Zero-quantum frequency-selective recoupling of homonuclear dipole-dipole interactions in solid state nuclear magnetic resonance

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We describe a method for measuring magnetic dipole-dipole interactions, and hence distances, between pairs of like nuclear spins in a many-spin system under magic-angle spinning (MAS). This method employs a homonuclear dipolar recoupling sequence that creates an average dipole-dipole coupling Hamiltonian under MAS with full zero-quantum symmetry, including both secular and flip-flop terms. Flip-flop terms are then attenuated by inserting rotor-synchronized periods of chemical shift evolution between recoupling blocks, leaving an effective Hamiltonian that contains only secular terms to a good approximation. Couplings between specific pairs of nuclear spins can then be selected with frequency-selective π pulses. We demonstrate this technique, which we call zero-quantum shift evolution assisted homonuclear recoupling, in a series of one-dimensional and two-dimensional ^{13}C NMR experiments at 17.6 T and 40.00 kHz MAS frequency on uniformly ^{13}C -labeled L-threonine powder and on the helix-forming peptide MB(i+4)EK, synthesized with a pair of uniformly ^{13}C -labeled L-alanine residues. Experimental demonstrations include measurements of distances between ^{13}C sites that are separated by three bonds, placing quantitative constraints on both sidechain and backbone torsion angles in polypeptides.

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I. INTRODUCTION

Solid state NMR has become a powerful tool for investigating the molecular structures of amorphous and polycrystalline biological solids. With high-resolution spectra obtained from magic-angle spinning (MAS) and heteronuclear decoupling, relatively simple solid state NMR techniques can be used to determine protein structures from a large number of qualitative structural constraints.^{1–4} Quantitative structural constraints on internuclear distances and torsion angles can be obtained from more sophisticated techniques involving dipolar recoupling methods,^{5–9} in principle allowing molecular structures to be determined more completely and with higher precision. Quantitative structural techniques that are applicable to uniformly ^{15}N , ^{13}C -labeled samples are particularly desirable,^{10–12} because in principle uniform isotopic labeling provides a faster route to structure determination than does labeling of specific sites in a large set of samples.^{13,14} However, even if the spectral resolution for a given sample of interest is sufficiently high to permit uniform isotopic labeling, the presence of many-like spins leads to “dipolar truncation” effects, in which the quantum mechanical noncommutivity of pairwise magnetic dipole-dipole couplings causes the stronger one-bond and two-bond couplings to effectively shut off the weaker, longer-range couplings. Since the weaker dipole-dipole couplings contain the most important structural information (e.g., constraints on

backbone torsion angles, sidechain conformations, and tertiary contacts), dipolar truncation prevents standard dipolar recoupling techniques from being useful as quantitative structural techniques for uniformly labeled samples. Dipolar truncation is particularly problematic for homonuclear recoupling techniques, e.g., techniques for measuring ^{13}C – ^{13}C dipole-dipole couplings under MAS.^{15–18}

Frequency-selective dipolar recoupling techniques represent one approach to overcoming dipolar truncation. Such techniques have the effect of switching on dipole-dipole couplings only between pairs of spins with specific NMR chemical shifts under MAS. The earliest frequency-selective homonuclear recoupling techniques were based on the rotational resonance (RR) effect^{6,19,20} and its extension to recoupling under continuous wave radio-frequency (rf) irradiation, through RR in the tilted rotating frame (RRTR).^{10,11} The RR and RRTR techniques are limited to relatively low MAS frequencies or spin pairs with relatively large chemical shift differences. More recent alternatives involve simultaneous recoupling of both dipole-dipole couplings and isotropic or anisotropic chemical shifts.^{21,22} Truncation of the recoupled homonuclear dipole-dipole couplings by chemical shift differences *within the recoupling period* (unlike the approach described below) produces couplings that can be manipulated by frequency-selective π pulses. These alternative approaches are limited by practical difficulties of implementing the simultaneous dipole-dipole/chemical shift recoupling techniques at higher MAS frequencies and removing residual heteronuclear couplings to abundant proton spins.

Recently, Paravastu and Tycko²³ introduced the shift evolution assisted selective homonuclear recoupling

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(SEASHORE) technique for frequency-selective double-quantum homonuclear recoupling. The SEASHORE technique consists of alternating blocks of nonselective double-quantum recoupling and blocks of free spin precession under isotropic chemical shifts, with the length of each block being a multiple of the MAS rotation period τ_R . For homonuclear couplings with double-quantum symmetry, the chemical shift precession periods have the effect of averaging out all pairwise couplings except those for which the net precession angle in each period is an integral multiple of 2π . In principle, the SEASHORE technique is quite general, as the desired frequency selectivity can be achieved for any spin system with any set of chemical shifts by adjusting the lengths of the chemical shift precession periods. In practice, the double-quantum SEASHORE technique depends on having a nonselective dipolar recoupling pulse sequence that produces pure double-quantum couplings at an appropriate MAS frequency over the experimentally relevant range of chemical shifts. The experiments of Paravastu and Tycko²³ employed the POST-C7 recoupling sequence of Hohwy *et al.*²⁴ at MAS frequencies in the 6–10 kHz range and at a 9.4 T magnetic field, but other double-quantum recoupling sequences could be employed at higher MAS frequencies and higher magnetic fields.

In this paper, we describe an approach to frequency-selective zero-quantum (ZQ) homonuclear recoupling that employs the SEASHORE principle of separating dipolar recoupling and chemical shift precession into discrete periods within a rf pulse sequence. Starting with homonuclear couplings with full ZQ symmetry, chemical shift precession periods (and, in some cases as described below, dipolar truncation itself) produce secular ZQ couplings that can be manipulated by frequency-selective π pulses, as suggested²³ or demonstrated^{21,22} in earlier work. Zero-quantum recoupling is achieved with the finite-pulse radio-frequency-driven recoupling (fpRFDR) sequence,^{9,25} which is effective at high MAS frequencies over large chemical shift ranges and produces adequate proton decoupling without proton irradiation at sufficiently high MAS frequencies.²⁵ Thus, the ZQ-SEASHORE approach has important practical advantages over previous approaches to frequency-selective homonuclear recoupling.

II. THEORY

A general ZQ recoupling sequence leads to an average Hamiltonian for a many-spin system under MAS of the form

$$H_{ZQ} = \sum_{i < j} a_{ij} I_{iz} I_{jz} + b_{ij} I_i^+ I_j^- + b_{ij}^* I_i^- I_j^+, \quad (1)$$

where $a_{ij} = 2s_{ij}\omega_d$ and $b_{ij} = s_{ij}\omega_d/2$, with the orientation-dependent scaling factor S_{ij} and $\omega_d = -(\gamma^2\hbar/r_{ij}^3)$. The distance between spins i and j is r_{ij} . I_{iz} is the z -component of spin angular momentum for spin i , and $I_i^\pm \equiv I_{ix} \pm iI_{iy}$. If a recoupling period of length $m\tau_R$ is followed by a free chemical shift evolution period of length $n\tau_R$ (during which dipolar interactions are averaged to 0 by MAS), the evolution operator for the spin system becomes

FIG. 1. (a) General form for a ZQ-SEASHORE pulse sequence. Periods of zero-quantum (ZQ) dipolar recoupling alternate with periods of spin precession at isotropic chemical shift (CS) frequencies, producing a truncated ZQ dipole-dipole Hamiltonian containing only secular terms. A frequency-selective π pulse in the middle of the ZQ-SEASHORE sequence decouples the selected spins from others. (b) In this paper, ZQ recoupling is achieved with the fpRFDR sequence, (Refs. 9 and 25) consisting of one π pulse per MAS rotor period τ_R , with XY-8 phases. (Ref. 27)

$$U = \exp\left(-in\tau_R \sum_i \omega_i I_{iz}\right) \exp(-iH_{ZQ}m\tau_R), \quad (2)$$

where ω_i is the NMR frequency of spin i relative to the rf carrier frequency, i.e., the resonance offset. After N repetitions, the total evolution operator for the ZQ-SEASHORE sequence becomes

$$\begin{aligned} U_{\text{tot}} &= U^N = \exp\left(-in\tau_R \sum_i \omega_i I_{iz}\right) \exp(-iH_{ZQ}m\tau_R) \\ &\quad \times \exp\left(-in\tau_R \sum_i \omega_i I_{iz}\right) \exp(-iH_{ZQ}m\tau_R) \dots \\ &\quad \times \exp\left(-in\tau_R \sum_i \omega_i I_{iz}\right) \exp(-iH_{ZQ}m\tau_R) \\ &= \left[\prod_{k=1}^N \exp(-i\tilde{H}_{ZQ}^{(k)}m\tau_R) \right] \\ &\quad \times \exp\left(-iNn\tau_R \sum_i \omega_i I_{iz}\right), \end{aligned} \quad (3)$$

where

$$\begin{aligned} \tilde{H}_{ZQ}^{(k)} &= \exp\left(-ikn\tau_R \sum_i \omega_i I_{iz}\right) H_{ZQ} \exp\left(ikn\tau_R \sum_i \omega_i I_{iz}\right) \\ &= \sum_{i < j} a_{ij} I_{iz} I_{jz} + b_{ij} \exp[-ikn\tau_R(\omega_i - \omega_j)] I_i^+ I_j^- \\ &\quad + b_{ij}^* \exp[ikn\tau_R(\omega_i - \omega_j)] I_i^- I_j^+. \end{aligned} \quad (4)$$

If the dipole-dipole couplings are sufficiently weak relative to the MAS frequency, Eq. (3) can be approximated as

$$U_{\text{tot}} \approx \exp\left(-i \sum_{k=1}^N \tilde{H}_{ZQ}^{(k)}m\tau_R\right) \exp\left(-iNn\tau_R \sum_i \omega_i I_{iz}\right). \quad (5)$$

Note that $1/N |\sum_{k=1}^N \exp[\pm ikn\tau_R(\omega_i - \omega_j)]|$ diminishes with increasing N , as long as $n\tau_R(\omega_i - \omega_j)$ is not a multiple of 2π . Therefore, the total evolution operator for N repetitions of the ZQ recoupling and chemical shift precession blocks is approximately

$$U_{\text{trunc}}(N) \approx \exp\left(-iNm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \times \exp\left(-iNn\tau_R \sum_i \omega_i I_{iz}\right), \quad (6)$$

where the full ZQ dipole-dipole coupling Hamiltonian is replaced by a truncated Hamiltonian that contains only prod-

ucts of z -components of spin angular momenta, i.e., the secular terms in the ZQ coupling Hamiltonian.

If a selective π pulse covering frequencies ω_s and ω_r is applied in the middle of the ZQ-SEASHORE sequence, as shown in Fig. 1(a), spins s and r are decoupled from all other spins but remain coupled to one another, as shown by

$$\begin{aligned} U'_{\text{trunc}}(N) &= U_{\text{trunc}}\left(\frac{N}{2}\right) \exp[i\pi(I_{sx} + I_{rx})] U_{\text{trunc}}\left(\frac{N}{2}\right) = \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i \omega_i I_{iz}\right) \exp[i\pi(I_{sx} + I_{rx})] \\ &\times \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i \omega_i I_{iz}\right) = \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i \omega_i I_{iz}\right) \\ &\times \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} g_i g_j I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i g_i \omega_i I_{iz}\right) \exp[i\pi(I_{sx} + I_{rx})] \\ &= \exp\left[-iNm\tau_R \left(a_{rs} I_{rz} I_{sz} + \sum_{i,j \neq r,s} a_{ij} I_{iz} I_{jz}\right)\right] \exp\left(-iNn\tau_R \sum_{i \neq r,s} \omega_i I_{iz}\right) \exp[i\pi(I_{sx} + I_{rx})], \end{aligned} \quad (7)$$

where $g_i \equiv (1 - 2\delta_{ir} - 2\delta_{is})$.

The net chemical shift precession of spins other than r and s in Eq. (7) can be removed by applying both a nonselective π pulse and a selective π pulse in the middle of the ZQ-SEASHORE sequence, as shown by

$$\begin{aligned} U''_{\text{trunc}}(N) &= U_{\text{trunc}}\left(\frac{N}{2}\right) \exp[i\pi(I_{sx} + I_{rx})] \exp\left(i\pi \sum_i I_{ix}\right) U_{\text{trunc}}\left(\frac{N}{2}\right) \\ &= \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i \omega_i I_{iz}\right) \exp\left(i\pi \sum_{i \neq r,s} I_{ix}\right) \\ &\times \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i \omega_i I_{iz}\right) \\ &= \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} I_{iz} I_{jz}\right) \exp\left(-\frac{i}{2}Nn\tau_R \sum_i \omega_i I_{iz}\right) \\ &\times \exp\left(-\frac{i}{2}Nm\tau_R \sum_{i < j} a_{ij} g_i g_j I_{iz} I_{jz}\right) \exp\left(\frac{i}{2}Nn\tau_R \sum_i g_i \omega_i I_{iz}\right) \exp\left(i\pi \sum_{i \neq r,s} I_{ix}\right) \\ &= \exp\left[-iNm\tau_R \left(a_{rs} I_{rz} I_{sz} + \sum_{i,j \neq r,s} a_{ij} I_{iz} I_{jz}\right)\right] \exp[-iNn\tau_R (\omega_r I_{rz} + \omega_s I_{sz})] \exp\left(i\pi \sum_{i \neq r,s} I_{ix}\right). \end{aligned} \quad (8)$$

III. MATERIALS AND METHODS

L-threonine with 27% uniformly ^{15}N , ^{13}C -labeled molecules was prepared by recrystallization of a mixture of labeled and unlabeled L-threonine from de-ionized water. The 17-residue helical peptide MB(i+4)EK (N-acetyl-AEAAAKEAAAKE AAKA-NH₂) was synthesized using standard Fmoc solid-phase methods as previously described,²⁶ with uniformly ^{15}N , ^{13}C -labeled L-alanine at the Ala9 and Ala10 positions. Both samples (approximately 3 mg each) were finely ground and center-packed in a 1.8 mm MAS rotor. NMR experiments were performed at 17.6 T (188.04 MHz ^{13}C NMR frequency), using a Varian Infinity spectrometer console and a three-channel high-speed

MAS probe built by the group of Dr. Ago Samoson (National Institute of Chemical Physics and Biophysics, Tallinn, Estonia). Rf pulse sequences are shown in Figs. 2 and 5. Zero-quantum ^{13}C - ^{13}C dipolar recoupling was performed with fpRFDR,²⁵ using 7.0 μs π ^{13}C pulses at 40.00 kHz MAS frequency, one π pulse per rotor period, and XY-8 phases.²⁷ Pulse sequences were actively synchronized to an MAS tachometer signal, with synchronization occurring at the beginning of each rotor period in the recoupling blocks. The π pulses were applied either at the beginning of a rotor period (A blocks), or after delays of $\tau_R/3$ (B blocks) or $2\tau_R/3$ (C blocks), as discussed below. Proton decoupling fields were 120 kHz, with two-pulse phase modulation²⁸ (TPPM) during chemical shift precession and signal detec-

FIG. 2. Pulse sequences used in experiments for Figs. 3 and 4. (a) Sequence for 2D ^{13}C - ^{13}C spectroscopy with longitudinal mixing and without frequency selectivity. ^{13}C spin polarization is prepared initially by ^1H - ^{13}C cross-polarization. Zero-quantum recoupling is applied during the mixing period for time $8N\tau_R$. TPPM decoupling is applied during t_1 and t_2 periods. For quadrature detection in t_1 , signals are acquired with $\phi=x$ and $\phi=y$. (b) Sequence for 2D ^{13}C - ^{13}C spectroscopy with longitudinal mixing under truncated ZQ dipole-dipole couplings. (c) Sequence for 2D ^{13}C - ^{13}C spectroscopy with transverse mixing under truncated ZQ dipole-dipole couplings. (d) Sequence for 2D ^{13}C - ^{13}C spectroscopy with frequency-selective transverse mixing under truncated ZQ dipole-dipole couplings. Under this sequence, transverse polarization transfers can occur within groups of spins that are inverted by the frequency-selective π pulses, or within groups that are unaffected by the frequency-selective π pulses. The length of these pulses is determined by the integer q . Vertical dashed lines indicate the beginning of each $2q\tau_R$ period.

tion periods, except as noted below. No proton rf was applied during fpRFDR periods, as the fpRFDR sequence on ^{13}C spins was found to provide adequate decoupling of ^{13}C from protons. The ^{13}C rf carrier frequency was set to the average of the CO and C_α chemical shifts during ZQ-SEASHORE periods in all experiments, except in Fig. 6(a) where the carrier frequency was set to the average of C_α and C_β chemical shifts. ^{13}C NMR chemical shifts in all spectra are relative to tetramethylsilane. Other details of NMR measurements are given below or in the figure captions.

IV. RESULTS

A. Spin polarization transfers under truncated and frequency-selective ZQ recoupling

Two-dimensional (2D) ^{13}C - ^{13}C NMR spectra of polycrystalline L-threonine were acquired with pulse sequences in Fig. 2. The 2D spectrum in Fig. 3(a) was acquired with untruncated fpRFDR recoupling during the mixing period and with longitudinal mixing [i.e., spin polarization transfers between z -components of angular momentum; pulse se-

quence in Fig. 2(a)]. Strong crosspeaks that connect isotropic chemical shifts of directly bonded ^{13}C sites are observed. Weaker two-bond crosspeaks (and even weaker three-bond crosspeaks) are also observed. The 2D spectrum in Fig. 3(b) was acquired with truncated fpRFDR recoupling during the mixing period and with longitudinal mixing [pulse sequence in Fig. 2(b)]. All crosspeaks are strongly suppressed, as expected if the effective coupling Hamiltonian contains only secular terms as in Eq. (6). If nonsecular terms are absent, the z -component of angular momentum of each individual spin is conserved, preventing longitudinal polarization transfers.

The 2D spectrum in Fig. 3(c) was acquired with truncated fpRFDR recoupling during the mixing period and with transverse mixing [i.e., spin polarization transfers between x - or y -components of angular momentum; pulse sequence in Fig. 2(c)]. Note that the pulse sequence in Fig. 2(c) includes a $\pi/2$ pulse in the center of the mixing period, phase-shifted by $\pi/2$ relative to the $\pi/2$ pulses at the beginning and end of the mixing period. This central pulse is required to produce transverse polarization transfers under a truncated ZQ cou-

FIG. 3. 2D ^{13}C – ^{13}C spectra of uniformly ^{15}N , ^{13}C -labeled L-threonine powder (27% labeled molecules diluted in unlabeled molecules by recrystallization) obtained with pulse sequences in Fig. 2, at 188.04 MHz ^{13}C NMR frequency and 40.00 kHz MAS frequency. ^{13}C π pulses in fpRFDR blocks were 7.0 μs . (a) Spectrum obtained with sequence in Fig. 2(a), with $N=12$. (b) Spectrum obtained with sequence in Fig. 2(b), with $N=8$ and $n=7$. (c) Spectrum obtained with sequence in Fig. 2(c), with $N=8$ and $n=7$. (d) Spectrum obtained with sequence in Fig. 2(d), with $N=64$ and $n=7$, and with frequency-selective π pulses (250 μs pulse length) applied to C_α and C_β spins to decouple them from CO and C_γ spins. 1D slices at NMR frequencies of CO, C_α , C_β , and C_γ (at 170.0, 60.2, 65.4, and 18.9 ppm, respectively) are shown to the right of each 2D spectrum.

pling Hamiltonian, as is well known from simple product-operator rules.²⁹ Note also that “relayed” polarization transfers (i.e., transfers between spin pairs that are not directly coupled, mediated by their couplings to a common third spin) should not occur under the secular coupling. Crosspeak patterns in Fig. 3(c) are similar to those in Fig. 3(a), except that two-bond crosspeaks are weaker, as expected in the absence of relayed polarization transfers.

The 2D spectrum in Fig. 3(d) was acquired with frequency-selective, truncated fpRFDR recoupling during the mixing period, and with transverse mixing [pulse sequence in Fig. 2(d)]. The frequency-selective, Gaussian-shaped π pulses in Fig. 2(d) were applied with the ^{13}C rf carrier frequency between the isotropic shifts of the C_α and C_β sites, so that only the $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ spins experience the π rotation. According to Eq. (8), this should leave the $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ spins coupled to one another and the ^{13}CO and $^{13}\text{C}_\gamma$ spins coupled to one another, but $^{13}\text{C}_\alpha$ – ^{13}CO , $^{13}\text{C}_\beta$ – $^{13}\text{C}_\gamma$, $^{13}\text{C}_\alpha$ – $^{13}\text{C}_\gamma$, and $^{13}\text{C}_\beta$ – $^{13}\text{C}_\gamma$ couplings should be absent. Note that two frequency-selective π pulses are required, one in each half of the mixing period, with a $\pi/2$ pulse in the center of the mixing period as discussed above. As expected, crosspeaks between CO and C_γ chemical shifts are observed in Fig. 3(d), while C_α /CO, C_β /CO, C_α / C_γ , and C_β / C_γ crosspeaks are suppressed. C_α / C_β crosspeaks and diagonal peaks for C_α and C_β are also attenuated, due to the relatively short T_2 relaxation times for these sites.

Results in Fig. 3 demonstrate that the ZQ-SEASHORE technique can be implemented successfully at high MAS frequencies and high fields, that this technique does indeed create secular ZQ couplings, and that frequency selectivity can be achieved with frequency-selective π pulses. Figure 4 compares experimental crosspeak volumes (plots on left side) with simulated crosspeak volumes (plots on right side)

FIG. 4. Dependence of experimental [(a)–(d)] and simulated [(e)–(h)] cross-peaks on mixing time, from 2D spectra as in Figs. 3(a)–3(d), respectively. Crosspeak volumes from experimental spectra are scaled to the CO diagonal peak volume at the shortest mixing time in each panel. Crosspeak volumes from simulations (i.e., polarization transfer amplitudes) are scaled to the initial spin polarization at a single site. Simulations include four ^{13}C spins with the geometry and NMR parameters of L-threonine in crystalline form.

as functions of the mixing time for the four types of 2D experiments in Figs. 2 and 3. Numerical simulations were performed with the SPINEVOLUTION program³⁰ and include the four ^{13}C spins in L-threonine with the geometry from the reported crystal structure,³¹ isotropic chemical shifts and chemical shift anisotropies (CSAs) reported for L-threonine,³² and the same ^{13}C rf pulse sequence as in experiments (except for the initial ^1H – ^{13}C cross-polarization step). Proton spins were not included in simulations. Agreement between experiments and numerical simulations is generally good. Comparison of Figs. 4(d) and 4(h) illustrates the effects of T_2 relaxation, which attenuates the C_α / C_β crosspeaks and (to a lesser extent) the C_γ /CO crosspeaks in experimental measurements at long mixing times.

B. Distance measurements by constant-time frequency-selective recoupling

Figures 5(a) and 5(b) show pulse sequences for quantitative measurements of ^{13}C – ^{13}C distances by frequency-

FIG. 5. Pulse sequences for ^{13}C - ^{13}C distance measurements by constant-time frequency-selective ZQ recoupling. (a) Sequence in which spins that are inverted by the frequency-selective π pulse are decoupled from other spins. Distances among spins that are unaffected by the frequency-selective π pulse are determined from the dependence of their NMR signals on the integer k . (b) Sequence for determining distances among spins that are inverted by the frequency-selective π pulse. (c) Sequence for indirect detection of internuclear distances by 2D spectroscopy. Transverse polarization of spins that are inverted by the frequency-selective π pulse (e.g., backbone ^{13}CO spins in a polypeptide) dephases under frequency-selected ZQ recoupling for a variable time $24k\tau_R$, and is then transferred to directly bonded spins (e.g., backbone $^{13}\text{C}_\alpha$ spins) during a longitudinal mixing period of length $8k'\tau_R$. After a t_1 evolution period, a second longitudinal mixing period of length $8k''\tau_R$ allows additional polarization transfers (e.g., to $^{13}\text{C}_\beta$ spins). Distances are extracted from the dependence of crosspeak volumes on k . Initial polarization is selected by taking the difference between signals acquired with $\zeta=x$ from signals acquired with $\zeta=y$. For quadrature detection in t_1 , signals are acquired with $\phi=x$ and $\phi=y$. ξ is adjusted empirically from its ideal value ($\xi=x$) to maximize signals. (d) Definition of A, B, and C blocks, which differ only by displacement of the ^{13}C π pulses in the fpRFDR sequence by multiples of $\tau_R/3$ relative to beginning of each MAS rotor period. According to symmetry principles, (Ref. 33) concatenation of the A, B, and C blocks results in cancellation of fpRFDR recoupling, so that ideally the “N-k” periods in a, b, and c do not contribute to the dephasing of transverse polarization.

selective ZQ recoupling. As discussed above, rotor-synchronized, finite-length π pulses (in groups of eight with XY-8 phases, and with each pulse starting $\tau_R/3$ after the beginning of a rotor period, comprising B blocks) are applied alternately with chemical shift precession periods of length $n\tau_R$, creating dipole-dipole couplings that contain only the secular terms to a good approximation. A frequency-selective π pulse [Fig. 5(b)] or both a frequency-selective and a non-selective π pulse [Fig. 5(a)] are applied in the middle of the ZQ-SEASHORE period, decoupling spin pairs of interest from other spins. To extract internuclear distances, the dipole-

lar dephasing time (determined by k in Fig. 5) is incremented, and the decay of ^{13}C NMR signals is measured and compared with simulations.

When the dephasing time extends over many milliseconds, the ^{13}C NMR signals also decay due to T_2 relaxation and incomplete proton decoupling. These extraneous contributions to signal decay can be minimized by performing the measurements in a “constant-time” manner, as previously demonstrated in related contexts.^{25,33,34} In Fig. 5, constant-time recoupling sequences are created by adding ABC blocks separated by chemical shift precession periods of length

FIG. 6. Constant-time frequency-selective dipolar dephasing data for uniformly ^{15}N , ^{13}C -labeled L-threonine powder, at 188.04 MHz ^{13}C NMR frequency and 40.00 kHz MAS frequency. (a) Dephasing of ^{13}CO signals by intramolecular coupling to $^{13}\text{C}_\gamma$ spins, using the pulse sequence in Fig. 5(a) with the frequency-selective π pulse applied to $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ spins. Experimental data were obtained with $n=7$ and $N=14$, 11, and 9 (squares, circles, and triangles, respectively). Solid lines are ideal simulations for ^{13}C spin pairs with purely secular dipole-dipole couplings, with internuclear distances from 2.5 to 3.5 Å in increments of 0.1 Å. Heavy dashed line is a simulation for the full pulse sequence and the full coupled four-spin ^{13}C system of L-threonine with realistic geometry and NMR parameters. (b) Dephasing of ^{13}CO signals under the pulse sequence in Fig. 5(b) with frequency-selective π pulses applied to ^{13}CO spins. Experimental data were obtained with $n=1$ and $N=14$, 11, and 9 (squares, circles, and triangles, respectively). Solid lines are ideal simulations for ^{13}C spin pairs with purely secular dipole-dipole couplings. Heavy dashed line is a full simulation for the four-spin system.

$3n\tau_R$ before and after the ZQ-SEASHORE period, where the A and C blocks differ from the B blocks only by displacements of the finite-length π pulses by $\pm\tau_R/3$. As previously shown, an ABC block produces no net dipolar recoupling, in the limit that the MAS frequency is large compared with chemical shift differences.³³ Therefore, the ABC blocks do not contribute to the net dipolar dephasing time. The dephasing time can be incremented without changing the overall pulse sequence duration and without changing the number of rf pulses, by incrementing k from 0 to N while keeping N constant.

Figure 6 shows experimental measurements of the dephasing of CO signals from uniformly ^{15}N , ^{13}C -labeled L-threonine. For Fig. 6(a), the pulse sequence in Fig. 5(a) was used, with the frequency-selective π pulse applied to $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ spins. The signal decay should then result from ^{13}CO – $^{13}\text{C}_\gamma$ dipole-dipole couplings. Comparison with numerical simulations of signal decay for ^{13}C spin pairs with ideal secular dipole-dipole couplings shows good agreement at a 3.1 Å internuclear distance, corresponding to the 3.09 Å intramolecular CO– C_γ distance in the L-threonine crystal structure.³¹ These simulations assume the ideal ZQ average Hamiltonian for fpRFDR,^{25,33} truncated to secular terms. The experimental data also agree well with numerical simulations that include the full rf pulse sequence with MAS and the four-spin system of dipole-coupled ^{13}C nuclei in L-threonine [heavy dashed lines in Fig. 6(a)]. Experiments were performed with total constant-time recoupling periods (equal to $48N\tau_R$, not including chemical shift precession periods) of 10.8, 13.2, and 16.8 ms. As shown in Fig. 6(a), experimental decay curves (normalized to the signals at zero dephasing time) are independent of the constant-time period.

For Fig. 6(b), the pulse sequence in Fig. 5(b) was used, with the frequency-selective π pulse applied to ^{13}CO spins. This should decouple ^{13}CO spins from $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, and $^{13}\text{C}_\gamma$ spins. Signal decay is significantly slower than in Fig. 5(a),

agreeing approximately with simulations for 5 Å internuclear distances. In crystalline L-threonine, each carboxylate carbon has two nearest neighbor carboxylates, at 3.845 Å distances.³¹ Given the dilution of isotopically labeled molecules in our sample, approximately 53% of ^{13}CO spins have no nearest neighbor ^{13}CO at this distance. Next-nearest neighbors are at 5.110 Å. Thus, data in Fig. 6(b) are in rough agreement with expectations based on the L-threonine crystal structure.

Data in Fig. 6(a) are corrected for natural-abundance ^{13}C contributions to the CO signals by subtracting a constant from the experimental signal amplitudes, equal to 22% of the signal at zero dephasing time for data at $48N\tau_R=16.8$ ms. This correction is larger than the 3% correction that would be based on the ratio of natural-abundance carboxylate ^{13}C nuclei to ^{13}C labels because the transverse spin relaxation rate for carboxylate ^{13}C nuclei in uniformly labeled L-threonine molecules is greater than the relaxation rate for natural-abundance carboxylate ^{13}C nuclei by a factor of 5.

Figure 7 shows constant-time frequency-selective recoupling data for lyophilized MB(i+4)EK, with uniform ^{15}N , ^{13}C -labeling of Ala9 and Ala10. As shown in Fig. 7(d), this sample contains a mixture of peptides with an α -helical conformation and peptides with an extended conformation,²⁶ in an approximate 7:1 ratio. Figure 7(a), obtained with the pulse sequence in Fig. 5(b) and with frequency-selective π pulses applied to ^{13}CO spins (~ 15 ppm excitation bandwidth), shows measurements of inter-residue ^{13}CO – ^{13}CO dipole-dipole couplings. The decay of helical CO signals indicates a CO–CO distance of 2.9 Å. The decay of nonhelical CO signals indicates a CO–CO distance of 3.3 Å. These distances are in good agreement with distances expected in α -helical (backbone torsion angle $\phi=-50^\circ$) and extended ($\phi=100^\circ$) conformations at Ala10. Note that the nonhelical MB(i+4)EK molecules are expected to adopt a distribution of conformations, so that the apparent CO–CO distance for

FIG. 7. Constant-time frequency-selective dipolar dephasing data for MB(i+4)EK with uniform ^{15}N , ^{13}C -labeled L-alanine at Ala9 and Ala10, at 188.04 MHz ^{13}C NMR frequency and 40.00 kHz MAS frequency. (a) Dephasing of ^{13}CO signals by ^{13}CO – ^{13}CO couplings, using the pulse sequence in Fig. 5(b) with frequency-selective π pulses applied to ^{13}CO spins. Data for helical (squares, up triangles, and plus symbols) and nonhelical (circles, down triangles, and asterisks) signal components are shown, obtained $n=1$ and $N=9$ (squares, circles), $N=7$ (up and down triangles), and $N=5$ (plus symbols and asterisks). Lines are ideal two-spin simulations for internuclear distances from 2.6 to 3.6 Å, in increments of 0.1 Å. (b) Dephasing of helical $^{13}\text{C}_\beta$ signals, using the pulse sequence in Fig. 5(b) with frequency-selective π pulses applied to $^{13}\text{C}_\beta$ spins. Data were obtained with $n=1$ and $N=9$, 7, and 5 (circles, squares, and triangles, respectively). (c) Dephasing of helical ^{13}CO signals by ^{13}CO – $^{13}\text{C}_\beta$ couplings, using the pulse sequence in Fig. 5(a) with frequency-selective π pulses applied to $^{13}\text{C}_\alpha$ spins. Data were obtained with $n=1$ and $N=9$, 7, and 5 (circles, squares, and triangles, respectively). (d) 1D ^{13}C NMR spectrum, showing helical (strong peaks) and non-helical (asterisks) signal components.

these molecules represents an average over the distribution. The distribution of ϕ values for α -helical molecules is expected to be in the range from -45° to -70° , based on earlier molecular dynamics simulations,²⁶ corresponding to distances in the range from 2.86 to 3.06 Å.

Figure 7(b), obtained with the pulse sequence in Fig. 5(b) and with frequency-selective π pulses applied to $^{13}\text{C}_\beta$ spins, shows measurements of inter-residue $^{13}\text{C}_\beta$ – $^{13}\text{C}_\beta$ dipole-dipole couplings. The slow signal decay indicates distances greater than 4.5 Å, as expected in both helical and extended conformations. The slow signal decay confirms that $^{13}\text{C}_\beta$ spins are well decoupled from ^{13}CO and $^{13}\text{C}_\alpha$ spins under these experimental conditions, despite the relatively short intra-residue C_α – C_β and CO – C_β distances. Figure 7(c), obtained with the pulse sequence in Fig. 5(a) and with frequency-selective π pulses applied to $^{13}\text{C}_\alpha$ spins, shows measurements of ^{13}CO – $^{13}\text{C}_\beta$ dipole-dipole couplings. The decay of both helical and nonhelical CO signals indicates a CO – C_β distance of 2.5 Å, in good agreement with the expected intrasidue distance (2.51 Å, independent of backbone conformation).

In experiments for Figs. 7(b) and 7(c), proton rf fields of 120 kHz were applied for proton decoupling during chemical

shift precession periods, but not during fpRFDR periods (A, B, or C blocks in Fig. 5). In experiments for Fig. 7(a), proton rf fields were not applied during both the chemical shift precession periods and the fpRFDR periods, as proton couplings to ^{13}CO spins are sufficiently weak that rapid MAS (40.00 kHz rotation frequency) provides adequate proton decoupling during the chemical shift precession periods.

Experimental data points in Figs. 6 and 7 were obtained from peak areas in one-dimensional (1D) ^{13}C NMR spectra, recorded at various values of the dephasing time. In most applications to biomolecular systems, the resolution in 1D spectra would be insufficient to permit measurements of signal amplitudes for individual sites and their dependence on the dephasing time. In particular, resolution in the CO region of 1D solid state ^{13}C NMR spectra of proteins and peptides is generally poor, necessitating higher-dimensional spectroscopy. Figure 5(c) shows a 2D version of the technique in Fig. 5(b), intended for measurements of site-specific backbone CO – CO distances in peptides and proteins with multiple uniformly labeled residues. After the constant-time frequency-selective ZQ-SEASHORE period, a period of longitudinal mixing under the fpRFDR sequence allows CO – C_α polarization transfers. After a subsequent t_1 evolution period, a sec-

FIG. 8. [(a) and (b)] 2D ^{13}C – ^{13}C NMR spectra of MB(i+4)EK obtained with the pulse sequence in Fig. 5(c) at 188.04 MHz ^{13}C NMR frequency and 40.00 kHz MAS frequency. Frequency-selective π pulses were applied to ^{13}CO spins, $n=1$, $N=7$, $k'=14$, and $k''=14$. The ^{13}C rf carrier frequency was set to 115 ppm during the ZQ-SEASHORE dephasing period, 115 ppm during the first longitudinal mixing period, and 33 ppm during the second longitudinal mixing period. Spectra are shown for $k=1$ (a) and $k=7$ (b). 1D slices at the helical (i) and nonhelical (ii) $^{13}\text{C}_\alpha$ chemical shift values are shown to illustrate the more rapid dephasing of helical signal components, due to the shorter ^{13}CO – ^{13}C distances in helical molecules. (c) Comparison of experimental C_α/C_β crosspeak volumes for helical (filled squares) and nonhelical (filled circles) signal components with ideal two-spin simulations for indicated internuclear distances. Open symbols are CO signal amplitudes from 1D experiments, as in Fig. 7(a).

ond period of longitudinal mixing allows C_α – C_β polarization transfers before ^{13}C NMR signals are detected in the t_2 period. In this way, the decay of transverse CO polarization under ^{13}CO – ^{13}CO dipole-dipole couplings (which depend on backbone conformation) can be monitored through the amplitude of C_α/C_β crosspeaks in a series of 2D spectra, obtained with different dephasing times [i.e., different values of k in Fig. 5(c)]. Depending on the spectral resolution and the longitudinal mixing conditions, CO/ C_α , CO/ C_β , or other crosspeaks can also be used.

2D spectra of the MB(i+4)EK sample with dephasing times of 1.2 and 8.4 ms are shown in Figs. 8(a) and 8(b). These spectra illustrate the more rapid decay of crosspeak (and diagonal) signals associated with helical MB(i+4)EK molecules, compared with signals associated with nonhelical molecules. The dependences of signal amplitudes on dephasing time in Fig. 8(c), determined from C_α/C_β crosspeak volumes, are nearly identical to the dependences from 1D spectra in Fig. 7(a), for both helical and nonhelical signals. This result demonstrates the feasibility of obtaining quantitative constraints on backbone conformation from ^{13}CO – ^{13}CO dipole-dipole couplings in measurements on multiply or uniformly labeled samples.

In Fig. 8, signals originating from CO polarization were selected by phase cycling of the frequency-selective π pulse in Fig. 5(c) (i.e., difference between signals acquired with $\zeta=x$ and $\zeta=y$). In addition, the phase ξ in Fig. 5(c) was adjusted empirically to maximize the signals, accounting for residual phase shifts under the frequency-selective ZQ-SEASHORE sequence.

Finally, because the chemical shift difference between labeled CO sites is quite small for helical MB(i+4)EK, it may be surprising that the experimental data in Figs. 7(a) and 8(c) agree well with simulations for ^{13}C spin pairs with the expected helical internuclear distance, given that the simulations assume purely secular ZQ dipole-dipole couplings. In fact, truncation of ^{13}CO – ^{13}CO couplings to secular terms is

brought about primarily by one-bond ^{13}CO – $^{13}\text{C}_\alpha$ couplings, which are stronger than and do not commute with the longer-range ^{13}CO – ^{13}CO couplings. Thus, CO chemical shift differences are not required in these experiments. To support this analysis, Fig. 9 shows simulations of frequency-selective ZQ-SEASHORE recoupling with the full pulse sequence in Fig. 5(b) for a six-spin system, representing all ^{13}C sites in two sequential alanine residues in a helical peptide. Simulations with and without a CO chemical shift difference are nearly identical, and agree well with two-spin simulations that assume a fully truncated coupling (solid line). Two-spin simulations with an untruncated ZQ coupling show a more rapid signal decay (dashed line).

FIG. 9. Comparison of full numerical simulations of ^{13}CO signals from a six-spin ^{13}C system, representing all carbon sites in two sequential alanine residues in an α -helical peptide (up and down triangles), with two-spin simulations that include only the CO sites (solid and dashed lines). All simulations use the pulse sequence in Fig. 5(b) with $n=1$ and $N=12$ at 188.04 MHz ^{13}C NMR frequency and 40.00 kHz MAS frequency. The CO–CO distance is 2.97 Å. In six-spin simulations, the chemical shift difference between the two CO sites is either 0 Hz (up triangles) or 400 Hz (down triangles). In two-spin simulations, the chemical shift difference is either 0 Hz (dashed line, corresponding to dephasing under full, untruncated ZQ dipole-dipole couplings) or 20.000 kHz (solid line, corresponding to truncated, secular ZQ dipole-dipole couplings). In six-spin simulations, truncation of ^{13}CO – ^{13}CO couplings to secular terms results primarily from noncommuting ^{13}CO – $^{13}\text{C}_\alpha$ couplings.

V. DISCUSSION

The theory presented above shows that effective homonuclear dipole-dipole couplings containing only secular ZQ terms can be created by alternation of ZQ dipolar recoupling periods (which create full ZQ couplings) and chemical shift precession periods (which truncate the couplings to secular terms). This is the ZQ-SEASHORE concept. Frequency-selective π pulses can then be used to refocus undesired couplings, allowing selected couplings of interest to be measured from dephasing curves (as in Figs. 6 and 7) or in principle from polarization transfer curves under transverse mixing (as in Figs. 3 and 4). Compared with previously proposed approaches to the creation of secular ZQ couplings,^{21,22} the ZQ-SEASHORE concept has the advantage of flexibility. Any ZQ recoupling sequence can be used, so the recoupling sequence can be adapted to suit the relevant chemical shift range, MAS frequency, magnetic field strength, available rf field strengths, or other experimental parameters. The relative magnitudes of dipole-dipole couplings and chemical shift differences, which determine the efficiency of truncation to secular terms, can be adjusted by varying the lengths of the recoupling and the chemical shift precession periods.

Experiments described above used the fpRFDR recoupling sequence^{9,25} because of its simplicity, compatibility with high fields and high MAS frequencies, and relatively good proton decoupling efficiency (either without proton rf irradiation at high MAS frequencies or with proton rf irradiation at lower MAS frequencies). In addition, constant-time recoupling techniques are readily constructed with the A, B, and C variants of fpRFDR as shown in Fig. 5, based on symmetry principles as previously described.³³ Constant-time techniques are necessary if experimental data are to be compared directly with numerical simulations to extract internuclear distances, as previously demonstrated.^{25,33,34}

Experiments in Figs. 3, 4, and 6–8 were performed with MAS at 40.00 kHz. This relatively high MAS frequency is required in ^{13}C NMR experiments on uniformly labeled peptides and proteins to ensure that the dipolar recoupling sequence averages out isotropic and anisotropic chemical shifts (which are on the order of 20 kHz at 17.6 T), producing a pure dipole-dipole Hamiltonian during the recoupling periods. At lower fields, somewhat lower MAS frequencies could be used. However, the MAS frequency must still be high enough that each dipolar recoupling period in the ZQ-SEASHORE sequence (minimally $4\tau_R$ for fpRFDR with XY-4 phases) is short compared with the dipole-dipole coupling time scale (approximately 500 μs for one-bond ^{13}C – ^{13}C couplings). Thus, the MAS frequency can not be much lower than 20 kHz in ^{13}C NMR experiments on uniformly labeled systems, even at lower fields.

The optimal MAS frequency for ZQ-SEASHORE is also dictated by the requirement that the precession angles for chemical shift differences during the $n\tau_R$ periods in Figs. 1, 2, and 5 not be close to integer multiples of 2π , especially for ^{13}C pairs that are separated by only one or two bonds. In peptides and proteins, CO– C_α and CO– C_β shift differences (typically 105–130 ppm and 100–160 ppm, respectively) demand MAS frequencies greater than 30 kHz at 17.6 T if

$n=1$. When C_α – C_β shift differences (or other relevant shift differences) are small, larger values of n may be required to ensure adequate truncation of one-bond C_α – C_β couplings. For typical sets of ^{13}C chemical shifts in peptides and proteins, we find that MAS frequencies near 40 kHz generally allow adequate truncation of the relevant ^{13}C – ^{13}C couplings in experiments at 17.6 T.

As demonstrated in Figs. 7(a) and 8, the ZQ-SEASHORE concept allows quantitative constraints on backbone ^{13}CO – ^{13}CO distances to be obtained for peptides and proteins that are uniformly ^{13}C -labeled, or are prepared with two or more sequential uniformly labeled residues. Backbone ^{13}CO – ^{13}CO distances depend on backbone ϕ torsion angles (although ψ torsion angles affect the angles between successive ^{13}CO – ^{13}CO internuclear vectors and therefore must also be considered when analyzing data for uniformly labeled samples). We have previously shown that constraints on backbone ^{15}N – ^{15}N distances, which depend on ψ torsion angles, can be obtained for uniformly ^{15}N , ^{13}C -labeled peptides and proteins with the PITHIRDS-CT technique.^{14,33} Thus, the ZQ-SEASHORE and PITHIRDS-CT techniques provide a route to obtaining full sets of quantitative backbone conformational constraints for uniformly labeled samples. As shown in Fig. 8 and in our earlier work,^{14,33} constraints on ^{13}CO – ^{13}CO or ^{15}N – ^{15}N distances can be obtained even when the ^{13}CO or ^{15}N NMR lines are poorly resolved, by transferring ^{13}CO or ^{15}N polarization to directly bonded $^{13}\text{C}_\alpha$ sites and employing multidimensional spectroscopy.

As demonstrated in Figs. 6(a) and 7(c), the ZQ-SEASHORE concept can also be used to measure distances between backbone and sidechain carbons, in principle placing constraints on sidechain conformations. Conformational information is contained in distances between backbone carbonyl and sidechain methyl sites (for methionine, isoleucine, leucine, valine, and threonine residues), between backbone α -carbon and sidechain methyl sites (for methionine, isoleucine, and leucine residues), between backbone carbonyl and sidechain carbonyl or carboxylate sites (for aspartate, asparagine, glutamate, and glutamine residues), between backbone α -carbon and sidechain carbonyl or carboxylate sites (for glutamate and glutamine residues), between backbone carbonyl and sidechain aromatic carbon sites (for phenylalanine, tyrosine, tryptophan, and histidine residues), and between backbone carbonyl or α -carbon sites and sidechain δ - or ϵ -carbon sites (for lysine and arginine residues). Conformational constraints from intraresidue carbon-carbon distances are complementary to constraints from intraresidue nitrogen-carbon distances that have been obtained with frequency-selective heteronuclear recoupling methods.^{12,35}

Finally, it may also be possible to obtain quantitative constraints on tertiary contacts or on intermolecular distances in supramolecular structures from ZQ-SEASHORE measurements. Together with quantitative constraints on backbone and sidechain conformations, such measurements would represent a significant improvement on the more qualitative approaches that have been employed in recent structural studies of proteins by solid state NMR.^{1–4} In particular, we note that information about tertiary contacts or intermolecular contacts

derived from proton-driven spin diffusion¹⁶ or proton-assisted three-spin effects³⁶ is useful for establishing proximities of amino acid sidechains or other aspects of protein structures, but that such measurements do not yield precise distances between specific pairs of nuclei.

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