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3D $^{15}N/^{15}N/^{1}H$ Chemical Shift Correlation Experiment Utilizing an RFDR-based $^{1}H/^{1}H$ Mixing Period at 100 kHz MAS

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Abstract

Homonuclear correlation NMR experiments are commonly used in the high-resolution structural studies of proteins. While $^{13}C/^{13}C$ chemical shift correlation experiments utilizing dipolar recoupling techniques are fully utilized under MAS, correlation of the chemical shifts of ^{15}N nuclei in proteins has been a challenge. Previous studies have shown that the negligible ^{15}N-^{15}N dipolar coupling in peptides or proteins necessitates the use of a very long mixing time (typically several seconds) for effective spin diffusion to occur and considerably slows down a $^{15}N/^{15}N$ correlation experiment. In this study, we show that the use of mixing proton magnetization, instead of ^{15}N, via the recoupled ^{1}H-^{1}H dipolar couplings enable faster $^{15}N/^{15}N$ correlation. In addition, the use of proton-detection under ultrafast MAS overcomes the sensitivity loss due to multiple magnetization transfer (between ^{1}H and ^{15}N nuclei) steps. In fact, less than 300 nL (~1.1 micromole quantity) sample is sufficient to acquire the 3D spectrum within 5 hours. Our results also demonstrate that a 3D $^{15}N/^{15}N/^{1}H$ experiment can render higher resolution spectra that will be useful in the structural studies of proteins at ultrafast MAS frequencies. 3D $^{15}N/^{15}N/^{1}H$ and 2D radio frequency-driven dipolar recoupling (RFDR)-based $^{1}H/^{1}H$ experimental results obtained from a powder sample of N-acetyla-L-^{15}N-valyl-L-^{15}N-leucine at 70 and 100 kHz MAS frequencies are presented.

Keywords

solid-state NMR; ultrafast MAS; proton-detection; RFDR; peptide

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Supporting Information Available

High-resolution 2D $^{1}H/^{1}H$ chemical shift correlation spectra of a powder sample of NAVL obtained at different RFDR mixing times under ultrafast MAS conditions.

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Introduction

Magic angle spinning (MAS) solid-state NMR spectroscopy has become an essential tool to obtain atomic-level structural and dynamic insights into folding/misfolding, aggregation, membrane interaction, ligand binding, and function of a variety of proteins.[1-8] In most such studies, like in a solution NMR approach, homonuclear chemical shift correlation is commonly used to assign peaks from multidimensional spectra of proteins.[9-12] While correlation of the chemical shifts of ^{13}C nuclei is easily accomplished using dipolar recoupling techniques,[13-16] correlation of ^{15}N chemical shifts in peptides and proteins has not been an easy task as the coherent dipolar coupling between ^{15}N nuclei in a protein is very small.[17] In fact, the ^{15}N-^{15}N distance in a peptide backbone is conformation dependent: ~2.8 Å in an α-helix with a ^{15}N-^{15}N dipolar coupling of ~56 Hz and ~3.5 Å in a β-strand with a ^{15}N-^{15}N dipolar coupling of ~29 Hz. Though it is possible to use spin diffusion to accomplish this task, the required very long mixing time - typically on the order of seconds – considerably reduces the sensitivity of the technique due to spin-lattice (T_1) relaxation of ^{15}N.[17-19] Previous studies on static, oriented solids and MAS studies demonstrated that it is possible to shorten the very long mixing time using proton spin diffusion and also using proton-assisted approaches.[17, 20-23] Another study demonstrated the use of mixing proton magnetization to speed up the spin-diffusion process in static solids.[24] . A recent study demonstrated an approach ^{15}N-BARE (Backbone REcoupling) that utilizes fpRFDR blocks to recouple ^{15}N-^{15}N dipolar couplings, and reported the use of the experimentally measured ^{15}N-^{15}N and ^{13}C-^{13}C dipolar couplings as restraints to improve the precision of the 3D structure of microcrystalline GB1 protein.[25]

In this study, we demonstrate a new proton-detected 3D experiment to overcome these difficulties – particularly at ultrafast (70 and 100 kHz) MAS frequencies. As shown in the 3D pulse sequence (Figure 1C), the ^{1}H transverse magnetization is transferred to ^{15}N nuclei by the first ramp-cross-polarization (ramp-CP) sequence [26] and then ^{15}N chemical shift is expressed in the t_1 period. The second ramp-CP sequence transfers the ^{15}N magnetization back to ^{1}H nuclei and the *z*-magnetization is allowed to spin-diffuse through the recoupled ^{1}H-^{1}H dipolar couplings. The ^{1}H magnetization is then transferred to ^{15}N nuclei by the third ramp-CP sequence to evolve under the ^{15}N chemical shift in the t_2 period. Then, the ^{15}N magnetization is transferred to ^{1}H magnetization by the fourth ramp-CP sequence. Finally, the ^{1}H NMR spectrum is acquired in the t_3 period. This method suffers from the magnetization loss during the four ramp-CP-based magnetization transfers between ^{1}H and ^{15}N nuclei. However, the ultrafast MAS, which has been demonstrated to be feasible to perform experiments up to spinning speed of 110 kHz [30-33], suppresses ^{1}H-^{1}H homonuclear dipolar interaction leading to narrow ^{1}H spectral lines and enhances both sensitivity and resolution even though a very small amount of sample (<300 nL containing about 0.3 mg sample) is used. Although ultrafast MAS suppresses ^{1}H-^{1}H spin diffusion, RF driven ^{1}H-^{1}H zero-quantum recoupling by the finite-pulse-RFDR (radio frequency driven dipolar recoupling) pulse sequence ensures a rapid spin diffusion process in the mixing period of the pulse sequence.[34-38] The efficiency of this method is experimentally demonstrated on a powder sample of N-acetyl-^{15}N-L-valyl-^{15}N-L-leucine (NAVL).

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Experimental

All NMR experiments were performed on a 600 MHz JNM-ECA600II solid-state NMR spectrometer equipped using a 0.75 mm ultrafast MAS probe (JEOL RESONANCE Inc.). NAVL was prepared as explained elsewhere.[19] Samples were packed in a 0.75 mm zirconia rotor and all experiments were performed at room temperature. About 1.1 micromole sample was packed in the 0.75 mm MAS rotor (volume of 290 nL). The pulse sequence used to obtain homonuclear correlation spectra are shown in Figure 1. We also applied finite-pulse RFDR pulse sequence with the $XY4^1_4$ phase cycling during the mixing time to recouple zero-quantum dipolar interactions as shown in Figure 1.[38] We also utilized the RFDR pulse sequence to reduce the repetition delay as explained below. Since the ^{1}H-^{1}H spin diffusion is highly suppressed under ultrafast MAS condition, the T_1 relaxation times of protons in NAVL are not uniform and varies from 0.98 to 8.3 s at 100 kHz MAS. The amide-protons have a T_1 relaxation time of 4.5 s. Therefore, we normally need to provide a repetition delay of 5 to 6 s between successive scans to avoid signal saturation, however it can be shortened by applying the RFDR pulse sequence in the proton channel to recouple ^{1}H-^{1}H dipolar couplings during the repetition delay.[33] We have applied six RFDR trains with the $XY4^1_4$ phase cycling in which each RFDR train consists of 640 π pulses. This approach successfully reduced the required repetition delay to 2 s. The RFDR condition during the repetition delay was optimized to maximize the signal-to-noise (*S/N*) ratio per unit time for amide protons. The *S/N* of amide protons was improved by a factor of two if we compare the signal intensities observed with a repetition delay of 2 s and with/without RFDR. The πpulse durations used in the RFDR sequence were 1.6 μs for ^{1}H and 6.5 μs for ^{15}N. All other experimental conditions used in this study are given in the figure caption.

Results and Discussion

We have chosen NAVL as a model system to demonstrate the new solid-state NMR approach presented in this study. By spinning the powder sample at an ultrafast MAS frequency, all line broadening interactions - including the dipolar couplings between protons - are averaged out. As a result, a very high-resolution ^{1}H chemical shift of NAVL is obtained (Figure 2A); spectral resolution achieved in this study is higher than that reported in a previous study on a uniformly-deuterated NAVL.[39] Then, we performed a 2D ^{1}H/^{1}H chemical shift correlation experiment using the pulse sequence given in Figure 1A. By using the finite-pulse RFDR pulse sequence with an efficient $XY4^1_4$ phase cycling to recouple ^{1}H-^{1}H dipolar couplings during the mixing time, an excellent 2D ^{1}H/^{1}H single-quantum correlation spectrum of NAVL was obtained as shown in Figure 2B. In spite of a very small amount of sample (290 nL) used for this experiment, the entire 2D ^{1}H/^{1}H spectrum was easily collected within 8.5 minutes by well utilizing the ultrafast MAS condition and proton-detection approach. The 2D spectrum of NAVL shown in Figure 2 is remarkable in that it is of a very high quality with excellent spectral resolution and a mixing time of 6.4 ms is sufficient to obtain total correlation of all proton resonances in the molecule; a series of ^{1}H/^{1}H 2D chemical shift correlation spectra of NAVL obtained at different mixing times given in Figure S1 (in the supporting information) indicate the fast spin diffusion process via the recoupled ^{1}H-^{1}H dipolar couplings. These results demonstrate

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the ability of the finite-pulse-RFDR-XY4$^1_4$ pulse sequence to efficiently recover ^{1}H-^{1}H dipolar couplings under ultrafast MAS conditions. We very recently reported a comprehensive analysis of the performance of XY-phase cycling based fp-RFDR for 2D ^{1}H/^{1}H chemical shift correlation experiments under ultrafast MAS conditions.[38] Even though the 2D ^{1}H/^{1}H spectrum shown in Figure 2B is well resolved, the resonances from two different amide-protons are not resolved (Figure 2C); spectra obtained at different mixing times are given in Figures S1 and S2 (in the supporting information). Reason for this could be the isotropic chemical shifts of amide-protons of Val and Leu residues in NAVL are quite similar within the achieved spectral resolution.

We then performed a proton-detected 3D ^{15}N/^{15}N/^{1}H experiment on the NAVL sample to quickly obtain the 2D ^{15}N/^{15}N chemical shift correlation spectrum using the pulse sequence given in Figure 1B. This pulse sequence utilized the XY4$^1_4$ phase cycling based finite-pulse RFDR pulse sequence to recouple ^{15}N-^{15}N dipolar couplings in the mixing time. However, we observed no cross peaks in the resultant spectrum as shown in Figure 3. The ^{15}N chemical shift values are in agreement with previous studies on NAVL.[19] The absence of cross peaks confirmed that there is no significant dipolar coupling between ^{15}N nuclei in NAVL, which is in complete agreement with previous studies.[17-19] These results further confirmed that the recoupling pulse sequences are not effective in achieving chemical shift correlation of ^{15}N nuclei – this is unlike the successful use of recoupling techniques to correlate the chemical shifts of ^{13}C nuclei in peptides and proteins. It may be noted that we recently demonstrated that XY4$^1_4$ phase cycling based finite-pulse-RFDR pulse sequence provides an optimum performance under various experimental conditions.[38]

To accomplish ^{15}N/^{15}N chemical shift correlation, we implemented a new 3D ^{15}N/^{15}N/^{1}H pulse sequence that is shown in Figure 1C. As mentioned earlier, this proton-detected 3D pulse sequence expresses the chemical shift of ^{15}N nuclei in the incrementable t_1 period, transfers the ^{15}N transverse magnetization to protons via CP, utilizes the finite-pulse RFDR with an efficient XY4$^1_4$ phase cycling to recouple the ^{1}H-^{1}H dipolar couplings during the mixing time, transfers the ^{1}H transverse magnetization to ^{15}N via CP, expresses the chemical shift of ^{15}N nuclei in the incrementable t_2 period, then finally transfers the ^{15}N transverse magnetization via cross-polarization to protons for detection. It is highly impressive that any loss of magnetization due to several magnetization transfer steps, between the ^{1}H and ^{15}N channels, in the sequence, is well compensated by the higher sensitivity gained by the proton-detection approach. In fact, 31% of the original signal intensity remains after the first two CP periods of the pulse sequence. Assuming the same transfer magnetization efficiency, there will be 10% of the original signal intensity before signal acquisition (i.e., after the four CP periods of the 3D pulse sequence). The ^{1}H-^{1}H spin diffusion process could further reduce the magnetization. Therefore, our experimental results show a survival of about 1.6% of the original signal intensity for detection. Most importantly, this amount of signal is strong enough to measure the 3D spectrum obtained with 8 scans in less than 5 hrs. The 2D ^{15}N/^{15}N correlation spectrum of NAVL obtained at 100 kHz using this 3D sequence is given in Figure 4. It is remarkable that the 2D spectrum clearly reveals the cross peaks and the connectivity of ^{15}N nuclei in NAVL. Unlike the long spin diffusion based mixing (typically several seconds), our experiment needed only 6.4 ms

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to accomplish the correlation of ^{15}N nuclei. In fact, a mixing time 3.2 ms was sufficient to obtain the 2D spectrum but the observed cross peak intensity is smaller than that obtained using a 6.4 ms mixing time. This 3D experiment was also performed at 70 kHz MAS and the resultant 2D ^{15}N/^{15}N spectrum is given in Figure S3. It is remarkable that the 2D ^{15}N/^{15}N spectra obtained at two different spinning speeds, 70 and 100 kHz, are very similar. This observation suggests that the proposed 3D ^{15}N/^{15}N/^{1}H pulse sequence could also be useful for studies at a lower spinning speed (like 70 kHz) with an added advantage of utilizing a large MAS rotor (1 or 1.3 mm) with a relatively more sample volume (1 to 3 μL). While performing this experiment at 70 kHz could potentially beneficial for samples like proteins, proton spectral resolution could depend on the nature of the sample. For example, experiments performed on a highly homogeneous sample at 100 kHz or higher MAS frequencies could provide a better proton spectral resolution and therefore sensitivity than that obtained with a lower spinning speed (for example, 70 kHz).

These results demonstrate the power of ultrafast MAS, proton detection, and RFDR-based ^{1}H-^{1}H dipolar recoupling utilized in this new method. The correlation in the new method is based on the spin diffusion driven by the coherent ^{1}H-^{1}H dipolar couplings; thus, this method is not restricted by the homonuclear dipolar interactions between the nuclei of interest (^{15}N nuclei in the present case). Therefore, this method can be applied to low-γ nuclei that exhibit a very small homonuclear dipolar coupling as demonstrated for ^{15}N in this study, as long as the two-way heteronuclear magnetization transfer efficiency is efficient.

Conclusions

While the need for high-resolution multidimensional spectra and proton-detected experiments for structural studies on biological solids have been fully realized, the chemical shift correlation of amide-^{15}N nuclei is not easy to achieve. In this study, we demonstrated a new 3D ^{15}N/^{15}N/^{1}H chemical shift correlation technique that can resolve amide-proton resonances and also quickly correlate the chemical shifts of ^{15}N nuclei under ultrafast MAS conditions. Our experimental results demonstrate the use of recoupled ^{1}H-^{1}H dipolar couplings to mix protons and hence to enable faster ^{15}N/^{15}N single quantum correlation. Though the use of multiple steps to transfer magnetization between ^{1}H and ^{15}N nuclei in the pulse sequence results in a loss of the net magnetization and reduces the overall sensitivity, about 31% magnetization survives after the first two CP periods of the 3D pulse sequence due to the high magnetization transfer efficiency and proton-detection under ultrafast MAS frequency utilized in this study. Therefore, we believe that this method would be valuable in the development of higher dimensional techniques to correlate ^{13}C, ^{15}N and ^{1}H chemical shifts for resonance assignment in the structural studies of proteins. While the proposed 3D ^{15}N/^{15}N/^{1}H method is complementary to other methods that are used in the assignment of resonances from a uniformly-^{15}N-labeled protein, like the ^{15}N-BARE based experiments [25], our approach would particularly be beneficial in the development of ^{15}N-based solid-state MAS experiments.

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Supplementary Material

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Acknowledgments

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Highlights

A new 3D $_{15}$N/$_{15}$N/$_{1}$H chemical shift correlation MAS technique is demonstrated.

$_{1}$H-mixing via the recoupled $_{1}$H-$_{1}$H dipolar couplings enable faster $_{15}$N/$_{15}$N correlation.

$_{1}$H-detection under ultrafast MAS renders fast acquisition of the 3D spectrum.

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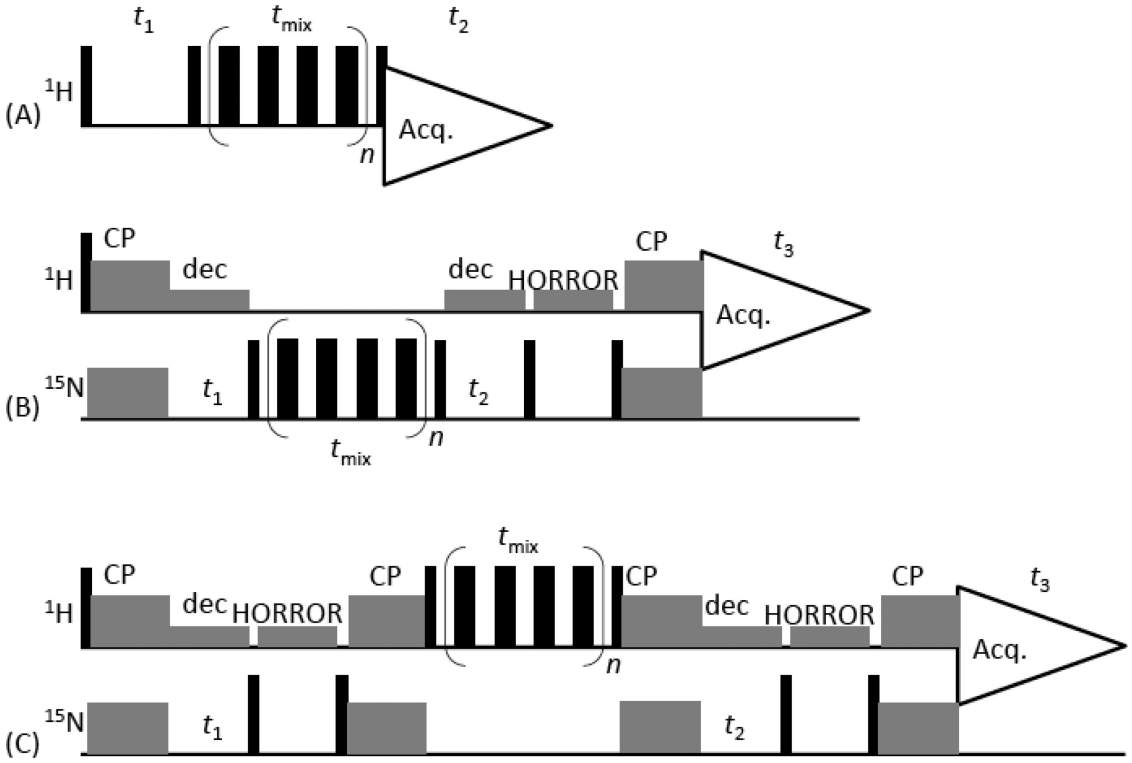

Figure 1. Proton-detected homonuclear correlation pulse sequences

(A) 2D ^{1}H/^{1}H pulse sequence that correlates the isotropic chemical shifts of protons. In this method, the RFDR recoupling technique is used in the mixing time to recouple ^{1}H-^{1}H dipolar couplings. (B) Proton-detected ^{15}N/^{15}N chemical shift correlation experiment with the RFDR sequence in the mixing period to recouple ^{15}N-^{15}N dipolar couplings. In this pulse sequence, protons are prepared and detected, and ramp-CP [26] is employed to transfer the transverse magnetization from ^{1}H to ^{15}N or ^{15}N to ^{1}H as indicated. (C) Proton-detected 3D ^{15}N/^{15}N/^{1}H experiment that correlates the isotropic chemical shifts of ^{15}N, ^{15}N and ^{1}H nuclei. Chemical shifts of ^{15}N nuclei are expressed during t_1 and t_2 periods, and z-magnetization of protons are exchanged during the mixing period via the ^{1}H-^{1}H dipolar couplings recoupled by RFDR. Protons are decoupled by a low-power CW decoupling [27] during the ^{15}N chemical shift evolution periods t_1 and t_2, and the HORROR sequence [28] is used to destroy the proton magnetization remaining in the ^{1}H channel [27, 29]. An echo sequence (tau-180°-tau = two MAS rotor period), before data acquisition, was used in (A) to suppress the background signal from the probe. Additional RFDR pulse sequences applied during the repetition time (not shown in the pulse schemes) considerably shorten the repetition delay (see the text). The XY4$^1_4$ phase cycling was used for all the RFDR schemes.

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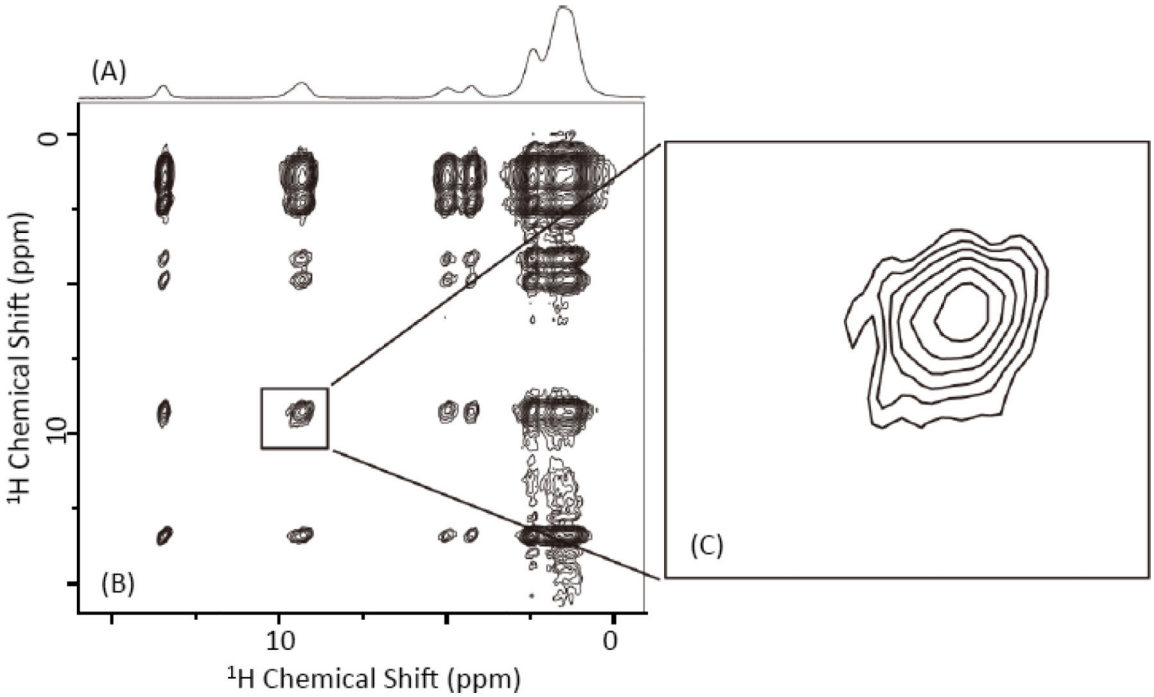

Figure 2. 2D chemical shift correlation of protons

(A) A 1D ^{1}H NMR spectrum of a powder sample of NAVL obtained at 100 kHz MAS. (B) A 2D ^{1}H/^{1}H chemical shift correlation spectrum of NAVL powder sample obtained using the pulse sequence given in Figure 1(A) with a 6.4 ms RFDR mixing time and XY4$^1_4$ phase cycling at 100 kHz MAS. 64 t_1 points with a recycle delay of 2 s were used. The total measurement time was 8.5 minutes. (C) An expanded amide-^{1}H chemical shift region of the 2D spectrum is shown (right). 2D spectra recorded at RFDR different mixing times are given in Figures S1 and S2 in the Supporting Information.

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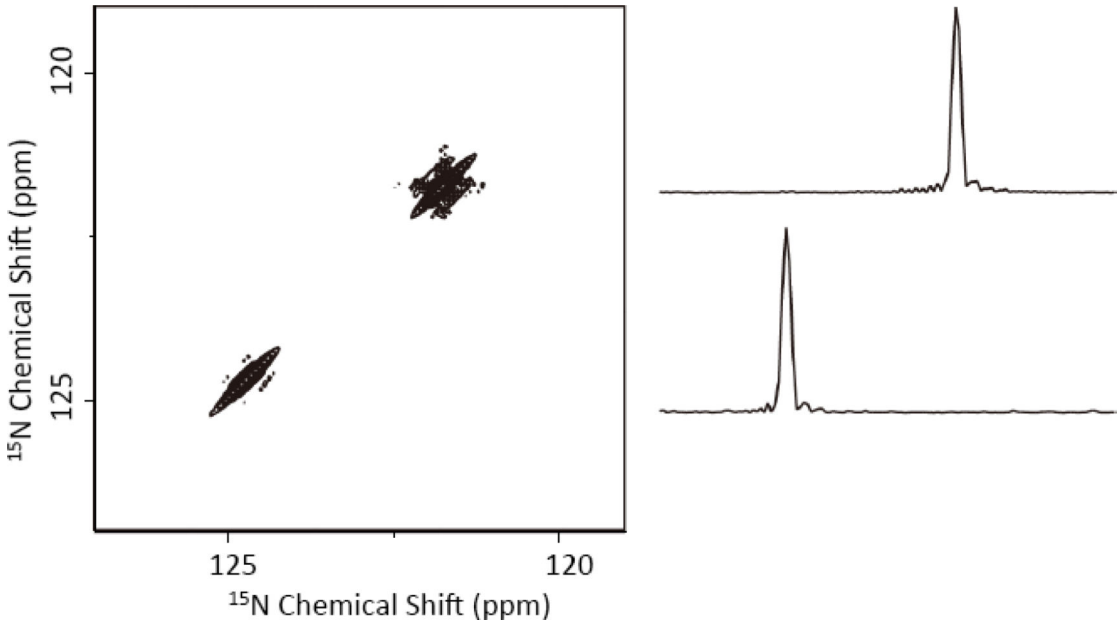

Figure 3. Proton-detected ^{15}N/^{15}N chemical shift correlation obtained with an RFDR mixing in the ^{15}N channel

A proton-detected 2D ^{15}N/^{15}N chemical shift correlation spectrum of NAVL powder sample obtained using the pulse sequence given in Figure 1(B) with 32 ms RFDR mixing time and XY4$^{1}_{4}$ phase cycling at 100 kHz MAS. 32 t_1 and 32 t_2 points were observed with a recycle delay of 2 s. The measurement time was 18.2 hour. The 2D spectrum is a project of the 3D ^{15}N/^{15}N/^{1}H spectrum on to the ^{15}N/^{15}N plane. 1D spectral slices extracted from the 2D ^{15}N/^{15}N spectrum are shown (right). A contact time of 1 ms was used for both CP transfers.

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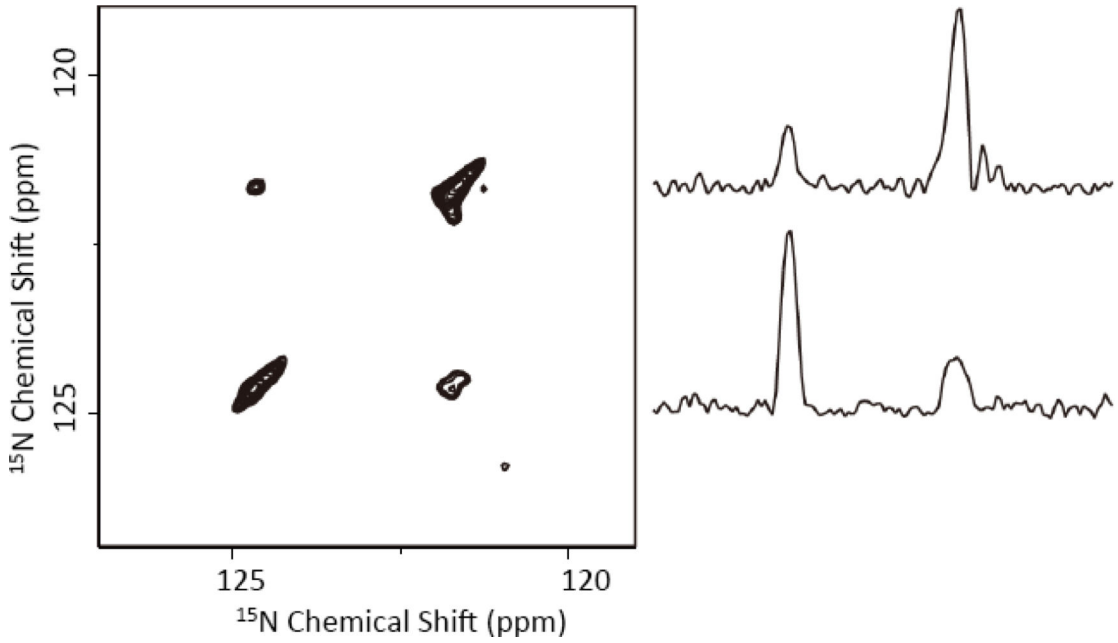

Figure 4. Proton-detected ^{15}N/^{15}N chemical shift correlation obtained with an RFDR mixing in the ^{1}H channel

A proton-detected 2D ^{15}N/^{15}N chemical shift correlation spectrum of NAVL powder sample obtained using the 3D ^{15}N/^{15}N/^{1}H pulse sequence given in Figure 1(C) with a 6.4 ms RFDR mixing time and XY4$^{1}_{4}$ phase cycling at 100 kHz MAS. 16 t_1 and 16 t_2 points were observed with a recycle delay of 2 s. The measurement time was 4.6 hour. The 2D spectrum is a project of the 3D ^{15}N/^{15}N/^{1}H spectrum on to the ^{15}N/^{15}N plane. 1D spectral slices extracted from the 2D ^{15}N/^{15}N spectrum are shown (right). A contact time of 1 ms was used for all the CP transfers.