to aliphatic sites, and not the reverse. This is a consequence of the use of a selective pulse in the pulse sequence, which eliminates all other ‘source’ peaks, and would be not be as suitable for uniform excitation of the aliphatic resonances. Another key difference has to do with the relative sensitivity or signal-to-noise (S/N) of the overall data set compared to that of individual 1D data. For typical Fourier-transformation based 2D spectra, when a 1D and a 2D spectrum are acquired over the same amount of overall time, the relative sensitivity of a (normal) 2D spectrum compared to the 1D [41], is given by:

$$\frac{sens_{2D}}{sens_{1D}} = \sqrt{\frac{1}{t_1^{\max}} \int_0^{t_1^{\max}} s_1^e(t_1)^2 dt_1} \quad (1)$$

Here $s_1(t_1)$ is the signal intensity in an individual (1D) FID, as a function of the evolution time t_1 . In the 2D spectrum the S/N increases with the addition of additional FIDs for the various evolution times. However, there is a relative loss of sensitivity compared to an equivalent 1D spectrum as a consequence of reduced signal intensity (s_1) in successive 1D FIDs as the evolution time t_1 increases (reflecting transverse relaxation T_2). Although this could suggest that our 1D-based reconstruction would thus necessarily be 'better', as there is no such t_1 -related relaxation, this 2D 'spectrum' does not reflect a summation of the underlying 1D data, but rather a concatenation of the individual spectra. Thus, although there is no T_2 relaxation during evolution, there is also no additive effect of the acquisition of additional 1Ds: if we consider a particular cross-peak (e.g. reflecting a crucial distance constraint in the hydrophobic core [21]), then its apparent S/N in the reconstruction should be the same as the S/N in the 1D that happens to feature the peak maximum. None of the other 1D slices acquired will contribute to that peak maximum. Instead, in a normal 2D spectrum, the S/N of a particular cross peak finds its origin in the summed intensity across all acquired 1Ds (accounting for relaxation losses as indicated above).

If one acquires a normal 2D and reconstructed 2D in the same time (i.e., from N_{total} scans), and considers how the above affects the apparent S/N for the cross-peak in question, then the following should apply. The reconstruction consists of $N_{\text{total}} = n_{\text{mas}} \times n_{1D}$ scans, whereas the normal 2D is acquired from $N_{\text{total}} = M_1 \times n_{\text{scan}}$. Here, n_{mas} is the number of MAS rates explored in the R^2W experiment, with n_{1D} scans acquired at each point; M_1 and n_{scan} are the number of t_1 points and number of scans per t_1 point, respectively. Taking into account the number of scans contributing to the specific peak's intensity relative to the noise $((S/N)_{\text{pk}})$ for both cases, we find:

$$\left(\frac{S}{N}\right)_{\text{pk, recon}} / \left(\frac{S}{N}\right)_{\text{pk, 2D}} = \frac{\sqrt{n_{1D}}}{\sqrt{n_{\text{scan}} M_1}} / \sqrt{\frac{1}{t_1^{\text{max}}} \int_0^{t_1^{\text{max}}} s_1^e(t_1)^2 dt_1} \quad (2)$$

This ratio reflects the relative likelihood to be able to distinguish a particular peak from the noise (rather than the overall information density of the spectrum). The ratio depends on the T_2 relaxation, choice of maximum t_1 evolution time, and number of MAS rates probed, leading to values ranging from 0.3 to around unity, for a various combinations of these parameters (including the experimental conditions employed here). This would thus indicate that by this simple measure, regular 2D data are as good or somewhat better in terms of recovering a specific cross peak out of the noise.

Comparison to other experiments

The above analysis showed that although there are definite time-savings over previously used 3D R^2W experiments featuring many 2D spectra, the ability to detect a particular cross-peak in these reconstructed spectra may well be inferior to spending the same time acquiring a 'normal' 2D experiment. Furthermore, broadbanded spectra (e.g. using DARR mixing) also tend to include many additional peaks, which further add to their *overall* S/N or information content. These observations indicate that in some applications, DARR spectra are the preferred way to obtain a large number of structural constraints. Instead, frequency-selective methods like R^2 are preferred in cases where one desires a higher level of precision and accuracy in the measurements of a *defined* set of ^{13}C - ^{13}C constraints. In this case, the tendency of broadbanded long-mixing 2D experiments to distribute magnetization over many additional sites may actually not be preferable. Furthermore, for many of the broadband techniques the magnetization losses *during mixing* are likely to be noticeably more severe than during the longitudinal R^2 mixing under optimized high-power decoupling. Indeed, the normalized cross-peak intensities in DARR data were significantly lower than

those where transfer occurs via frequency selective R^2 (Fig. 4). Moreover, the relayed polarization transfer seen in DARR (and other) experiments, also obfuscates the correlation between peak intensity and internuclear distance (Fig. 4b). Thus, frequency selective and broad-banded recoupling methods serve complementary purposes, and their suitability has to be evaluated on a case-by-case basis.

As with other R^2 -based experiments, the nuclei that can practically be recoupled are dictated by the accessible MAS rates, which usually imply a minimum chemical shift difference. Although there have been some cases where R^2 experiments were used to measure inter-aliphatic distances [14, 16, 42], it usually means the recoupling of interactions between carbonyl and aliphatic sites [13–20, 22, 42–44] or between aromatic and aliphatic sites [5, 18, 21]. Compared to broadbanded spectra, such frequency-selective experiments by necessity feature fewer peaks, and thus ‘information’. Nonetheless, there are a number of observations to be made. First, in many cases the useful information of a 2D spectrum is contained in the cross-peaks, and often even in a specific subset of (long-distance) cross-peaks [40, 45]. The scenarios in which R^2 -based experiments (including the experiment described herein) would be optimally employed, are in order to detect a desired set of long-distance contacts, rather than a large collection of cross-peaks, most of which would be short- to medium-range. Moreover, a key feature of $R^2(W)$ experiments is the ability to not only *detect* long ^{13}C - ^{13}C distances, but also to measure them with good accuracy and precision—facilitated by the well-defined polarization transfer pathway (Fig. 4).

Conclusions

R^2 -based methods have the potential for providing valuable long-distance ^{13}C - ^{13}C constraints, with good accuracy and precision, but they have also been particularly time-consuming to apply to *multiple* distance constraints. In such applications, one would acquire numerous multidimensional spectra at different MAS rates, many of which would feature very few (if any) informative cross-peaks. Here we make use of the frequency-information encoded in the matching condition, which allowed us to use judicious processing of 1D data to yield 2D spectral reconstructions that can be analyzed as conventional 2Ds, in the NMR analysis software of choice. Thanks to the frequency-selective transfer process, in these spectra the effects of dipolar truncation are largely avoided and long-range distances are observed. Moreover, as expected for this frequency selective recoupling method, this generates intense cross-peaks over long distances, with a clear distance dependence. This approach partly ameliorates one inherent disadvantage of frequency selective polarization transfer: the low information density of any particular spectrum. This caveat is to still present, but is less of a practical concern here due to the reduced time-demands of relying on 1D instead of 2D data. The obtained data can be analyzed for extracting distance constraints, or act as a precursor to multidimensional R^2W experiments. In the latter case, this experiment efficiently provides crucial reference data that reliably reflect the polarization transfer behavior one can anticipate in the 3D R^2W experiment. We expect that analogous processing may be of use in a variety of other MAS-rate dependent experiments, in particular for applications featuring similarly narrow-banded recoupling [23, 46–49] as well as in future applications of swept or variable MAS rates enabled by ongoing development in MAS NMR instrumentation [48, 49].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Rotational resonance width experiments were performed across a range of MAS rates.

Matched MAS rates encode the resonance frequencies of recoupled nuclei.

We describe a protocol that reconstructs 2D data from variable MAS 1D spectra.

Application of this protocol to peptide and protein samples is demonstrated.

Substantial time savings versus normal variable-MAS multidimensional experiments.

Fig. 1. One-dimensional R^2W data on the N-Ac-VL sample. (a-d) Selected 1Ds acquired at different MAS rates, as indicated. In absence of R^2 matching (e.g. in c), only the carbonyl signals are present (obtained via a selective z-filter). Asterisks indicate spinning side-bands. (e) Systematic array of 1D spectra acquired between 12.5 and 8.315 kHz MAS, at equidistant intervals of 15 Hz, as a pseudo-2D. The vertical axis shows the MAS rate employed. The slanted lines near the middle reflect $n=1$ spinning side-bands, also marked with asterisks in (a-d). Cross-peaks in the aliphatic region occur at selective MAS rates, reflecting $n=2$ R^2 matching conditions. The arrows at the far right indicate the location of the individual 1Ds highlighted in panels a-d. The dashed box indicates the spectral region shown in Fig. 2a.

Fig. 2.

Spectral reconstruction protocol. (a) The aliphatic region with longer-distance cross-peaks is extracted, featuring peaks of interest, minimal peak distortion and relatively narrow matching conditions. The vertical axis is multiplied by the R^2 matching condition (here $n=2$). (b) Based on the $n=2$ R^2 matching and the linear correlation between MAS rate and chemical shift difference of the two nuclei contributing to each peak, a shearing operation is applied as described in the text. (c) Finally, the y-axis of the resulting sheared spectrum is recalculated to reflect the chemical shift of the corresponding polarization source on the Y-axis, analogous to conventional 2D spectra.

Fig. 3. Application to GB1 microcrystals, targeting aromatic-methyl contacts in the hydrophobic core. (a-c) Selected 1Ds from a set of 40 1D spectra obtained over a MAS rate range of 11.0 down to 10.025 kHz. (d) 2D spectral region reconstructed from the 1D data. Long-distance cross-peaks between the Tyr C resonances and various methyl groups are indicated in the spectrum. Peak maxima visible in the 1Ds in (a-c) are marked accordingly in (d). (e) Observed contacts in GB1's hydrophobic core are shown in the 3D structure, in views rotated by 90° [21, 30].

Fig. 4.

Distance dependence. (a) Peak maxima in N-Ac-VL R^2W data plotted against the corresponding distance in the X-ray structure [29], for R^2 mixing times of 10 ($\diamond$), 20 ($\square$), 30 ($\triangle$), and 40ms ($\times$). (b) Cross-peak volumes in DARR spectra obtained at 13kHz MAS, with 25 ($\diamond$), 50 ($\square$), 100 ($\triangle$), and 250ms ($\times$) mixing. In (a) and (b) peak intensities are normalized relative to the corresponding zero-mixing carbonyl peak intensity. (c) Width of the R^2W resonance condition in the N-Ac-VL data (Fig. 2), plotted against the X-ray distance.