Dipolar truncation in magic-angle spinning NMR recoupling experiments

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Quantitative solid-state NMR distance measurements in strongly coupled spin systems are often complicated due to the simultaneous presence of multiple noncommuting spin interactions. In the case of zeroth-order homonuclear dipolar recoupling experiments, the recoupled dipolar interaction between distant spins is attenuated by the presence of stronger couplings to nearby spins, an effect known as dipolar truncation. In this article, we quantitatively investigate the effect of dipolar truncation on the polarization-transfer efficiency of various homonuclear recoupling experiments with analytical theory, numerical simulations, and experiments. In particular, using selectively ¹³C-labeled tripeptides, we compare the extent of dipolar truncation in model three-spin systems encountered in protein samples produced with uniform and alternating labeling. Our observations indicate that while the extent of dipolar truncation decreases in the absence of directly bonded nuclei, two-bond dipolar couplings can generate significant dipolar truncation of small, long-range couplings. Therefore, while alternating labeling alleviates the effects of dipolar truncation, and thus facilitates the application of recoupling experiments to large spin systems, it does not represent a complete solution to this outstanding problem. © 2009 American Institute of Physics.

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I. INTRODUCTION

In the last decade, high resolution solid-state NMR has emerged as the method of choice for structural investigations of complex, insoluble systems such as membrane and amyloid proteins. Advances in solid-state NMR methodology that are essential for these experiments include the improved resolution available with magic-angle spinning (MAS), heteronuclear decoupling schemes, and optimized sample preparation techniques. Significant advances have also been achieved with the development of efficient dipolar recoupling schemes, which enable the measurement of internuclear distances by reintroducing the dipole-dipole coupling otherwise averaged out by MAS.¹ In particular, a large number of heteronuclear and homonuclear recoupling pulse sequences are now available and applicable to a variety of experimental conditions.^{2–21} Most of the recoupling sequences generate in a zeroth-order approximation an effective dipolar-coupling Hamiltonian that contains zero-quantum (ZQ) or double-quantum (DQ) two-spin operators. In this publication we focus primarily on zeroth-order sequences. Second-order or higher-order recoupling sequences^{22–25} have different properties because they contain cross terms between two interactions and the effective coupling constants depend on both interactions.

In spite of the advances in pulse sequence design, the successful application of recoupling methods to the accurate

measurement of ¹³C–¹³C distance in macromolecules has typically been possible only when employing either specifically labeled spin-pair samples^{26–37} or frequency-selective recoupling methods,^{38–47} experiments in which a single internuclear distance is measured at a time. Although highly accurate ($\pm 5\%$ is not difficult to achieve), both of these approaches are of limited practical utility in protein structure determinations that benefit from the availability of hundreds of constraints even for small proteins. For instance, the most efficient rotational resonance based methods^{43,46,48} yield only a modest number of relevant constraints,⁴⁸ and are thus most useful at the refinement stage of a structure determination process and for addressing specific mechanistic questions. On the other hand, broadband recoupling schemes applied to uniformly labeled samples offer the possibility of measuring many internuclear distances simultaneously. However, the deleterious effects of multiple-spin interactions interfere with the efficiency and analysis of zeroth-order broadband homonuclear dipolar recoupling schemes.^{11,15,49–52} In particular, the recoupling of a small dipolar coupling between a distant spin pair is severely attenuated in the presence of a third spin strongly coupled to one of the first two, an effect known as dipolar truncation.⁵² As a result of dipolar truncation, the multiple-spin recoupled dipolar interactions are dominated by the strong dipolar coupling, prohibiting the measurement of the long-range distances that are important in constraining structures.

The dipolar truncation effect has impeded the application of many highly efficient zeroth-order broadband homonuclear recoupling sequences to the simultaneous measurement of multiple long internuclear ¹³C–¹³C distances in uni-

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formly labeled proteins. Therefore, a potential approach to attenuating dipolar truncation effects is to employ sparse isotopic labeling methods, such as the alternating labeling scheme introduced by LeMaster and Kushlan,⁵³ which produces few directly bonded pairs of ^{13}C nuclei. Indeed, this labeling scheme has been employed in solid-state protein NMR with the intention of eliminating dipolar truncation by generating spin systems without strong dipole-dipole couplings for most amino acid residues.^{54–56} However, as we will see below, the remaining two-bond couplings (0.5 kHz as opposed to 2.2 kHz) already result in significant attenuation of the structurally interesting long-range constraints.

In this paper we present a study of the dipolar truncation effect in homonuclear dipolar recoupling experiments for long-range polarization transfer. First, we present a theoretical description of dipolar truncation, and then we investigate its effect on various zeroth-order recoupling pulse sequences via numerical simulations. Finally, using a set of three selectively labeled samples, we demonstrate dipolar truncation experimentally and illustrate its dependence on relative dipolar-coupling magnitudes. The model systems studied allow us to compare the extent of dipolar truncation observed in homonuclear dipolar recoupling of protein spin systems produced by uniform and alternating ^{13}C labeling.

II. THEORY

In this paper we focus on the effects of the reintroduction of dipolar interactions using rotor-synchronized pulse sequences¹⁷ on the time evolution of the spin system during MAS. Depending on the form of the effective Hamiltonian,

dipolar recoupling sequences are classified into DQ and ZQ experiments. In the case of DQ recoupling sequences, the recoupled zeroth-order effective Hamiltonian for a three-spin system has been shown to be^{4,6,7}

$$\bar{H}_{\text{DQ}} = \sum_{\substack{i,j=1 \\ i < j}}^3 C_{ij,\text{DQ}} [I_i^+ I_j^+ + I_i^- I_j^-], \quad (1)$$

where $C_{ij,\text{DQ}}$ represents the scaled dipolar coefficient between spins i and j and is a function of several variables including the spin-system parameters as well as the scaling factors due to the rf pulses. In a similar vein the recoupled zeroth-order effective Hamiltonian in the ZQ case is represented by^{8,14,38}

$$\bar{H}_{\text{ZQ}} = \sum_{\substack{i,j=1 \\ i < j}}^3 C_{ij,\text{ZQ}} [I_i^+ I_j^- + I_i^- I_j^+]. \quad (2)$$

Since, the recoupled dipolar Hamiltonians [Eqs. (1) and (2)] among the various spin pairs are homogeneous in nature (i.e., the interactions do not commute with one another), the resulting spin dynamics is more complicated and difficult to analyze.

To elucidate the effects of dipolar truncation and the mechanism of polarization transfer in a strongly coupled spin system, we employ a state-space approach (or indirect method) to describe the spin dynamics. Using the standard basis functions for a three-spin system, the ZQ and DQ spin Hamiltonians derived in the appropriate interaction frame to zeroth order is represented by Eqs. (3) and (4), respectively.

	$ \beta\beta\beta\rangle$	$ \alpha\beta\alpha\rangle$	$ \beta\alpha\alpha\rangle$	$ \alpha\alpha\beta\rangle$	$ \alpha\alpha\alpha\rangle$	$ \beta\alpha\beta\rangle$	$ \alpha\beta\beta\rangle$	$ \beta\beta\alpha\rangle$
$\langle\beta\beta\beta $	0	0	0	0				
$\langle\alpha\beta\alpha $	0	0	C_{12}	C_{23}				
$\langle\beta\alpha\alpha $	0	C_{12}	0	C_{13}				
$\langle\alpha\alpha\beta $	0	C_{23}	C_{13}	0				
$\langle\alpha\alpha\alpha $					0	0	0	0
$\langle\beta\alpha\beta $					0	0	C_{12}	C_{23}
$\langle\alpha\beta\beta $					0	C_{12}	0	C_{13}
$\langle\beta\beta\alpha $					0	C_{23}	C_{13}	0

(3)

	$ \beta\alpha\beta\rangle$	$ \alpha\alpha\alpha\rangle$	$ \beta\beta\alpha\rangle$	$ \alpha\beta\beta\rangle$	$ \alpha\beta\alpha\rangle$	$ \beta\beta\beta\rangle$	$ \alpha\alpha\beta\rangle$	$ \beta\alpha\alpha\rangle$
$\langle\beta\alpha\beta $	0	C_{13}	0	0				
$\langle\alpha\alpha\alpha $	C_{13}	0	C_{12}	C_{23}				
$\langle\beta\beta\alpha $	0	C_{12}	0	0				
$\langle\alpha\beta\beta $	0	C_{23}	0	0				
$\langle\alpha\beta\alpha $					0	C_{13}	0	0
$\langle\beta\beta\beta $					C_{13}	0	C_{12}	C_{23}
$\langle\alpha\alpha\beta $					0	C_{12}	0	0
$\langle\beta\alpha\alpha $					0	C_{23}	0	0

(4)

In this representation of the effective Hamiltonian, the recoupling sequences are assumed to be ideal, i.e., the chemical shifts are ignored and the Hamiltonian is block diagonal. If the coupling between spins 1 and 3 is set to zero ($C_{13}=0$), then we have a representation for the Hamiltonian that is identical for both ZQ and DQ recoupling sequences. Such an approach allows derivation of analytical formulas that are common to both types of sequences. Following the

standard procedure, the time dependence of any observable is calculated by

$$\langle O \rangle(t) = \text{Tr}[O\rho(t)] = \text{Tr}[OU(t,0)\rho(0)U^{-1}(t,0)]. \quad (5)$$

Since the effective Hamiltonian is block diagonal, the effective propagator $U(t,0)$ [i.e., $U(t,0)=\exp(-i\bar{H}t)$] for both types of sequences can be represented as depicted in Eq. (6).

$$U(t,0) = \begin{array}{c|cccc} & |1\rangle & |2\rangle & |3\rangle & |4\rangle \\ \hline \langle 1| & 1 & 0 & 0 & 0 \\ \langle 2| & 0 & \cos(\omega t) & -i\frac{C_{12}}{\omega}\sin(\omega t) & -i\frac{C_{23}}{\omega}\sin(\omega t) \\ \langle 3| & 0 & -i\frac{C_{12}}{\omega}\sin(\omega t) & \frac{C_{12}^2}{\omega^2}\cos(\omega t) + \frac{C_{23}^2}{\omega^2} & \frac{C_{12}C_{23}}{\omega^2}(\cos(\omega t) - 1) \\ \langle 4| & 0 & -i\frac{C_{23}}{\omega}\sin(\omega t) & \frac{C_{12}C_{23}}{\omega^2}(\cos(\omega t) - 1) & \frac{C_{23}^2}{\omega^2}\cos(\omega t) + \frac{C_{12}^2}{\omega^2} \end{array} + \begin{array}{c|cccc} & |5\rangle & |6\rangle & |7\rangle & |8\rangle \\ \hline \langle 5| & 1 & 0 & 0 & 0 \\ \langle 6| & 0 & \cos(\omega t) & -i\frac{C_{12}}{\omega}\sin(\omega t) & -i\frac{C_{23}}{\omega}\sin(\omega t) \\ \langle 7| & 0 & -i\frac{C_{12}}{\omega}\sin(\omega t) & \frac{C_{12}^2}{\omega^2}\cos(\omega t) + \frac{C_{23}^2}{\omega^2} & \frac{C_{12}C_{23}}{\omega^2}(\cos(\omega t) - 1) \\ \langle 8| & 0 & -i\frac{C_{23}}{\omega}\sin(\omega t) & \frac{C_{12}C_{23}}{\omega^2}(\cos(\omega t) - 1) & \frac{C_{23}^2}{\omega^2}\cos(\omega t) + \frac{C_{12}^2}{\omega^2} \end{array} \quad (6)$$

The ket vectors $|1\rangle, |2\rangle \dots |8\rangle$, are identical to the basis functions employed in the corresponding zero and DQ Hamiltonians illustrated in Eqs. (3) and (4), respectively. The coupling between spins 1 and 3 is set to zero.

With this formulation, the mechanism of polarization transfer and the effects of dipolar truncation are transparent. Starting with polarization on spin 2 only [i.e., $\rho(0)=I_{2z}$], the expectation values for ZQ polarization transfer to spins 1 and 3 from spin 2 are given by

$$\begin{aligned} \langle I_{1z} \rangle(t) &= \frac{C_{12}^2}{(C_{12}^2 + C_{23}^2)} \sin^2(\omega t), \\ \langle I_{2z} \rangle(t) &= \cos^2(\omega t), \\ \langle I_{3z} \rangle(t) &= \frac{C_{23}^2}{(C_{12}^2 + C_{23}^2)} \sin^2(\omega t), \end{aligned} \quad (7)$$

where $\omega = \sqrt{C_{12}^2 + C_{23}^2}$. The equations illustrated above are identical for DQ transfer, except for a minus sign in the denominator of the expressions for $\langle I_{1z} \rangle$ and $\langle I_{3z} \rangle$. It is important to note that there is only a single frequency present in the time evolution under such a zeroth-order effective Hamil-

tonian. This is even true if all three dipolar couplings are present in the three-spin system. Only the relative intensities will change with a change in the relative magnitude of the dipolar coefficients. Neglecting relative orientations, when the two dipolar couplings are of equal magnitude ($C_{23} = C_{12}$), polarization transfer is identical between the two pairs of coupled spins. However, when one of the dipolar couplings is stronger, for instance, between spins 2 and 3, it dominates the dynamics and causes polarization transfer to occur mostly from spin 2 to spin 3, reducing the extent of transfer from spin 2 and spin 1. For example, if $C_{23}=2C_{12}$ then 80% of the polarization from spin 2 is transferred to spin 3 and only 20% is transferred to spin 1. Moreover, if the couplings differ by an order of magnitude then the extent of transfer drops by two orders of magnitude, even in the quasiequilibrium state.⁵⁷

The above description of dipolar truncation illustrates the magnitude of the expected effects in an idealized three-spin system that was initially outlined by Costa in 1996.⁵² Detailed equations for the full three-spin system under DQ recoupling can be found in Hohwy *et al.*¹¹ However, a more realistic analysis of dipolar truncation requires consideration

of higher-order contributions to the effective Hamiltonians, which contain cross terms between different interactions in the spin-system Hamiltonian. For this reason, we performed numerical simulations and dipolar recoupling experiments on model systems, the results of which are described in Sec. IV. The zeroth-order average Hamiltonian generated by radio frequency-driven recoupling^{3,8} (RFDR) is of the general form shown in Eq. (2) with an effective coupling constant that depends not only on the magnitude of the dipolar coupling but also on the ratio of the isotropic chemical shift differences and the spinning frequency.¹⁷

III. EXPERIMENTAL METHODS

A. NMR experiments

NMR experiments were performed on a custom designed spectrometer operating at a field of 11.7 T (500.125 MHz for ^1H , courtesy of D. J. Ruben) using a Chemagnetics triple resonance MAS probe equipped with a 4.0 mm spinning module. The peptide samples (~ 20 mg) were packed in the center third of the rotor to reduce the inhomogeneity of the rf field to $\sim 5\%$. All the experiments consisted of ^1H – ^{13}C cross polarization (CP) followed by a mixing period and detection. In two-dimensional (2D) RFDR experiments, a chemical shift evolution period was inserted between the CP and the mixing period. The RFDR mixing period consisted of rotor-synchronized 50 kHz ^{13}C π pulses, with concurrent ^1H cw decoupling fields of 115 and 90 kHz during ^{13}C pulses and the windows between them, respectively, optimized to avoid polarization losses due to heteronuclear interference.^{8,21} During chemical shift evolution and detection periods, 83 kHz two-pulse phase-modulated⁵⁸ (TPPM) decoupling was used applied. For each 2D RFDR spectrum, 280 t_1 points, with four scans each, were collected, with 512 points in the direct dimension. Data processing included shifted sine bell apodization and zero filling to 1024 points in each dimension. Spectra were processed and analyzed using NMRPIPE.⁵⁹

B. Selectively labeled samples

The experimental results reported in this article were obtained utilizing three selectively labeled tripeptides illustrated in Fig. 1: Ala-Gly-Gly (Ref. 60) labeled as Ala-[2- ^{13}C]Gly-[1- ^{13}C]Gly (AGG-2) and Ala-[1,2- $^{13}\text{C}_2$]Gly-[1- ^{13}C]Gly (AGG-3), and Gly-Gly-Val (Ref. 61) labeled as [1- ^{13}C]Val-[2- ^{13}C]Gly-[1- ^{13}C]Gly (GGV-3). All three nitrogen sites were ^{15}N labeled in all three compounds.

The AGG-2 sample is a weakly coupled isolated spin pair with a dipole-dipole coupling of 66 Hz and serves as a reference system for polarization-transfer efficiency. The samples AGG-3 and GGV-3 are three-spin systems formed by incorporating a third spin into the two-spin system of AGG-2. In AGG-3, the third spin is directly bonded to spin 2, thus exhibiting a strong ^{13}C – ^{13}C coupling (2.150 kHz) while the coupling between spins 1 and 3 is only 150 Hz. The third spin in GGV-3 is two bonds away from spin 2 and thus represents a moderate (550 Hz) coupling. AGG-3 and GGV-3 are simple models for the geometries encountered in

FIG. 1. (Color) Labeling schemes employed in the model tripeptides used to illustrate the effect of dipolar truncation. AGG-2 (Ala-[2- ^{13}C]Gly-[1- ^{13}C]Gly) is a two-spin system with a dipolar coupling of 66 Hz, corresponding to a distance of 4.86 Å between nuclei 1 and 2. AGG-3 (Ala-[1,2- $^{13}\text{C}_2$]Gly-[1- ^{13}C]Gly) is a three-spin system formed by adding a third labeled nucleus (spin 3) to AGG-2, forming a strong dipolar coupling of 2.15 kHz with spin 2. The coupling between spins 1 and 3 is 150 Hz. GGV-3 ([1- ^{13}C]Val-[2- ^{13}C]Gly-[1- ^{13}C]Gly) is a three-spin system similar to AGG-3, but with the third labeled nucleus (spin 3) two bonds away from spin 2. GGV-3 has a weak coupling of 80 Hz corresponding to a distance of 4.56 Å between spins 1 and 2, and a medium coupling of 550 Hz corresponding to a distance of 2.43 Å between spins 2 and 3.

uniform and alternating labeling of amino acids, respectively. All the samples were diluted to 10% in natural abundance lattice to minimize the effects of intermolecular ^{13}C – ^{13}C dipolar couplings.

C. Numerical simulations

To evaluate dipolar truncation in various homonuclear recoupling sequences, numerical simulations were programmed in C++ using the GAMMA spin-simulation environment.⁶² All simulations were powder averages of 1154 orientations, where the individual orientations were determined by the method of Cheng *et al.*⁶³ and Conroy.⁶⁴ The time dependence of the Hamiltonian was approximated by subdividing each rotor period into at least 100 time steps with a time-constant Hamiltonian. Only the ^{13}C spins were simulated. The chemical shift anisotropy (CSA) parameters were estimated using the SIMMOL package,⁶⁵ based on the crystal structures.^{60,61} In addition, the SPINEVOLUTION program⁶⁶ was used to simulate the experimental RFDR data. Because of the efficiency of this program, this second set of simulations included not only ^{13}C spins, but several ^1H spins and the exact rf-irradiation scheme employed in the experiments in order to reproduce the experimentally observed polarization-transfer profiles as closely as possible.

FIG. 2. (Color) Simulated buildup curves of magnetization transfer from spin 2 (blue) to spin 1 (red) and spin 3 (green) in model spin systems AGG-2, AGG-3, and GGV-3 with various recoupling sequences. ZQ sequences: RFDR, $\omega_r/2\pi=10$ kHz, $\omega_1=50$ kHz; $SR4_4^1$, $\omega_r/2\pi=30$ kHz, $\omega_1=30$ kHz. DQ sequence: $R12_2^5$, $\omega_r/2\pi=15$ kHz, $\omega_1=90$ kHz. Mixed sequence: DRAWS, $\omega_r/2\pi=10$ kHz, $\omega_1=85$ kHz.

IV. RESULTS AND DISCUSSION

A. Dipolar truncation in homonuclear recoupling schemes: Numerical simulations

The results of numerical simulations demonstrating the effect of dipolar truncation for various recoupling sequences are presented in Fig. 2. These simulations start with magnetization on spin 2 and monitor the transfer to the distant spin (spin 1) and to the near spin (spin 3). The polarization-transfer efficiency in an isolated spin pair (AGG-2 in Fig. 2) varies for different recoupling schemes depending on their particular characteristics such as scaling factor and compensation for chemical shift terms, as examined previously. Recoupling of the weak dipole-dipole coupling corresponding to the 4.86 Å internuclear distance in the AGG-2 spin system requires long mixing times, which makes the contributions from small error terms that appear in higher-order contributions to the effective Hamiltonian more visible. Nevertheless, a variety of recoupling schemes show significant polarization transfer in such an isolated two-spin system.

Upon introduction of a third spin (spin 3) presenting a one-bond coupling to one of the spins in the original spin pair (spin 2), a dramatic reduction in polarization transfer to the distant spin (spin 1) is observed, as the simulations for AGG-3 in Fig. 2 illustrate. This happens despite the fact that in AGG-3 a relay polarization-transfer pathway from spin 2 via spin 3 to spin 1 is possible and most likely faster than the direct polarization transfer from spin 2 to spin 1. Indeed, dipolar truncation of a weak coupling by a one-bond ^{13}C – ^{13}C coupling has a severe effect in all the recoupling schemes we studied, with polarization transfer to the distant ^{13}C spin reduced to a few percent in the most favorable cases ($SR4_4^1$ and RFDR). In the case of a third spin with a two-bond coupling (GGV-3 in Fig. 2), the transfer efficiency to the distant spin increases for all schemes compared to the one-bond dipolar truncation case, with considerable improvements for the ZQ recoupling sequences RFDR and

$SR4_4^1$. However, comparing the simulations for GGV-3 to those for the isolated distant spin pair (AGG-2), it is evident that a two-bond coupling is sufficient to cause significant dipolar truncation of the weak dipolar coupling in this model three-spin system. These results are in agreement with the expectations from the analytical solutions, which predict significant truncation effects for two dipolar couplings that differ by a factor of 7. However, the process that finally leads to the transfer to the distant spin is not described by the zero-order three-spin model because it has a slower characteristic time constant than the direct transfer, in contrast to the expectations from this model Hamiltonian which predicts all transfers to proceed with the same frequency.

Similar results were obtained from simulations of other pulse sequences such as SPC-5,¹¹ POST-C7,⁹ $R16_2^7$ and $R20_2^9$.¹⁹ The pulse sequences presented in Fig. 2 were selected to represent various classes of homonuclear recoupling sequences, namely, ZQ π -pulse: RFDR;^{3,8} ZQ symmetry-based; $SR4_4^1$,^{19,67} DQ symmetry-based $R12_2^5$,^{19,68} and mixed zero- and DQ: DRAWS.^{2,69} We restricted our investigation to zeroth-order dipolar recoupling sequences that produce a zeroth-order recoupled dipolar Hamiltonian, which are the ones typically used to obtain accurate internuclear distance measurements. We specifically avoided the treatment of proton-driven spin diffusion,^{70–72} DARR/RAD,^{73–75} homonuclear TSAR^{22,23} techniques and MIRROR²⁵ recoupling, which perform polarization transfer via higher-order mechanisms that are more tolerant to dipolar truncation effects in multiple-spin systems, as shown previously,^{22,51} but typically yield ambiguous distance constraints due to the complexity of the mechanisms.

B. Experimental characterization of dipolar truncation in RFDR spectra

We investigated the dipolar truncation effect on our model three-spin systems experimentally using the RFDR

FIG. 3. (Color) Cross sections of 2D RFDR correlation spectra through the diagonal peak of spin 2 for three different mixing times. The arrow indicates the cross peak between spin 2 (a C α carbon at 44 ppm) and the distant spin 1 (a carboxyl carbon at 175 ppm). The intensity of this cross peak is diminished in AGG-3 and GGV-3 compared to AGG-2, demonstrating the attenuation of polarization transfer due to the dipolar truncation effect.

pulse sequence.^{3,8,21} Polarization-transfer efficiencies (defined as cross peak intensity divided by diagonal intensity at zero mixing time) were measured in a series of broadband 2D correlation spectra with increasing RFDR mixing times following CP preparation of all ^{13}C nuclei. Experimental data from three representative mixing times are shown in Fig. 3 as cross sections of 2D spectra through the diagonal peak of spin 2 (the $^{13}\text{C}\alpha$). The arrow in Fig. 3 indicates the cross peak arising from polarization transfer between the weakly coupled spin pair (from spin 2 to spin 1), present in all three samples, while the adjacent cross peak arises from the coupling that is the source of the dipolar truncation (from spin 2 to spin 3). The cross sections in Fig. 3 show the sensitivity we were able to obtain with RFDR mixing in these challenging spin systems. Indeed, we attempted similar recoupling experiments and more time-efficient one-dimensional variants with several other pulse sequences often with less satisfactory results. We note that the 4.86 Å internuclear carboxyl/methylene distance of AGG-2 proved challenging for several recoupling sequences due to deleterious effects such as the accumulation of pulse imperfections and magnetization losses due to inadequate heteronuclear decoupling during the long mixing period. In such cases, the additional signal attenuation due to dipolar truncation made the experimental shortcomings only more evident. These technical difficulties, which vary from one recoupling

scheme to another, rendered the experimental evaluation and fair comparison of dipolar truncation for several pulse sequences rather difficult. We, therefore, present experimental data for a single recoupling scheme, RFDR, which afforded ample sensitivity to measure long-range polarization transfer and to, therefore, directly assess the dipolar truncation effect.

Figures 4(a)–4(c) present experimental curves of RFDR polarization transfer in AGG-2, AGG-3, and GGV-3. The data points correspond to integrated peak volumes from 2D spectra for the diagonal ^{13}C nucleus (spin 2) and its cross peaks to the distant carboxyl nucleus (spin 1) and the nearby carbonyl nucleus (spin 3), normalized to the intensity of the diagonal peak at zero mixing time. Numerical simulations including all parameters used in the experiments, depicted in Figs. 4(d)–4(f), are in good agreement with our experimental results.

Polarization transfer over the 4.86 Å distance of the two-spin system of AGG-2, shown in Fig. 4(a), was characterized by significant magnetization loss due to insufficient proton decoupling during the mixing period and, to a lesser degree, pulse imperfections. The maximum magnetization transfer observed in this weakly coupled spin pair was approximately 19.0%, obtained after 29 ms of RFDR mixing, the longest mixing time employed. Simulations for longer mixing periods (to 60 ms) show that the polarization builds up in AGG-2 plateaus after ~ 30 ms.

FIG. 4. (Color) Top: Experimental RFDR buildup curves for AGG-2, AGG-3, and GGV-3. Each data point is the integrated peak intensity of 2D correlation peaks normalized to the diagonal peak at zero mixing. RFDR experiments employed 50 kHz ^{13}C π pulses and 115 and 90 kHz ^1H decoupling fields during the mixing period and were performed at a MAS frequency of 8.929 kHz. Bottom: Numerical simulations of polarization transfer in AGG-2, AGG-3, and GGV-3 showing long-term behavior of RFDR mixing. These simulations included ^{13}C CSA parameters, eight neighboring ^1H spins, and experimental parameters identical to those performed in our experiments.

In the three-spin system of AGG-3, polarization transfer over the weakly coupled spin pair is considerably decreased compared to that of AGG-2, experimentally demonstrating the effect of dipolar truncation. The RFDR buildup curve of AGG-3, shown in Fig. 4(b), presents a maximum transfer efficiency of 2.7% between spins 1 and 2, with a direct dipolar coupling of 66 Hz. There is of course also relay polarization transfer from spin 2 via spin 3 to spin 1 which may be faster than the direct transfer from spin 2 to spin 1. Thus, the strong one-bond dipolar coupling between spins 2 and 3 (2.15 kHz) dominates the RFDR recoupling dynamics and causes a sevenfold reduction in transfer efficiency between the distant spin pair.

Compared to AGG-3, Fig. 4(c) shows that the effect of dipolar truncation is diminished in the GGV-3 spin system, in which the RFDR recoupling dynamics are dominated by a dipolar coupling of moderate magnitude (two-bond coupling, 550 Hz) instead of the one-bond coupling present in AGG-3. The maximum polarization transfer achieved in the weakly coupled spin pair (spins 1 and 2) of GGV-3 was 7.0%, almost a factor of 3 smaller than the results for AGG-2 but a considerable improvement in long-range polarization transfer over the AGG-3 case. Even though the weak coupling in GGV-3 is slightly larger than that of AGG-3 (80 and 66 Hz, respectively), the increased transfer efficiency is primarily the result of the smaller dipolar coupling effecting truncation in GGV-3, as simulations similar to those in Fig. 2 indicate (data not shown).

However, in spite of the improvement in GGV-3 over AGG-3, the approximately twofold reduction in long-range recoupling efficiency compared to AGG-2 shows that dipolar truncation is still significant in the GGV-3 spin system. These results demonstrate that dipolar truncation of weak couplings is diminished in the absence of the strong couplings formed by directly bonded nuclei, and yet, it is not fully attenuated in the presence of medium-strength couplings such as those arising from two-bond ^{13}C – ^{13}C interactions. Therefore, the majority of homonuclear recoupling schemes can be expected to suffer partial dipolar truncation during long-range ^{13}C polarization transfer in proteins whether they are enriched uniformly or with the alternating scheme, although to different degrees. On the other hand, the attenuation of dipolar truncation by removal of one-bond couplings yields favorable spin systems and allows efficient long-range recoupling, as exemplified by our RFDR results.

V. CONCLUSIONS

We have demonstrated the effect of dipolar truncation in model three-spin systems and shown its dependence on the magnitude of the dominating dipolar coupling. The samples we used were selected to model spin systems encountered in proteins produced with uniformly and alternating labeling. Our results indicate that the deleterious effect of dipolar truncation in many zeroth-order homonuclear recoupling schemes is moderately alleviated, yet it is still significant, in the alternating labeling scenario. Therefore, while alternating

labeling should facilitate the application of schemes that typically show dipolar truncation effects, it does not represent a complete solution to the problem.

As our experimental results suggest, the inefficiency of long-range dipolar recoupling with existing recoupling schemes is due in part to the limitations typically associated with implementing lengthy mixing times, such as intense heteronuclear decoupling fields. In this regard, recoupling techniques that function well in the absence of ^1H decoupling^{16,20,21} may offer an important improvement over other schemes. Beyond practical considerations, dipolar truncation is a fundamental problem in homonuclear dipolar recoupling with a severe deleterious effect, particularly in uniformly labeled systems. Addressing dipolar truncation has led to the design of a variety of interesting experimental schemes^{29,46,76–79} and remains an active area of research in our and other laboratories. Finally it should be noted that, in the large spin systems of proteins,⁸⁰ the multitude of dipolar interactions can be expected to present complexities in addition to dipolar truncation (e.g., relayed polarization transfer) that further complicate the analysis of dipolar recoupling experiments in such systems and will need to be addressed in future work.

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