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Progress in Proton-detected Solid-state NMR (SSNMR): Super-fast 2D SSNMR Collection for Nano-mole-scale Proteins

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Abstract

Proton-detected solid-state NMR (SSNMR) spectroscopy has attracted much attention due to its excellent sensitivity and effectiveness in the analysis of trace amounts of amyloid proteins and other important biological systems. In this perspective article, we present the recent sensitivity limit of ^1H -detected SSNMR using “ultra-fast” magic-angle spinning (MAS) at a spinning rate (ν_R) of 80–100 kHz. It was demonstrated that the high sensitivity of ^1H -detected SSNMR at ν_R of 100 kHz and fast recycling using the paramagnetic-assisted condensed data collection (PACC) approach permitted “super-fast” collection of ^1H -detected 2D protein SSNMR. A ^1H -detected 2D ^1H – ^{15}N correlation SSNMR spectrum for ~27 nmol of a uniformly ^{13}C - and ^{15}N -labeled GB1 protein sample in microcrystalline form was acquired in only 9 s with 50% non-uniform sampling and short recycle delays of 100 ms. Additional data suggests that it is now feasible to detect as little as 1 nmol of the protein in 5.9 h by ^1H -detected 2D ^1H – ^{15}N SSNMR at a nominal signal-to-noise ratio of five. The demonstrated sensitivity is comparable to that of modern solution protein NMR. Moreover, this article summarizes the influence of ultra-fast MAS and ^1H -detection on the spectral resolution and sensitivity of protein SSNMR. Recent progress in signal assignment and structural elucidation by ^1H -detected protein SSNMR is outlined with both theoretical and experimental aspects.

Graphical Abstract

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Keywords

^1H detection; solid-state NMR; ultra-fast MAS; super-fast 2D NMR

Introduction

^1H -detected high-resolution solid-state NMR (SSNMR) is gaining increasing attention for its high sensitivity and broad application in the study of biological systems such as amyloid proteins^{1–5} and membrane-bound protein assemblies,^{3–7} including ones in native cell membranes.⁸ ^1H -indirect detection was originally introduced to solution NMR in the early 1970's.^{9–11} Because of its higher sensitivity, ^1H detection has become an indispensable tool for biological solution NMR.^{12–13} In contrast, due to the limited resolution and sensitivity in ^1H SSNMR, ^1H -detection in high-resolution SSNMR did not provide any substantial sensitivity advantage until the recent development of fast magic angle spinning (MAS), over 30–40 kHz.^{1, 14–16} The recent introduction of MAS at a spinning speed of 80 kHz or higher, which is often termed “ultra-fast MAS” (UFMAS), has dramatically improved the ^1H resolution and sensitivity of SSNMR.^{2, 17–19} The simplicity of this approach has prompted us to achieve additional sensitivity gains by a series of complementary sensitivity enhancement methods, such as the use of paramagnetic relaxation enhancement,²⁰ a higher static magnetic field,²¹ more efficient probe circuits with small sample coils, and extensive deuteration.^{3, 22–23} Although the sensitivity gain achieved by each method is moderate, the cumulative sensitivity gain can reach a couple of orders of magnitude. Thus, despite initial concerns about the limited sample volume ($\sim 1\ \mu\text{L}$) for a fast MAS rotor, the UFMAS approach has been proven to be highly effective for the assignment and structural characterization of a broad range of proteins.^{2, 18–19, 24}

In this perspective article, we explored the current sensitivity limit of ^1H -detected protein SSNMR using UFMAS at a spinning rate (ν_R) of 100 kHz, a high field, and paramagnetic assisted condensed data collection (PACC).²⁰ The high sensitivity of ^1H -detected SSNMR and fast recycling in the PACC approach allowed for “super-fast” data collection of multi-dimensional SSNMR for $\sim 27\ \text{nmol}$ of a protein within only 9 s. Then, we summarized recent developments in ^1H -detected SSNMR methods and other related methods, primarily through introducing recent protein SSNMR data collected at our laboratories. Sequential assignment and structural elucidation strategies in ^1H -detected protein SSNMR are also outlined.

Theory

We compare the frequency-domain signal-to-noise (S/N) ratio (per root-square of the time) obtained by the direct detection of a spin-1/2 system (nuclei X) with that obtained by indirect ^1H detection. Following ref. 14, the sensitivity enhancement factor ξ for the 2D ^1H indirect detection over 1D direct detection of the X nuclei was given by

$$\begin{aligned}\xi &\equiv \frac{(S/N)_{ID}}{(S/N)_{DD}} \\ &= \frac{f}{\sqrt{2}} \left(\frac{\gamma_H}{\gamma_X} \right)^{3/2} \frac{\langle s_H w_H \rangle \left(t_2^{\max} \right)^{1/2}}{\langle w_H^2 \rangle^{1/2} \left(t_1^{\max} \right)} \left(\frac{Q_H}{Q_X} \right)^{1/2},\end{aligned}\quad [1]$$

where γ_M is a gyromagnetic ratio for the M nuclei ($M = \text{H}, \text{X}$), s_H is an envelope function of a ^1H time-domain signal and w_H is a window function for the ^1H signal, $\langle a \rangle$ denotes the time-averaged signal intensity of a , and $t_2^{\max}$ and $t_1^{\max}$ are the maximum t_2 and t_1 periods in the 2D indirect data collection. We assume that the signal length $t_{\max}$ collected in the 1D direct detection is $\sim t_1^{\max}$ and that the same window functions are applied to the indirectly collected X signal in the t_1 domain and the directly collected 1D X signal. Q_M is a circuit Q -factor for the M-channel of the probe, for which the single coil probe circuit double-tuned for H and X is assumed. When a matched window function is applied to s_H , eq. [1] is reduced to

$$\xi = \frac{f}{\sqrt{2\kappa}} \left(\frac{\gamma_H}{\gamma_X} \right)^{3/2} \left(\frac{W_X}{W_H} \right)^{1/2} \left(\frac{Q_H}{Q_X} \right)^{1/2} \quad [2]$$

with

$$\kappa \equiv 2\pi t_1^{\max} W_X \quad [3]$$

$$W_H \equiv (2\pi \langle s_H^2 \rangle t_2^{\max})^{-1} \quad [4]$$

$$W_X \equiv (2\pi \langle s_X^2 \rangle t_1^{\max})^{-1}, \quad [5]$$

The expression W_M represents a general NMR line width (in units of Hz), which matches with full-width-at-half-height (FWHH) in a Lorentzian line shape or the FWHH times

$(2\pi\ln 2)^{-1/2}$ or 0.479 in Gaussian line shape. With a matched filter condition, $t_1^{\max}$ is typically selected to be $1/(2W_X)$ and thus $\kappa = \pi$. In a typical setting using UFMAS at 100 kHz, $f \sim 0.6$, and $Q_H/Q_X = 2-3$. Assuming the line widths of $W_X \sim 100$ Hz (0.5 ppm) and $W_H \sim 160$ Hz (0.2 ppm) at a ^1H frequency of 800 MHz for ^{13}C (X) and ^1H and the Q-ratio of 2, we safely obtain $\xi = 2.1$ for ^1H indirect detection of ^{13}C . For ^1H indirect detection of ^{15}N , we obtain $\xi = 6.0$ assuming that $W_X \sim 40$ Hz (0.5 ppm) and $W_H \sim 160$ Hz (0.2 ppm). As discussed below, ^1H line widths of 0.1–0.2 ppm are reported for fully protonated GB1 and other microcrystalline protein with UFMAS.^{2, 19} Thus, notable sensitivity enhancements are expected, even when 2D ^1H detection is compared with 1D X detection. In modern protein SSNMR, matched window functions are rarely used for optimum spectral resolution. However, the factor estimated by eq. [2] is still reasonable in modern SSNMR applications, for which data processing of the indirect dimension is often optimized for better resolution with non-uniform-sampling (NUS). Suppose that we lose some sensitivity by removing the matched window function in the ^1H direct dimension for better resolution. The loss is equivalent to a sensitivity gain by applying “matched” NUS²⁵ in the indirect dimension for X. Thus, if the data are adequately collected with NUS, eq. [2] still provides a reasonable estimate for resolution optimized ^1H detected data.

When 2D ^1H detection is compared with 2D X detection, the sensitivity enhancement factor is given by

$$\xi = f \left(\frac{\gamma_H}{\gamma_X} \right)^{3/2} \left(\frac{W_X}{W_H} \right)^{1/2} \left(\frac{Q_H}{Q_C} \right)^{1/2}. \quad [6]$$

For the above-mentioned condition, we obtain enhancement factors of 5.4 and 13.4 for X of ^{13}C and ^{15}N , respectively. Thus, in terms of the required experimental time, notable time saving by a factor of 25–170 is expected.

Estimating the ratio of the Q-factors (Q_H/Q_X) in eqs. [1] and [2] from the circuit's tuning profiles may be sometimes misleading since a probe circuit often involves a parasitic inductance/impedance, especially for a probe with a small sample coil with a long lead wire. Based on the formula by Clark,^{26–27} the amplitude of the oscillating magnetic field $2B_1$ from a RF coil is described by

$$B_{1M} \sim \frac{3}{10} \sqrt{\frac{P_M Q_M}{V_s \nu_{0M}}}, \quad [7]$$

where M = H or X, P_M is a transmitter power in watts, V_s is a coil volume in cubic centimeters, and ν_{0M} is the NMR resonance frequency in Hz, and B_{1M} denotes the amplitude of a rotating RF field in tesla. Thus,

$$\sqrt{\frac{Q_H}{Q_X}} = \frac{\omega_{1H}}{\omega_{1X}} \sqrt{\frac{\omega_{0H} P_X}{\omega_{0X} P_H}}, \quad [8]$$

where we assume that the coil volume is the same for the circuits of H and X, and ω_{1M} denotes a nutation angular frequency due to B_{1M} , and $\omega_{0M} = 2\pi \nu_{0M}$. All the parameters at the right side of the equation can be estimated from the NMR parameters. This relationship suggests that improving a probe-circuit power efficiency for ^1H detection also improves sensitivity in the ^1H detection.

Results

Super-fast 2D Protein SSNMR and the Current Sensitivity Limit of ^1H Detection

We first discuss the current sensitivity limit of ^1H -detected SSNMR and prospects for the UFMAS approach. Figure 1(a) shows a 2D ^{15}N - ^1H correlation SSNMR spectrum for a microcrystalline sample of uniformly ^{13}C - and ^{15}N -labeled GB1 (ca. 180 μg). With the high sensitivity of UFMAS at a spinning frequency (ν_R) of 100 kHz and a ^1H NMR frequency of 750.2 MHz, the spectrum was obtained in only 5 min for ~ 27 nmol of the protein sample. Based on the excellent S/N (~ 16 on average), we estimate that analysis by ^1H -detected 2D SSNMR for 1 nmol of the protein sample is feasible within only 5.9 h at a minimal S/N of 5. This is a notable achievement, considering that until a decade ago collecting ^{13}C -detected 2D protein SSNMR data generally required as much as 1 μmol of an isotope labeled sample and a few days of experimental time, even for the very best data in biological SSNMR at that time.^{28–29}

With this encouraging data, it was appropriate to compare the sensitivity of ^1H -detected SSNMR with that of solution NMR. Thus, we performed 2D ^1H - ^{15}N HSQC for a comparable amount of the labeled GB1 sample (~ 150 μg) with an 800 MHz NMR equipped with a 5-mm inverse triple-resonance room-temperature probe, at the University of Illinois at Chicago (UIC) the Center for Structural Biology. The sample was dissolved in 200 μL of a 50 mM phosphate buffer with 10% D_2O and 0.02 w/v % NaN_3 at pH 5.6 in a 3-mm NMR tube. The S/N ratio of the spectrum obtained within 5 min was approximately 10 (data not shown). Thus, the sensitivity of a modern ^1H -detected SSNMR has reached a level that is comparable to that of solution NMR.

By taking advantage of the excellent sensitivity of ^1H -detected SSNMR, we propose fast data collection of 2D protein SSNMR. In protein solution NMR, fast 2D data collection has been achieved through various creative methods, such as excitation with a short flip angle or simultaneous data collection for different t_1 points under a magnetic field gradient.^{30–32} Here, we combined the benefit of fast recycling (100–200 ms/scan) with the PACC method²⁰ and the high sensitivity of ^1H -detected SSNMR, with a view to implementing “super-fast” collection of 2D SSNMR for micro-screening of protein samples in a very straightforward manner. Figure 1(b) and (c) shows super-fast 2D SSNMR spectra collected for the GB1 sample in (b) 19 s and (c) 9 s (b) without and (c) with 50% NUS, respectively. Nearly all of the peaks for directly-bonded amide ^{15}N - ^1H pairs are observed, except for a few peaks, with

a minimal experimental time of 9 s in (c). The average S/N ratio for the resolved signals was ~6 for (c). The spectrum reasonably well reproduced the peak positions, as indicated in the 1D slices for the three spectra (Fig. 1(d–l)). It should be noted that fast 2D NMR in solution protein NMR typically requires ~1 μmol of a protein sample,^{30, 32} unless an advanced DNP implementation is employed.^{33–34} Our approach is straightforward and potentially useful for characterizing real-time structural changes in a variety of proteins in solids or semi-solids with minimal sample requirements. Applications for larger protein systems are feasible with additional sensitivity gain, for example, by use of a higher field at a ^1H frequency over 1 GHz.^{35–36}

^1H Resolution and Spinning Speed

Next, we discuss the prospect of further sensitivity enhancement by ^1H -detected SSNMR with faster MAS. Figure 2(a–d) shows MAS frequency (ν_R) dependence of ^1H SSNMR spectra of uniformly ^{13}C - and ^{15}N -labeled alanine (~0.3 mg) obtained at a ^1H NMR frequency of 900 MHz with a JEOL 0.75-mm triple-resonance CPMAS probe. Excellent resolution was achieved in (d) at ν_R of 90 kHz. ^1H resonances for the CH and CH_3 groups show improved resolution with ^{13}C WALTZ decoupling, as shown in Figure 2(e); the line widths for the CH and CH_3 groups were as narrow as 0.27 ppm and 0.36 ppm at a ν_R of 90 kHz, respectively. The resolution is satisfactory for the analysis of small organic compounds³⁷ without sophisticated homonuclear-decoupling sequences.^{38–40} For the NH_3^+ group, there appears to be additional broadening, which is consistent with previous studies.³⁹ Figure 2(f) shows the ν_R dependence of the signal intensities. The data show linear dependence of the normalized peak intensities on ν_R with minor deviation for the NH_3^+ group. These data imply that UFMAS beyond 100 kHz will further improve the resolution and sensitivity of ^1H -detected SSNMR. It should be noted that the data were collected without ^{13}C decoupling, for simplicity. For more mobile hydrated proteins in a microcrystal system, sharper lines are often observed, as is discussed below. Table 1 summarizes the sample volume and dimensions of fast MAS rotors provided by Bruker Biospin, JEOL RESONANCE, and Tallinn University of Technology. Various types of fast MAS probes are now available depending on the purpose of experiments.

A Basic Pulse-sequence Scheme for ^1H -detected SSNMR

In this section, we review basic pulse sequences of ^1H -detected 2D $^1\text{H}/^{13}\text{C}$ experiments in a fast MAS approach at ν_R of 40–100 kHz. Figure 3(a) and (b) compares (b) a modern low-power scheme for ^1H -detected 2D $^1\text{H}/^{13}\text{C}$ correlation SSNMR with (a) a traditional high-power scheme for 1D CPMAS experiments with ^{13}C direct detection. In (a), ^1H polarization is transferred to ^{13}C spins with a standard adiabatic or ramped cross polarization (CP) block; then, the ^{13}C signals are directly observed under high-power decoupling at a RF field strength of 70–100 kHz. For an efficient CP scheme, the average RF angular nutation frequency for ^1H $\langle\omega_{\text{H-CP}}\rangle$ should be matched to that of ^{13}C $\langle\omega_{\text{C-CP}}\rangle$ so that they satisfy the Hartman-Hahn condition for rotating solids ($\langle\omega_{\text{H-CP}}\rangle - \langle\omega_{\text{C-CP}}\rangle = n\omega_R$, where $\omega_R = 2\pi\nu_R$ and $n = \pm 1$ or 2. Note that this CP sequence requires high-power RF irradiation under fast MAS because either $|\langle\omega_{\text{H-CP}}\rangle|$ or $|\langle\omega_{\text{C-CP}}\rangle|$ should exceed ω_R . The sequence is typically repeated every 1–5 s so that sample degradation or probe arcing is prevented under high-power RF irradiation. Thus, 95–99 % of SSNMR machine time is typically consumed

for long recycle delays that are limited by this factor rather than ^1H T_1 values of the sample with only 1–5% of the time remaining for collection of spectral information.

A pulse sequence in (b) shows an example of a ^1H -detected SSNMR scheme using the UFMAS approach. Experiments were typically repeated with much shorter recycle delays (100–300 ms) in an approach called paramagnetic-assisted condensed data collection (PACC), which combines the use of a low-power pulse sequence, faster recycling, and paramagnetic T_1 relaxation enhancement with dopants such as Cu-EDTA.²⁰ To reduce ^1H T_1 values of proteins, 10–100 mM Cu-EDTA, or other paramagnetic agents, are doped with hydrated proteins; paramagnetic relaxation enhancement effects with the controlled doping reduce ^1H T_1 values down to 30–100 ms, allowing for faster recycling without noticeably compromising the spectral resolution.^{20, 41} The signal acquisition can be as much as 30% of the experimental time. Below, we outline a scheme when the X nucleus is ^{13}C , but the arguments here are generally applicable to most low γ spin-1/2 nuclei, including ^{15}N . In (b), ^1H polarization is first transferred to ^{13}C spins with a double-quantum cross polarization (DQ-CP) sequence, in which the sum of $\langle\omega_{\text{H-CP}}\rangle$ and $\langle\omega_{\text{X-CP}}\rangle$ is matched to $n\omega_{\text{R}}$. This allows for low-power implementation of cross polarization (that is, $|\langle\omega_{\text{H-CP}}\rangle|, |\langle\omega_{\text{X-CP}}\rangle| < \omega_{\text{R}}$). After DQ-CP, ^{13}C signal evolution during the t_1 period is monitored under low-power ^1H decoupling, a concept which was originally introduced with original ^1H -detected SSNMR.¹⁴ At $\nu_{\text{R}} = 40$ kHz, phase-modulated low-power ^1H decoupling schemes such as TPPM,⁴² XiX,⁴³ and SPINAL-64⁴⁴ with a ^1H RF nutation frequency (ν_{dec}) of 5–20 kHz exhibit excellent performance, which is comparable to that of high-power ^1H decoupling.⁴² At ν_{R} of 100 kHz, our study indicates that WALTZ-16 ^1H decoupling at ν_{dec} of 5–20 kHz, which was designed for solution NMR, offers superior or comparable resolution to that obtained by the aforementioned ^1H decoupling sequences designed for SSNMR, with minimal need for the adjustment of decoupling parameters.²⁴ After the t_1 period, the ^{13}C polarization is stored along the Z-axis parallel to the static magnetic field. Any ^1H background signal is suppressed by a background-suppression sequence such as the MISSISSIPPI scheme⁴⁵ during the “Z-filter” period. Then, ^{13}C polarization is transferred back to ^1H spins by the second DQ-CP scheme. Assuming that a fraction f of the ^{13}C polarization is transferred back to ^1H spins, the bulk magnetic moment for ^1H is typically enhanced by a factor of $f\gamma_{\text{H}}/\gamma_{\text{C}}$ over that of ^{13}C . Since the voltage induced across the sample coil caused by the bulk magnetic moment is proportional to $f\gamma_{\text{H}}/\gamma_{\text{C}}$, the Larmor frequency ω_{0H} , and the circuit Q-factor,⁴⁶ the resultant NMR signal is typically much stronger for ^1H detection than ^{13}C detection. With UFMAS, a ^1H time-domain signal can continue much longer than that with slower MAS. Thus, the overall sensitivity of ^1H -detected SSNMR can be greatly enhanced under UFMAS, especially when combined with a PACC approach.²⁰ As discussed above, ^1H -detected 2D SSNMR can now be considered feasible for isotope-labeled protein analysis at the nano-mole level.

Sensitivity & Resolution Enhancement by ^1H detection

Next, we examine the sensitivity and resolution advantages of ^1H -detected protein SSNMR using UFMAS at 80–100 kHz. Figure 4 shows a comparison of ^1H -detected 2D $^1\text{H}/^{15}\text{N}$ correlation spectra obtained at ν_{R} values of (a) 80 kHz and (b) 50 kHz for a lysine-reverse-labeled GB1 microcrystalline sample (~0.5 mg or ~80 nmol) doped with 20 mM Cu-EDTA.

This fully protonated sample was uniformly ^{13}C - and ^{15}N -labeled, except for six lysine (Lys) residues, as will be discussed later when exploring spectral editing experiments. The pulse sequence detailed in Figure 3(b) was used for the experiments, with recycle delays of 0.2 s. Although the spectrum collected at ν_{R} of 50 kHz in (b) displays a modest resolution, the resolution was greatly enhanced in (a) by UFMAS at 80 kHz. The signals were nearly completely resolved in the 2D spectrum. Similar levels of resolution were obtained for a handful of fully protonated proteins with UFMAS over 80 kHz.⁴

Figure 5(a) and (b) shows (a) ^1H -detected and (b) ^{13}C -detected 2D $^{13}\text{C}/^1\text{H}$ correlation spectra of ubiquitin that was selectively labeled with stereo-array-isotope-labeled (SAIL) Ile (~0.5 mg). A 1D ^{13}C CPMAS spectrum of the same sample is displayed in Figure 5(c). All the data were collected at ν_{R} of 80 kHz and a ^1H NMR frequency of 750.2 MHz with a JEOL 1-mm quad-resonance probe, using low-power CP and decoupling schemes.¹⁸ The recycle delays for (a–c) were set to 0.54 s, which was matched to short ^1H T_1 values (0.25 s) of this sample, even without paramagnetic dopants. The short ^1H T_1 values can likely be attributed to the presence of CHD_2 groups in Ile and the ^1H - ^1H dipolar network within the residue. For a sensitivity comparison, each of (a–c) was collected in ~5 min. In a SAIL labeling scheme, all the CH_2 and CH_3 groups of the relevant amino acid residues were replaced with CHD and CHD_2 , respectively, allowing for improved resolution in the ^1H SSNMR spectra.⁴⁷ The observed line widths W_{H} were in the 0.1–0.2 ppm range. In (a), signals for all seven Ile residues are clearly resolved. Superior sensitivity in the ^1H -detected data in (a) over the ^{13}C -detected data is obvious. In a comparison of the peaks (orange arrows) in the slices (d, e) of the 2D ^1H -detected with (c), the corresponding peaks (orange arrows) in the 1D ^{13}C CPMAS spectrum indicated a sensitivity enhancement factor of 2.1–5.0.¹⁸

Signal Assignment and Spectral Editing under Ultra-fast MAS

In this section, we evaluate signal assignment strategies in ^1H -detected protein SSNMR. Various approaches using sequential assignments were introduced to assign protein main chain and side chain signals in ^1H -detected SSNMR using fast MAS.^{2, 4, 7, 16, 19} In previous protein SSNMR studies, a suit of 3D ^1H -detected experiments such as 3D (H)CANH, (H)CA(CO)NH, (H)CACO(N)H, and (H)CXCA(N)H (polarization transfer diagrams in Figure 6(a) to (d)) were shown to be useful for the sequential assignment of the main chain signals of small or moderate-sized proteins such as GB1.^{7, 16} In this case, nearly complete resolution is needed in a 2D ^{15}N - $^1\text{H}_{\text{N}}$ correlation spectrum in order to connect $^1\text{H}_i$ - $^{15}\text{N}_i$ resonances to $^{13}\text{CA}_i$ as well as $^{13}\text{CA}_{i-1}$ resonances for sequential assignments. Alternatively, with sufficient resolution in a 2D ^{13}CA - ^1HA spectrum, $^1\text{HA}_i$ - $^{13}\text{CA}_i$ resonances can be connected to $^{15}\text{N}_i$ and $^{15}\text{N}_{i-1}$ for sequential assignments using schemes such as those shown in Figure 6(e) to (h).⁴ However, NMR signals often substantially overlap when a protein sequence carries repeats of the same amino-acid types in a common secondary structure. In such a case, establishing starting points of sequential assignments or connecting resonances of next-neighboring residues may be difficult. To address this spectral overlap issue in ^1H -detected SSNMR, we recently introduced an approach called HIGHLIGHT (HIGHspeed-spinning-optimized Labeled-residue-IGNited Hetero-nuclear Total spectral quenching).^{2, 48} Figure 7(a) shows a 2D NH correlation spectra of Lys-reverse-labeled GB1 (blue) without and (red) with HIGHLIGHT REDOR quenching. In this HIGHLIGHT experiment, ^{15}N

signals of the residues that are next to ^{13}C -labeled residues were nearly completely quenched by recoupled ^{13}CO – ^{15}N dipolar couplings; thus, the HIGHLIGHT REDOR experiment highlighted amide ^{15}N – ^1H signals only for the six residues next to unlabeled lysine residues (see the sequence in (d)), with one Trp-43 side chain signal.

With this strategy, we performed sequential assignments using 3D CANH and CA(CO)NH with the HIGHLIGHT approach. Figure 7(b) shows overlaid strip plots for 3D CANH (green) and 3D CA(CO)NH (blue) spectra of the Lys-reverse-labeled GB1 sample, along with those for 3D CANH obtained with HIGHLIGHT REDOR (red) for the same sample. The highlighted signals for Leu-5 and Gly-14 (red in Figure 7(b) and (c)) indicate the starting points of the sequential assignments for the residues following lysine residues ((b) Leu-5 to Gly-9, (c) Gly-14 to Val-21) as an excellent example of the approach. Based on these results only from the two data sets, we completed main-chain ^1H , $^{13}\text{C}_\alpha$, and ^{15}N signal assignments, except for lysine and other unassigned residues in the loop region (Ala-23 and Asp-47).² Torsion angles (ϕ , ψ) predicted by TALOS+ software⁴⁹ from the obtained ^1H , ^{15}N , and ^{13}C shifts showed excellent agreement with those from the crystal structure for most of the residues, except for those around unassigned residues. For complete main-chain assignments, we successfully obtained 3D CONH data. Nominal 3D experimental times needed to obtain average S/N ratios of ~6 were only 2.5 min, 20.5 min, and 28.5 min for 3D CANH, 3D CA(CO)NH and HIGHLIGHT 3D CANH experiments for ~100 nmol of the GB1 protein sample, respectively.

Side-chain signal assignment of a protein by ^1H -detected SSNMR is not so straightforward. Although side-chain assignment by ^1H -detected SSNMR has been attempted for some fully protonated proteins,⁴ many amino-acid side-chain groups carry CH_2 groups, which often offer limited ^1H resolution due to the presence of magnetically non-equivalent vicinal ^1H and strong ^1H – ^1H dipolar coupling within the vicinal group, even with UFMAS. For a per-deuterated protein, excellent resolution can be achieved for the back-exchanged amide ^1H , especially with fast MAS at 40 kHz or above.^{3, 7} However, the lack of aliphatic ^1H limits the starting point of 3D experiments only to the amide ^1H (or other exchangeable ^1H in the side chain), making side-chain assignment generally very difficult and less sensitive. To address these problems, we recently introduced the SAIL scheme⁴⁷ as an isotope-labeling scheme suited for ^1H -detected protein SSNMR. Figure 8(a) shows a 2D ^{13}C – ^{13}C projection of a ^1H -detected 3D CCH correlation of SAIL-Ile labeled ubiquitin. Because of seven Ile residues in the protein sequence, signals overlap substantially in the 2D ^{13}C – ^{13}C projection. For example, in the region selected in Figure 8(b), projected 2D signals originating from three Ile residues (Ile-3, -13, and -44) overlap severely. However, these signals can be nicely separated by ^1H shifts of isoleucine. For example, in Figure 8(c) to (e), three isoleucine signals can be clearly separated from distinctive $^1\text{H}_\beta$ resonances for (c) Ile-3, (d) Ile-13, and (e) Ile-44. Figure 8(f) presents assignments for all the side chain resonances of Ile-61. Because of the narrow ^1H resonances, all the isoleucine side-chain resonances were well resolved. The data illustrates an excellent example of side-chain assignment by 3D ^{13}C – ^{13}C – ^1H correlation for SAIL-labeled proteins.

Structure & Distance Restraints in ^1H -detected SSNMR

Lastly, we discuss structural determination by ^1H -detected SSNMR. Recent SSNMR-based elucidation of high profile amyloid-fibril structures^{28, 50–54} have ensured that SSNMR is recognized as a powerful tool in structural biology. Among ~100 SSNMR-based structures reported in the Protein Data Bank (PDB), nearly all were determined by traditional ^{13}C -detected SSNMR, which offers a variety of options in elucidating long-range distance restraints. Available methods for this purpose include DARR,⁵⁵ frequency-selective REDOR,⁵⁶ PARR,⁵⁷ and CHHC/CHHN schemes.⁵⁸ In contrast, for ^1H -detected SSNMR using fast MAS, there are limited options for collecting long-range distance restraints.^{22, 59} For this reason, there are only a handful of recent examples of de-novo protein structural determinations by ^1H -detected SSNMR under UFMAS.^{59–60} An earlier study established the first structural determination on a small model protein, uniformly ^2H , ^{13}C , and ^{15}N -labeled protein GB1 with back-exchanged amide ^1H .⁵⁹ Here, we introduce two recent examples, which provide more generic routes for structural determinations of small proteins by ^1H -detected SSNMR. The first example was performed on a microcrystalline sample of a model protein ubiquitin at a ^1H frequency of 850 MHz.⁶⁰ The assignments, torsion angles from chemical shifts, and ^1H – ^1H contacts were obtained for uniformly ^2H , ^{13}C , ^{15}N -labeled ubiquitin for which amide ^2H was back exchanged to ^1H . The ^1H – ^1H contacts, including 36 long-range and 50 medium-range contacts, were obtained by ^1H -detected 3D ^{15}N -resolved ^1H – ^1H spin diffusion experiments [3D HN(H)–H] at ν_{R} of 99.2 kHz, where H–H stands for long-range ^1H – ^1H mixing in the pulse sequence. The ^1H – ^1H rotating-frame spin diffusion involved 20 ms cw spin-lock at 13 kHz for optimum ^1H $T_{1\rho}$ values. Additional ^1H – ^1H contacts (68 long-range and 5 medium-range) were collected at a ν_{R} of 94.5 kHz for uniformly ^2H ^{15}N -labeled ubiquitin, for which one of the methyl groups of deuterated Ile, Leu, and Val were replaced with $^{13}\text{CHD}_2$, while the other was labeled with CD_3 .⁶¹ The ^1H – ^1H contacts were obtained with the 4D HSQC-DREAM-HSQC sequence [4D HC(H)–(H)CH],⁶¹ in which a $^1\text{H}_{\text{A}}$ – $^{13}\text{C}_{\text{A}}$ correlation was obtained during the t_1 and t_2 periods for one methyl residue and an additional $^1\text{H}_{\text{B}}$ – $^{13}\text{C}_{\text{B}}$ correlation was obtained during the t_3 and t_4 periods for another methyl residue, after long-range $^1\text{H}_{\text{A}}$ – $^1\text{H}_{\text{B}}$ polarization transfer using a 8-ms ^1H -DREAM mixing sequence at 47.2 kHz. The deuteration method is a practical approach to identify long-range contacts without excess spectral crowding.

More recently, structures of fully protonated proteins were determined for GB1 as well as a microcrystalline Acinetobacter phage 205 coat protein (AP205CP), a 28-kDa dimer in a 2.5MDa viral capsid assembly, at a ^1H NMR frequency of 1 GHz.¹⁹ Long-range ^1H – ^1H contacts were collected for GB1 and AP205CP by 3D (H)CH–H and 3D (H)NH–H sequences. Side-chain assignments were more straightforward for these samples. ^1H – ^1H long-range contacts of 236 and 410 were obtained for GB1 and AP205CP, respectively. Compared with heavily deuterated samples, a much larger number of side-chain long-range contacts were elucidated. It is not clear at this point how this approach can be effective for more structurally heterogeneous or complex biological systems. Nevertheless, considering that a high level of deuteration is very difficult for many such proteins of biological significance, the study presents a promising prospect for protein structural determination by ^1H -detected SSNMR.

Conclusion

^1H -detected SSNMR approach has provided unparalleled sensitivity in SSNMR for hydrated protein samples when employed together with UFMAS, a high field, the PACC approach, and selective/extensive deuteration schemes. Tremendous successes using this approach in the past decade have resulted in the sequential assignments and structural elucidation of small/moderate-sized proteins in ^1H -detected SSNMR. Nevertheless, the widespread applications of ^1H -detected SSNMR to solve serious biological problems remain largely unexplored. Additional efforts are needed to move ^1H -detected protein SSNMR schemes to the next level. In the next few decades, further advances in ^1H -detected SSNMR are likely to provide additional sensitivity gains through the development of ultra-high field magnets using high-temperature superconductive materials³⁵, faster MAS at 200 kHz or higher, and dynamic nuclear polarization methods.⁶² A SSNMR analysis of sub-nano-mole- or pico-mole-scale proteins would be within our scope with such advancements.

Experimental Section

The SSNMR experiments for Figure 1 were performed on a Bruker Avance III 750MHz spectrometer using a JEOL 0.75 mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}/^2\text{H}$ quad-resonance MAS probe at the UIC Center for Structural Biology. The MAS spinning rate was set at $100\text{ kHz} \pm 50\text{ Hz}$. The SSNMR experiments for Figure 2 were performed on a JEOL ECZR 900 MHz spectrometer equipped with an Oxford magnet and a JEOL 0.75 mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple-resonance MAS probe at the Yokohama RIKEN NMR facility. Details of all other data are described in the captions and the relevant references.

For the data in Figure 1, a pulse sequence based on that shown in Figure 3(b) was used with ^{15}N as the X channel and ^{13}C as an additional channel. First, the ^1H polarization was excited by a ^1H $\pi/2$ -pulse with a pulse width of $2.5\text{ }\mu\text{s}$. During the first adiabatic DQ-CP period, the ^1H RF field strength was ramped from 59.2 kHz to 98.7 kHz , while the ^{15}N RF field was kept constant at 21.7 kHz . For the second adiabatic DQ-CP period, the ^1H RF field was ramped up from 46.5 kHz to 77.5 kHz , while the ^{15}N RF field was kept at 32.5 kHz . During the t_1 period, WALTZ-16 ^1H decoupling was applied with a RF nutation frequency of 10 kHz and a ^{13}C π -pulse with a pulse width of $6\text{ }\mu\text{s}$ was applied in the middle of the t_1 period. During the t_2 period, ^{15}N and ^{13}C WALTZ-16 decoupling was applied with RF nutation frequencies of 6 kHz and 9 kHz , respectively. As the water signal was removed sufficiently by a minimum phase cycle of 2 scans, no water suppression sequence was employed. The GB1 microcrystal sample was prepared as described previously.^{2, 63}

For the data in Figure 2, a rotor synchronous echo pulse sequence was employed. The sequence started with a ^1H excitation $\pi/2$ -pulse with a pulse width of $0.6\text{ }\mu\text{s}$. It was followed by an echo period of twice the rotor cycle, in the middle of which a π -pulse with a pulse width of $1.2\text{ }\mu\text{s}$ was applied. Then, a ^1H signal was acquired during 8 ms with or without ^{13}C WALTZ-16 decoupling at a nutation frequency of 10 kHz .

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Highlights

- First demonstration of super-fast ^1H -detected 2D high-resolution solid-state NMR for a nano-mole-scale protein.
- First demonstration of collecting a 2D solid-state NMR spectrum for a 1 nmol of protein in less than 6 h.
- Perspective review of recent studies of signal assignments and structural elucidation by ^1H -detected protein solid-state NMR using ultra-fast MAS

Figure 1.

A comparison of (a) a traditional ^1H -detected 2D $^{15}\text{N}/^1\text{H}$ correlation spectrum (16 scans for each t_1 point) with (b, c) super-fast 2D spectra collected (2 scans for each t_1 point) at 100 kHz MAS (b) without using NUS and (c) with NUS at 50 % random sampling rate for uniformly ^{13}C - and ^{15}N -labeled GB1 protein in a microcrystal sample ($\sim 180\ \mu\text{g}$) with 20 mM Cu-EDTA. Recycle delay was set to 0.2 s for (a), and 0.1 s for (b) and (c). The experimental times are indicated in the figure. (d–l) 1D ^1H slices of the 2D NH spectra obtained at the ^{15}N chemical shifts of (d–f) 107.23 ppm, (g–i) 116.57 ppm, and (j–l) 124.36 ppm.

Figure 2.

(a–d) Magic angle spinning (MAS) frequency (ν_R) dependence of ^1H spectra of uniformly ^{13}C - and ^{15}N -labeled L-alanine. The spinning frequencies are indicated in the figure. (e) ^1H MAS spectrum for the same sample collected at ν_R of 90 kHz with WALTZ-16 ^{13}C decoupling with a RF nutation frequency of 10 kHz. (f) MAS frequency dependence of the ^1H signal intensities for the L-alanine sample. The data were obtained without ^{13}C -decoupling. The intensities were normalized to those obtained at ν_R of 90 kHz.

Figure 3.

(a) A pulse sequence for a standard CP scheme for direct-detection of spin-1/2 X nuclei such as ${}^{13}\text{C}$ and ${}^{15}\text{N}$. After cross polarization, signals from X spins are detected under a high-power ${}^1\text{H}$ decoupling scheme such as SPINAL-64. Experiments are typically recycled every 1–5 s. (b) A low-power pulse sequence for ${}^1\text{H}$ -detected 2D indirect-detection of the X nuclei. After the DQ-CP period from ${}^1\text{H}$ to X spins, the signals for the X spins are monitored indirectly during the t_1 period.

Figure 4.

A comparison of 2D $^{15}\text{N}/^1\text{H}$ correlation spectra collected under MAS at (a) 80 kHz and (b) 50 kHz for the lysine-reverse-labeled GB1 microcrystal sample (0.5 mg or ~ 80 nmol) doped with 20 mM Cu-EDTA. The data were collected with recycle delays of 0.2 s with a total experimental time of 4.5 min each. The pulse sequence is comprised of two adiabatic CP schemes with contact times of 1.5 ms (see Figure 3(b))². The spin polarization was transferred from ^1H to ^{15}N by the first adiabatic CP; then the ^{15}N signal was monitored in the t_1 period. After the t_1 period, the polarization was transferred back from ^{15}N to ^1H spins by the second adiabatic CP scheme for ^1H detection. For the data in (a), during the first adiabatic DQ-CP period, the ^{15}N RF field strength was ramped from 18.6 kHz to 31.0 kHz with an average RF field at $\sim \nu_R/3$, while the ^1H RF field was kept constant at 55 kHz ($\sim 2\nu_R/3$). For the second adiabatic CP period, the ^{15}N RF field was ramped down from 33.3 kHz to 19.9 kHz, while the ^1H RF field was kept at 55 kHz. For the data in (b), during the first adiabatic CP period, the ^{15}N RF field was swept from 10.6 kHz to 17.7 kHz with an average RF field at $\sim \nu_R/4$, while the ^1H RF field strength was kept at 35 kHz ($\sim 3\nu_R/4$). In the second adiabatic CP period, the ^1H RF field was ramped from 9.7 kHz to 16.1 kHz, while the ^{15}N RF field amplitude was kept at 37 kHz. (c) 1D slices of the 2D NH spectra for the spinning speeds of 80 kHz (red) and 50 kHz (blue) for resolution comparison. The spectra were modified from the data in ref. 2.

Figure 5.

A comparison of (a) ^1H -detected and (b) ^{13}C -detected 2D $^{13}\text{C}/^1\text{H}$ correlation SSNMR spectra of SAIL-Ile labeled ubiquitin obtained at ν_R of 80 kHz. A comparison of (c) a 1D ^{13}C CPMAS spectrum of the same sample and (d–g) slices from (d, e) ^1H -detected and (f, g) ^{13}C -detected 2D correlation spectra. The figures are modified from ref. 18. For a sensitivity comparison, both experiments were performed with low-power pulse sequences and the same recycle delays (0.54 s/scans), without paramagnetic doping.

Figure 6.

Diagrams of polarization transfer in (a–d) $^1\text{H}_\text{N}$ -detected and (e–h) $^1\text{H}_\alpha$ -detected 3D SSNMR experiments for main-chain protein sequential assignment. (a) (H)CANH, (b) (H)CA(CO)NH, (c) (H)CACO(N)H, (d) (H)CXCA(N)H, (e) (H)NCAH, (f) (H)N(CO)CAH, (g) (H)COCAH, and (h) (H)CXCAH schemes.

Figure 7.

(a) 2D ^1H -detected $^{15}\text{N}/^1\text{H}$ correlation spectra (blue) without and (red) with HIGHLIGHT REDOR, for the micro-crystalline sample of Lys-reverse-labeled GB1, along with the sequence shown in (d). (b, c) Overlaid 3D strip plots for sequential assignments from (a) Leu-5 through Gly-9 and (b) Gly-14 through Val-21 for a micro-crystalline sample of Lys-reverse-labeled GB1 protein obtained by 3D CANH (green), CA(CO)NH (blue), and 3D CANH with 4.2 ms HIGHLIGHT REDOR mixing data (red). The recycle delays were 0.3 s, 0.4 s, and 1 s for 3D CANH, 3D CA(CO)NH, and 3D CANH with HIGHLIGHT REDOR, respectively. All experiments were performed at a MAS spinning speed of 80 kHz with ~0.5 mg of sample. The total experimental times were 20 min for 3D CANH, 3.7 h for 3D CA(CO)NH, and 1 h for 3D CANH with HIGHLIGHT REDOR (for details, see the Experimental section and Figure S2 of ref. 2). The figure was modified from ref. 2.

Figure 8.

Resolution and side-chain assignments from 3D $^{13}\text{C}/^{13}\text{C}/^1\text{H}$ SSNMR of SAIL-ubiquitin. (a, b) 2D $^{13}\text{C}/^{13}\text{C}$ 2D projection spectra from a ^1H -detected 3D $^{13}\text{C}/^{13}\text{C}/^1\text{H}$ SSNMR of SAIL-ubiquitin at a MAS frequency of 80 kHz. All peaks, including minor ones in (a), can be attributed to intra-residue cross peaks within the Ile residues. (c–e) Representative 2D $^{13}\text{C}/^{13}\text{C}$ slices corresponding to ^1H chemical shifts of (c) 1.57 ppm, (d) 1.73 ppm, and (e) 1.41 ppm. The data show clear separation of signals for (c) Ile-3, (d) Ile-13, and (e) Ile-44 by ^1H shifts. The spectrum was processed with 45°- and 60°-shifted sine-bell window functions in the ^1H and ^{13}C dimensions, respectively. (f) $^{13}\text{C}/^1\text{H}$ assignments for Ile-61 from the 3D data. The pulse sequence can be found in ref. 18. The figure was modified from ref. 18.

Table 1

A comparison of MAS rates and sample volume for different fast MAS rotors

Outer diameter (mm)	1.9	1.3	0.7	2.0	1.0	0.75	1.8	0.81
Inner diameter (mm)	1.5	0.9	0.5	1.55	0.5	0.35	_{e)}	_{e)}
Sample volume (μL) ^{a)}	10.4	1.5	0.37	12.6	0.55	0.19	15	0.8
Max spinning rate (kHz)	42	67	111	40	80	100 ^{b)}	50	105
Manufacture	Bruker Biospin ^{c)}		JEOL RESONANCE			NMR Institute, Tallinn University of Technology ^{d)}		

^{a)}The sample volume denotes that within a sample coil.

^{b)}The spinning speed is based on the product specification rather than the maximum rate (~120 kHz).

^{c)}The information on the Bruker rotors was kindly provided by Dr. Frank Engelke at Bruker Biospin.

^{d)}The information on Tallin Univ. rotors was kindly provided by Dr. Ago Samoson.

^{e)}The data were not available.