Low-Power Solid-State NMR Experiments for Resonance Assignment under Fast Magic-Angle Spinning

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Solid-state NMR has evolved in the past decade into a powerful technique for the characterization of biomolecular structure and dynamics. Micro-crystalline globular proteins, amyloid fibrils, and membrane proteins can now be routinely studied using solid-state NMR techniques. This was made possible in part due to the development of 2D and 3D homonuclear and heteronuclear experiments that correlate $^{13}$C and $^{15}$N spins for resonance assignment as well as for obtaining long-range distance restraints in structure elucidation.

Remarkable developments in magic-angle spinning (MAS) probe technology also contributed to this success. Now, a new generation of commercially available 1.3 mm probes can reach above 60 kHz of MAS. This allows for more efficient averaging of strong dipolar couplings, hence providing better resolution in highly crowded protein spectra.

On the other hand, fast spinning reduces the effectiveness of many of the routinely used NMR experiments for obtaining resonance assignments. For example, at low MAS ($\approx$ 15 kHz), $^{13}$C-$^{13}$C correlations are often measured by proton-driven spin diffusion (PDSD). Under very fast MAS, efficient averaging of dipolar couplings renders PDSD experiments ineffective. Instead, selective dipolar recoupling of spins becomes necessary to allow for efficient polarization transfer.

Herein, we introduce a complete set of low-power solid-state NMR experiments sufficient for protein resonance assignment under fast MAS (>$60$ kHz), including sequential $^{15}$N-$^{13}$C correlation experiments. The low rf (radio frequency)-field requirements of our experiments prevent considerable heating of the sample, thus avoiding protein degradation and making this approach well-suited for the investigation of temperature-sensitive biomolecules.

As an application, NCA, N(CO)CA, and $^{13}$C-$^{13}$C correlation spectra were recorded at 60 kHz MAS on less than 1 mg of $\text{[}^{13}$C, $^{15}$N$\text{]}$ isotope-labeled sample. We also demonstrate that our approach can be readily performed on protein samples in which the $^1$H $T_1$ relaxation times are shortened by means of paramagnetic doping. Here, the reduced recycle delay enhances the sensitivity but requires the use of NMR sequences with low-power deposition, as described herein.

Figure 1 presents the different pulse schemes that were used to obtain $^{15}$N-$^{13}$C and $^{13}$C-$^{13}$C correlations. At low MAS, the initial cross-polarization (CP) transfer from protons to low-$\gamma$ nuclei generally requires high power irradiation on both channels. In contrast, under fast MAS, efficient CP transfer is also possible at low rf fields.

Our pulse schemes use second-order cross-polarization (SOCP) for the initial magnetization transfer. SOCP at the $n = 0$ Hartman–Hahn condition relies on second-order cross-terms between homonuclear and heteronuclear couplings. SOCP works efficiently at low rf fields if sufficient care is taken to avoid detrimental dipolar and/or CSA recoupling conditions at the used rf-field amplitudes. We employed rf fields of 9 kHz—well below all resonance conditions. SOCP is intrinsically band-selective as only weak rf fields are applied. The rf fields employed here are sufficient to excite all $^{15}$N protein backbone

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resonances in $^{15}$N–$^{13}$C correlation experiments but give rise to a band-selective transfer to Cα or CO in $^{13}$C–$^{13}$C correlation experiments. For the $^{15}$N–$^{13}$C transfer, we make use of a SPECIFIC-CP\textsuperscript{21} step. SPECIFIC zero quantum (ZQ) or double quantum (DQ) transfer is possible whenever one of the following conditions is fulfilled (Eq. (1)):

$$\sqrt{\Omega_I^2 + \omega_I^2} \pm \sqrt{\Omega_S^2 + \omega_S^2} = n\omega_r; \quad n = \pm 1, \pm 2$$

where + and − on the left-hand side of the equation apply to DQ and ZQ transfer, respectively. $\Omega_I$ and $\omega_I$ are chemical shift offsets, $\omega_I$ and $\omega_S$ are rf-field amplitudes for I and S spins, respectively, and $\omega_r$ is the spinning frequency. In the absence of chemical shift offsets, the above equation is the familiar selection rule for DQ and ZQ transfer in normal CP experiments. At high MAS, DQ CP can be easily matched with low rf fields. The DQ CP condition was first investigated by Meier\textsuperscript{22} and more recently exploited by Emsley and co-workers\textsuperscript{23} in the context of band-selective $^1$H–$^{13}$C polarization transfer in the high MAS regime. We used the low rf-field capabilities of DQ CP for $^{15}$N–$^{13}$C heteronuclear magnetization transfer similar to recent work by Ishii and co-workers\textsuperscript{17}.

For homonuclear polarization transfer from CO to Cα in the N(CO)CA experiment (see Figure 1 b), a ramped pulse (100 to 80%) around the HORROR\textsuperscript{24} dipolar recoupling condition—corresponding to 30 kHz rf field at 60 kHz MAS—is utilized. On an 800 MHz spectrometer, the rf-field amplitude employed here is sufficient to cover both Cα and CO resonances and compensates for chemical shift offsets. In the DREAM\textsuperscript{14} $^{13}$C–$^{13}$C correlation experiment (see Figure 1 c), a tangential amplitude sweep on $^{13}$C is applied.

The experiments described here use low rf fields for all the magnetization transfer periods. Accordingly, only low-power proton XIX\textsuperscript{25} (12 kHz) decoupling was used during the $t_1$ and $t_2$ periods. Note that no proton decoupling had to be employed during DQ SPECIFIC-CP, ramped $^{13}$C–$^{13}$C mixing, and DREAM dipolar recoupling periods.

As a first application of our fast MAS approach to resonance assignment, NCA, N(CO)CA, and $^{13}$C–$^{13}$C correlation spectra of PEG-precipitated uniformly $[^{13}$C,$^{15}$N] labeled ubiquitin were recorded at 60 kHz MAS. The sample contained less than 1 mg of isotope-labeled protein packed in a 1.3 mm rotor. The resulting spectra are shown in Figure 2 and exhibit excellent S/N (signal-to-noise) and resolution. Sequential correlations can be easily obtained from a combination of all three spectra as exemplified for residues V26-K27 and I30-Q31 in sequential walks (represented in Figure 2 by green lines).

Our low-power approach for resonance assignment under fast MAS is ideally combined with paramagnetic optimized relaxation times for sensitivity enhancement. As an example, we present data that was recorded on approximately 1 mg of CuII–EDTA (Cu–EDTA) doped uniformly $[^{13}$C,$^{15}$N] labeled paired

Figure 2. $^{15}$N–$^{13}$C and $^{13}$C–$^{13}$C correlation spectra of ubiquitin at 60 kHz MAS. a) NCA spectrum. b) Cα and CO regions of N(CO)CA spectrum. c) Cα–Cβ and Cα–CO regions of $^{13}$C–$^{13}$C DREAM spectrum. Carrier frequencies were 4.1 ppm on $^1$H, 119 ppm on $^{15}$N, 56 ppm on $^{13}$C for NCA and $^{13}$C–$^{13}$C, and 100 ppm on $^{13}$C for N(CO)CA except for the tangential sweep where the carrier was set to 175 ppm. Negative contours are shown in red and positive contours in blue. Sequential correlations (indicated by green lines) can be easily obtained from these spectra as exemplified for residues V26-K27 and I30-Q31. 
helical filaments (PHFs) formed from the tau construct K19. The presence of tau aggregates in neurons is a pathological hallmark of Alzheimer’s disease.\textsuperscript{[26,27]}

The reduced T\textsubscript{1} relaxation time in the Cu-EDTA-doped sample enabled us to shorten the interscan delay in our experiments to 400 ms. The NCO spectrum of K19 PHFs, shown in Figure 3, compares favourably to results obtained at lower speed both in terms of S/N and resolution.\textsuperscript{[28]}

Figure 3. NCO spectrum of uniformly \textsuperscript{13}C, \textsuperscript{15}N isotope-labeled paired helical filaments formed from the tau construct K19 doped with 150 mM Cu-EDTA. The spectrum was recorded at 60 kHz MAS on a standard-bore 800 MHz spectrometer.

In conclusion, we have shown that all the experiments needed for sequential resonance assignment, including \textsuperscript{15}N-\textsuperscript{13}C correlation experiments, can be measured at very high spinning speed and with low rf power deposition. With the full set of correlation experiments available, solid-state NMR assignment of proteins is now possible at high spinning speeds. By exploiting the better resolution at higher spinning speed, the low-power approach to resonance assignment under fast MAS described here—possibly in combination with paramagnetic optimized relaxation times—promises to be of general use for the characterization of protein structure and dynamics.

**Experimental Section**

Sample Preparation: Uniformly \textsuperscript{13}C, \textsuperscript{15}N labeled ubiquitin was recombinantly expressed in *E. coli* and purified as described before.\textsuperscript{[28]} Around 0.5–1 mg of PEG-precipitated protein sample was filled into a 1.3 mm rotor.

Tau-PHFs from the construct K19 were obtained using previously published procedures.\textsuperscript{[28]} For paramagnetic doping, about 1 mg of PHFs was incubated in 150 mM Cu-EDTA solution for two days. After centrifugation, the pellet was subsequently transferred into a 1.3 mm rotor.

Solid-State NMR Experiments: All spectra were recorded on a Bruker Avance II 800 MHz standard-bore spectrometer equipped with a 1.3 mm triple-resonance probe. MAS frequencies of 60 kHz were used in all experiments. The probe temperature was set to \(-30^\circ\text{C}\), corresponding to a sample temperature of \(\approx 30^\circ\text{C}\).

Initial polarization transfer to \textsuperscript{15}N/\textsuperscript{13}C was achieved using SOCP with rf-field amplitudes of \(\approx 9\) kHz on both channels (contact time = 4 ms for \textsuperscript{1}H–\textsuperscript{15}N and 2.5 ms for \textsuperscript{1}H–\textsuperscript{13}C, respectively). \textsuperscript{15}N-to-\textsuperscript{13}C transfers (mixing time = 5 ms) used a tangential amplitude sweep from \(23\) to \(27\) kHz on carbons (\(\Delta\text{rf} = 2\) kHz) and an rf-field amplitude of about 35 kHz applied on \textsuperscript{15}N. Double-quantum \textsuperscript{13}C–\textsuperscript{13}C mixing was accomplished by a linear amplitude ramp around 30 kHz for the N(CO)CA (mixing time = 4.5 ms) and by a tangential amplitude sweep from \(25\) to \(35\) kHz (mixing time = 4 ms, \(\Delta\text{rf} = 5\) kHz) for the DREAM \textsuperscript{13}C–\textsuperscript{13}C experiment. The total experimental times for N(CO)CA and NCA spectra were 128 and 61 h, respectively. The DREAM \textsuperscript{13}C–\textsuperscript{13}C spectrum was recorded in 14 h and the NCO spectrum of Cu-EDTA doped K19 PHFs in 19 h.

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High resolution at low power: A complete set of low-power solid-state NMR pulse schemes needed for resonance assignment of proteins under fast magic-angle spinning (> 60 kHz) is presented. The approach leads to high-resolution spectra using less than 1 mg of isotope-labeled sample (see picture).