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Ultra-high Resolution in Proton Solid-State NMR Spectroscopy at High Levels of Deuteration**

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Structure investigations of biological solids by high-resolution magic-angle spinning (MAS) solid-state NMR spectroscopy has rapidly progressed in the last few years and resulted in complete structure elucidation of several peptides and small proteins.^[1–4] Successful spectral assignment and determination of structural constraints in isotopically enriched materials (mostly ¹³C, ¹⁵N) is, however, still limited by resolution and sensitivity. A gain in sensitivity in solid-state NMR (ssNMR) experiments can in principle be achieved using direct proton detection. This technique makes use of the high gyromagnetic ratio γ of protons, a property which however, leads to broad resonance lines. Several approaches have been suggested to achieve line narrowing. Application of windowed homonuclear decoupling schemes^[5,6] yield a rescaled ¹H line width on the order of 140–400 Hz, but require large receiver bandwidths, which allows radio-frequency (RF) noise to fold into the spectral region which finally compromises overall sensitivity. In addition, the applied pulse sequences scale the ¹H chemical shift. In recent years, high-speed (35–60 kHz) MAS instrumentation has become available.^[7–9] However, even at these high spinning rates, fully protonated samples still have homogeneously broadened lines (> 500 Hz).

Alternatively, ¹H line narrowing could be achieved by isotopic spin dilution at moderate (10–20 kHz) MAS frequencies.^[10–14] Dilution is achieved by perdeuteration of the sample and subsequent back-exchange of deuterons by protons. In these experiments, the ¹H line width of most of the resonances is typically on the order 150–250 Hz or 80–150 Hz in the absence and in the presence of homonuclear ¹H,¹H decoupling, respectively. This labeling strategy allows, in addition the determination of long-range H^N–H^N distances,^[12,15,16] detection of dynamic water molecules in the protein structure^[16,17] and the characterization of protein side-chain dynamics.^[18,19]

Herein, we demonstrate that a further increase in the degree of deuteration by using a 10:90 H₂O:D₂O mixture for

recrystallization results in significant narrowing of the proton line width without loss in sensitivity. A ¹H line width on the order of 17–35 Hz can be achieved at moderate spinning frequencies (8–24 kHz) without application of homonuclear decoupling. The experiments are carried out using a perdeuterated, ¹⁵N-enriched microcrystalline sample of the SH3 domain from chicken α -spectrin. To our knowledge, this strategy yields the most dispersed ¹H correlation spectra in the solid state reported to date.

Figure 1 represents a comparison of the 2D-¹H,¹⁵N correlation spectra for the reference sample (Figure 1 B, D) and the H^N dilute sample (Figure 1 A, C). The ¹H-detected experiments (Figure 1 A at 400 MHz, Figure 1 C at 600 MHz) and the ¹⁵N-detected experiments (Figure 1 B at 400 MHz, Figure 1 D at 600 MHz) were acquired using the NMR pulse sequence shown in Figure 4 A and Figure 4 B, respectively (see also the Experimental Section). The spinning frequency for the ¹H experiments was set to 13 kHz and 10 kHz for the ¹⁵N experiments. For clarity, Figure 1 B and D are displayed such that the proton dimension appears on the horizontal axis. The proton line width is not sensitive to moderate changes in the spinning rate under homonuclear dipolar decoupling,^[6,20] if broadening conditions which arise from interference between MAS and the periodicity of the decoupling sequence are avoided.^[20] Clearly, the resolution in the proton dimension is clearly improved for the H^N dilute sample yielding a fully resolved ¹⁵N–¹H correlation spectrum of the protein even at 400 MHz. Figure 2 shows the ¹H line-width dependence as a function of the rotor period for selected residues. We find that the line width is inversely proportional to the spinning rate. This result is in agreement with previous studies which show that in the fast-spinning regime the residual dipolar line width depends linearly on the rotational frequency.^[7,21] In an investigation of protonated alanine which was embedded in a deuterated alanine matrix, Rienstra and co-workers found that the slope of the MAS-dependent ¹H line width depends only on the average proton concentration in the sample.^[22] The slope varies from 6728 Hz ms^{–1} to 970 Hz ms^{–1} depending on the degree of protonation. We observe in our studies a slope of 80.8 Hz ms^{–1} and of 144 Hz ms^{–1} for G51H^N and A56H^N, respectively. In spin systems that behave purely inhomogeneous (based on the criteria of Maricq and Waugh^[23]), the line width should not depend on the MAS rotational frequency, if ω_r is comparable or larger than the size of the interaction. Non-zero slopes of the spinning-frequency dependence of the line width indicate therefore that ¹H,¹H dipolar couplings are not totally suppressed despite the high level of deuteration. The fitted lines in Figure 2 have non-vanishing y-intercepts, implying that line broadening cannot be removed even at infinite MAS rates. The inherent transverse relaxation time T_2 , sample heterogeneity, static-field inhomogeneity (ca. 7 Hz, based on the ¹H line width of a water sample which was used for shimming the probe) might be responsible for this observation. Given the observed resolution, major hardware improvements, especially in view of the ²H lock system will be required in the future. We attribute differences in the line widths, y intercepts, and slopes for different residues to site-to-site variations in the local proton density and to local backbone dynamics.^[24,25]

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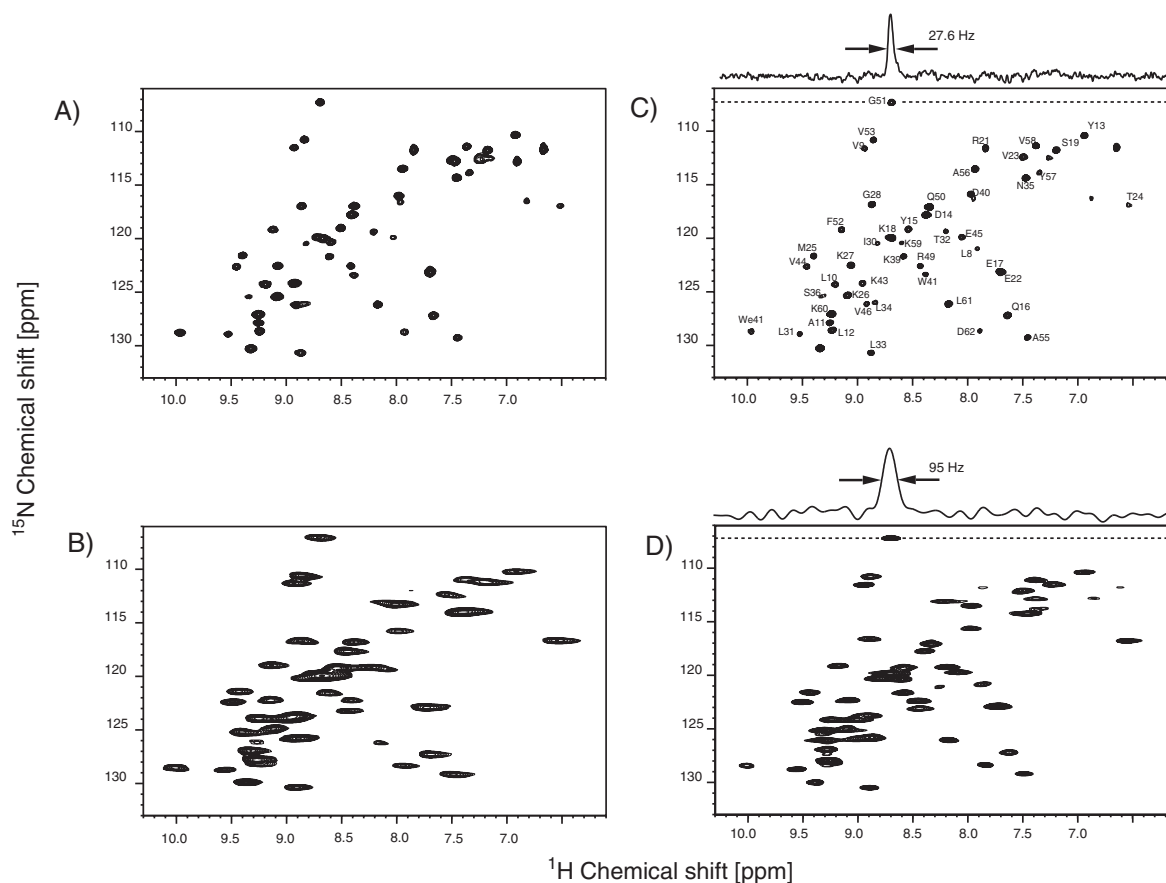


Figure 1. A,C) ^1H -detected 2D ^{15}N - ^1H correlation spectra of the H^{N} dilute sample (deuterated SH3, 10% ^1H at labile proton positions) recorded at 400 MHz (A) and 600 MHz (C). (64 scans per increment; $t_1^{\text{max}}(^{15}\text{N}) = 26.4$ ms; $t_2^{\text{max}}(^1\text{H}) = 100.0$ ms; total experimental time = 3.8 h.) B,D) ^{15}N -detected 2D ^{15}N - ^1H correlation experiments for the reference sample (deuterated SH3, 100% ^1H at labile proton positions) recorded at 400 MHz (B) 600 MHz (D). (8 scans per increment; $t_1^{\text{max}}(^1\text{H}) = 17.2$ ms; $t_2^{\text{max}}(^{15}\text{N}) = 37.0$ ms; total experimental time = 0.6 h). Acquisition of more increments in the indirect ^1H dimension did not result in a higher resolution in the ^1H dimension. All spectra were apodized using a 5 Hz lorentzian broadening in both dimensions.

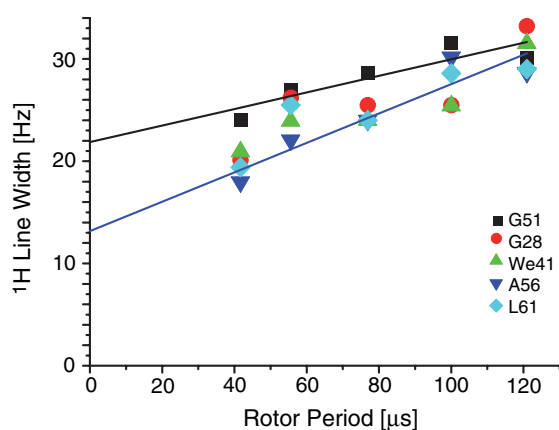


Figure 2. Dependence of the ^1H line width on the inverse MAS spinning frequency for selected residues in perdeuterated H^{N} dilute SH3.

While the ^1H line width for the H^{N} dilute sample is on the order of 17–35 Hz, the effective line width of the reference sample under phase-modulated Lee–Goldberg (PMLG) con-

ditions amounts to 80–150 Hz taking chemical shift scaling resulting from PMLG into account. The resolution in the proton dimension is therefore improved by a factor of 4–5. The ^{15}N line width in both experiments is on the order of 20–30 Hz, and is limited by the acquisition time employed. In case of the ^{15}N detected experiment, two pulsed phase modulation (TPPM) decoupling is applied during 30–37 ms in each scan. This irradiation induces significant sample heating, which reduces the life time of the sample even under good cooling conditions in case of short repetition delays.^[26] The ^1H -detected NMR experiments presented herein for the H^{N} dilute sample do not require homonuclear or heteronuclear decoupling, thus, reducing sample heating, set-up time, and possible experimental missettings. In addition, the proposed scheme does not require rescaling of the proton chemical shift, which is often problematic when spectra are acquired with homonuclear decoupling.

Figure 3 shows the experimental data for the ^1H T_1 measurements. ^1H T_1 times were found to be equal to 0.98 s (reference sample) and 1.76 s (H^{N} dilute sample), respectively. The H^{N} dilute sample has an unexpectedly short inversion recovery time T_1 , allowing a recycle delay of 2.2 s,

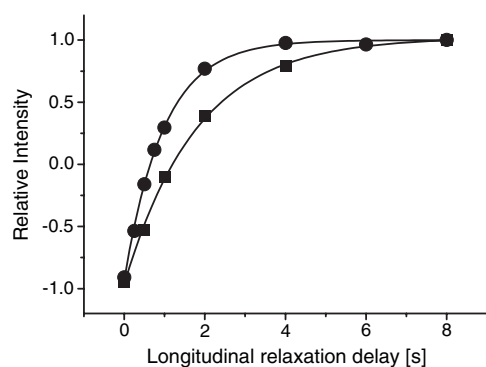


Figure 3. Experimental data for a ^1H inversion recovery experiment of $^1\text{H}^{\text{N}}$ bulk signal of the H^{N} dilute (■; ^1H T_1 = 1.76 s) and the reference sample (●; ^1H T_1 = 0.98 s).

while for the reference sample a repetition delay of 2.3 s was used to allow for dissipation of heat and to avoid sample degradation.

The improvement in ^1H line width is achieved by dilution of the proton spin density by a factor of 10. This results in a decrease of the Boltzmann magnetization in the same proportion and in an increase of the longitudinal ^1H T_1 relaxation time. These drawbacks in sensitivity are compensated by using protons for detection which yields a gain in sensitivity by a factor of 5–9.^[21] In addition, the ^1H line width is decreased by approximately a factor of 4. The signal-to-noise ratio for the ^{15}N - and ^1H -detected experiments amount to 10.4:1 and 25.3:1, respectively (determined for a ^1H cross section through the cross peak G51, as indicated in Figure 1 C and D). Taking into account the different amount of material and experimental time, the normalized signal-to-noise ratio for the H^{N} dilute sample is reduced by only a factor of 1.2 compared to the reference sample. The experiments therefore demonstrate that a high degree of deuteration leads to ultra-high-resolution proton spectra, which could not be achieved to date despite many attempts to improve homonuclear decoupling schemes and the development of fast MAS technologies. We expect that this labeling approach will enable a straight forward assignment strategy, making an additional backbone nucleus available for resonance dispersion.

Experimental Section

The employed 2D pulse scheme using proton detection is illustrated in Figure 4A. Effective suppression of the dominant water resonance was achieved by modification of the constant time (CT) experiment suggested by Zilm and co-workers.^[14] After magnetization transfer from ^1H to ^{15}N , polarization is stored along the z -axis during a variable delay ($\tau - t_1/2$), which precedes and follows the ^{15}N evolution period t_1 . Two variable delays are required to achieve J decoupling in the indirect dimension and to keep the experiment constant time with respect to water magnetization. The fixed delay τ_w (60–120 ms) which follows the CT period, is optimized for water signal suppression. After back-transfer of magnetization to ^1H , magnetization is acquired using Waltz-16 ($\omega_1 = 1.6$ kHz) for heteronuclear scalar decoupling.^[14]

Figure 4B shows the pulse sequence which was employed for the ^{15}N -detected ^1H - ^{15}N correlation spectra. PMLG-9^[27] was imple-

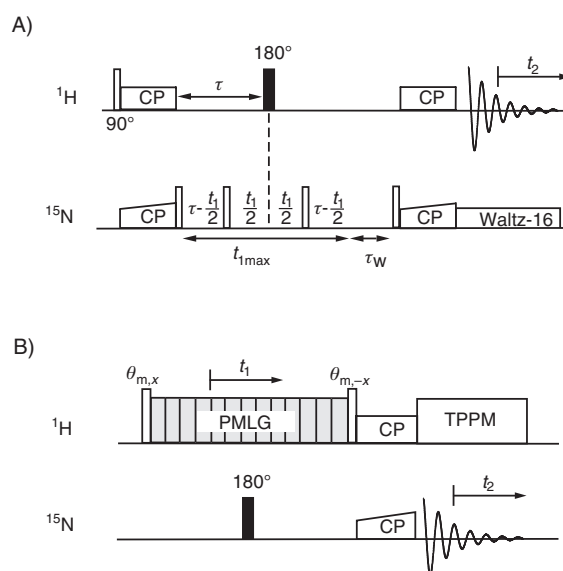


Figure 4. Pulse sequences employed for A) ^1H -detected and B) ^{15}N -detected ^{15}N - ^1H correlation experiments. CP = cross polarization.

mented in the indirect ^1H evolution period to achieve ^1H - ^{15}N dipolar decoupling ($\omega_1 = 81$ kHz). A 180° pulse on the ^{15}N channel is applied in the center of t_1 for heteronuclear J decoupling. During ^{15}N detection, TPPM proton decoupling was applied using a RF field of $\omega_1 = 90$ kHz.

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