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Proton-Detected Polarization Optimized Experiments (POE) Using Ultrafast Magic Angle Spinning Solid-State NMR: Multi-acquisition of Membrane Protein Spectra

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Abstract

Proton-detected solid-state NMR (ssNMR) spectroscopy has dramatically improved the sensitivity and resolution of fast magic angle spinning (MAS) methods. While relatively straightforward for fibers and crystalline samples, the routine application of these techniques to membrane protein samples is still challenging. This is due to the low sensitivity of these samples, which require high lipid:protein ratios to maintain the structural and functional integrity of membrane proteins. We previously introduced a family of novel polarization optimized experiments (POE) that enable to make the best of nuclear polarization to obtain multiple-acquisitions from a single pulse sequence and one receiver. Here, we present the ¹H-detected versions of POE using ultrafast MAS ssNMR. Specifically, we implemented proton detection into our three main POE strategies, H-DUMAS, H-MEIOSIS, and H-MAeSTOSO, achieving the acquisition of up to ten different experiments using a single pulse sequence. We tested these experiments on a model compound N-Acetyl-Val-Leu dipeptide and applied to a six transmembrane acetate transporter, SatP, reconstituted in lipid membranes. These new methods will speed up the spectroscopy of challenging biomacromolecules such as membrane proteins.

Graphical abstract

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Keywords

Polarization Optimized Experiments; Multi-acquisition; Solid-State NMR; Ultra-fast Magic Angle Spinning; SIM-CP; H-DUMAS; H-MEIOSIS; H-MaESTOSO; Membrane Proteins

1. INTRODUCTION

Solid-state NMR spectroscopy is a powerful tool to study the structure and dynamics of biomacromolecules that are recalcitrant to crystallization such as fibrillar and integral membrane proteins^{1–11}. In the past decades, heteronuclear detected experiments (direct detection) have represented the main approach to determine resonance assignments and obtain internuclear distances for structure calculations^{10, 12–16}. However, direct detection methods with ¹³C or ¹⁵N acquisition are inherently insensitive and require longer experimental times^{10, 17}. On the other hand, inverse detection (*i.e.*, ¹H-detected experiments) has represented the method of choice for solution NMR spectroscopy¹⁸. However, strong ¹H–¹H dipolar couplings, hampered the success of inverse detection for ssNMR applications. This problem has been overcome by the introduction of fast MAS technology, which enabled dramatic sensitivity enhancement for multi-dimensional experiments particularly for microcrystalline and fibrillary protein preparations^{19–26}. To achieve fast spinning rates, it is necessary to use rotors with smaller diameters which reduces the operative sample volumes to a range of 1–4 µl, lengthening significantly acquisition times. For membrane proteins, this problem is exacerbated by the presence of lipids and bulk water^{3, 27}. While low lipid-to-protein ratios and hydration levels are often used for ssNMR experiments, it is unlikely that membrane proteins maintain their functional and structural integrity under these conditions.

During the past decade, our group has been developing ¹³C-detected Polarization Optimized experiments (POE) aiming to speed up the spectroscopy of membrane proteins reconstituted with lipid-to-protein ratios close to physiological conditions^{9, 28–36}. *Our overall goal has been to adapt ssNMR spectroscopy samples to closely resemble physiological conditions, rather than adapting the samples to the needs of spectroscopy.* POE utilize orphan (or

undetected) spin operators that are discarded in classical multidimensional NMR experiments and recover them, allowing the acquisition of multiple 2D and 3D experiments in a single pulse sequence^{29–30, 32}. A similar approach has also been utilized for solution NMR experiments (e.g., NOAH sequences) for simultaneous acquisition of 2D spectra of small organic compounds³⁷. Unlike liquid samples, the longitudinal relaxation (T_1) times of solids or solid-like biomacromolecules are significantly longer for ^{15}N and ^{13}C nuclei. Therefore, it is possible to store ^{15}N and/or ^{13}C polarization along the longitudinal axis (N_z or C_z) and subsequently utilize it for multiple acquisitions^{29, 34}. The ease of storing the polarization in solids makes POE (e.g., DUMAS, MEIOSIS, and MAeSTOSO) unique tools for simultaneous acquisition of various types of triple-resonance experiments in ssNMR³⁸. Another key element for POE is the simultaneous cross-polarization (SIM-CP) scheme that matches the Hartmann-Hahn conditions for ^1H , ^{13}C , and ^{15}N , simultaneously^{29–30, 39}. The beauty of POE comes from its straightforward implementation on commercial spectrometers equipped with only one receiver and Low-E or E-free MAS probes^{40–41}. As a result, POE are becoming more popular and have been successfully implemented for ^1H -detected experiments, including hybrid pulse sequences with a combination of CP and INEPT polarization transfer periods^{28, 42–46}.

In this work, we further expand our POE family and show the implementation of novel and efficient pulse sequences for multi-acquisitions using ^1H -detection at ultra-fast magic angle spinning rates of up to 65 kHz. Specifically, we present the ^1H -detected versions of the three main POE strategies we previously introduced^{29, 31, 33}, *i.e.*, proton-detected DUMAS, MEIOSIS and MAeSTOSO (H-DUMAS, H-MEIOSIS, and H-MAeSTOSO). While specific experiments are presented in this paper, the DUMAS, MEIOSIS and MAeSTOSO strategies enable one to concatenate several different pulse sequences³². We demonstrate the application of these methods using the standard dipeptide, N-Acetyl-Val-Leu (NAVL), and a six-pass transmembrane protein that is responsible for transporting succinate and acetate in bacteria (succinate-acetate permease or SatP), which was reconstituted in lipid membranes⁴⁷.

2. EXPERIMENTAL

Uniformly ^{13}C and ^{15}N labeled succinate-acetate permease (SatP) was expressed and purified from *E. coli* bacteria using a SUMO construct as a fusion. The bacteria were cultured in M9 media enriched with ^{13}C glucose and ^{15}N ammonium chloride as reported previously⁴⁷. The final sample consisting of approximately 0.8 mg of SatP was reconstituted in 1:40 protein to lipid ratio with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes and packed into a 1.3 mm Bruker MAS rotor using our standard protocols.

All the experiments were implemented on a Bruker 600 MHz spectrometer equipped with 1.3 mm fast MAS probe. The CP and SIM-CP spectra reported in Figure 1 were acquired at 12 kHz MAS rate using 20 ms ^{13}C and ^{15}N acquisition times under 100 kHz SPINAL-64 decoupling and a 2 s recycle delay⁴⁸; whereas all the remaining spectra were acquired using a MAS rate (ω_r) of 65 kHz with ^1H acquisition time set to 8.3 ms and recycle delay of 2 s. The heat induced by fast spinning on the sample was compensated by lowering the temperature. Specifically, at 65 kHz the temperature of the RF coil was set to -20°C , which

corresponds to an effective sample temperature of 25 °C as measured from the water resonance position. The RF carrier frequencies for ^1H , ^{13}C , and ^{15}N were set to 4.7, 40, and 110 ppm, respectively. During ^1H acquisition, a 10 kHz WALTZ16 decoupling was applied on ^{13}C or ^{15}N channels⁴⁹. The 90° pulse length on ^1H was set to 1.25 μs , whereas 3 μs 90° pulses were used for ^{13}C and ^{15}N . During CP and SIM-CP, the RF amplitudes on ^{13}C and ^{15}N were set to 40 kHz, and the ^1H RF pulse was linearly ramped from 90 to 100% to obtain maximum signal at $n=1$ Hartmann-Hahn matching condition⁵⁰. The optimized ^1H amplitudes during CP and SIM-CP periods were 51.2 and 102 kHz at MAS rates of 12 and 65 kHz, respectively. The suppression of the ^1H signals arising from water and lipids was achieved by phase-switched pulses (MISSISSIPPI)⁵¹, with 30 kHz ^1H RF amplitude applied for 200 ms (t_{supp}). For the 2D experiments, the SIM-CP contact time was set to 750 μs . The durations of back CP transfers from ^{13}C to ^1H (T_{CP}^{C}) and ^{15}N to ^1H (T_{CP}^{N}) were set to 150 and 200 μs , respectively. The SPECIFIC-CP transfer between ^{15}N and ^{13}C was obtained by using a linear ramp (85 to 100%) pulse on ^{13}C applied at 60 ppm and a constant amplitude pulse on ^{15}N ⁵². The optimized RF amplitudes for SPECIFIC-CP on ^{13}C and ^{15}N were 18.3 and 43.3 kHz, respectively. During t_1 evolution, 10 kHz WALTZ16 decoupling was applied on ^1H . Simultaneous (or parallel) ^{13}C and ^{15}N t_1 evolution periods were synchronized by using 100 ^{13}C t_1 increments and two sets ($n=2$ in Figures 2, 4, 7 and 8) of 50 t_1 increments for ^{15}N ²⁹. The dwell-times for ^{13}C and ^{15}N were set to 100 and 200 μs that corresponds to a maximum t_1 evolution of 10 ms for both ^{13}C and ^{15}N . A four-step phase cycle was applied for all the pulse sequences with $\phi_1 = y, -y$, $\phi_2 = x, x, -x, -x$, and receiver phase $\phi_{\text{rec}} = x, -x, -x, x$. The t_1 dimension of 2D spectra were acquired in States mode by switching the phase of ϕ^* pulse between y and $-x$. For the 1D data reported in Figure 1, ^{13}C CP and SIM-CP spectra were acquired with 32 scans, and ^{15}N CP and SIM-CP spectra were acquired with 128 scans. ^{13}C -detected 1D MEIOSIS spectra of Figure 3 were acquired with 256 scans using 20 ms ^{13}C acquisition time under 10 kHz ^1H WALTZ decoupling. Proton 1D spectra in Figure 5 were acquired with 16 scans. The H-DUMAS (Figure 2), H-MAeSTOSO-4 (Figure 7) spectra on SatP were acquired using 16 and 40 scans, respectively, whereas the H-MEIOSIS (Figure 4) spectrum was acquired with 40 scans and two interleaved data sets obtained with $\phi = x$ and $-x$. The total experimental times for SatP 2D H-DUMAS, H-MEIOSIS, and H-MAeSTOSO4 spectra in Figures 2, 4, and 7 were 2, 10, and 5.3 hrs, respectively. The 2D H-MAeSTOSO-10 spectra of NAVL in Figure 8 were acquired using 24 scans for a total experimental time of 6.2 hrs. During HH RFDR, ^1H 180° pulse length was set to 2.5 μs . Mixing times for HH RFDR were set to 0.74 or 1.47 ms⁵³, whereas the CC TOCSY mixing (or $T_{\text{WALTZ}}^{\text{C}}$) time in Figure 8 was set to 9.6 ms. All the spectra were processed using NMRpipe scripts as described in references^{34, 54} for multiple acquisition data sets. Both t_1 and t_2 dimensions were processed with 90° shifted sine bell window functions.

To avoid the sharing of WALTZ16 pulse profile, we recommend using different names for each WALTZ16 period in the pulse sequences. Bruker TOPSPIN pulse programming allows up to nine CPD names (CPD1, CPD2, ..., CPD9) that can be recalled during the decoupling periods of a pulse program. For example, in Figures 5A and 5B the duration of $T_{\text{WALTZ}}^{\text{C}}$ on ^{13}C (or $T_{\text{WALTZ}}^{\text{N}}$ on ^{15}N) channels was varied from 0 to 10.2 ms followed by back-CP and another ^{13}C (or ^{15}N) WALTZ16 period. If the two ^{13}C WALTZ16 decoupling sequences

(1st and 2nd acquisitions of Figure 5A) on ^{13}C channel are used in a pulse sequence with the same name, then the shape or pulse profile of WALTZ16 is shared between two periods depending on the length of each period. An alternative way is to have the WALTZ16 sequence to complete an entire cycle, setting 'n' cycles of WALTZ16 (= 2.4, 4.8, 7.2, and 9.6 ms). In this case, the WALTZ16 profile for two periods do not overlap.

3. RESULTS

3.1 Simultaneous cross-polarization (SIM-CP)

In POE, the initial ^{13}C and ^{15}N polarization is obtained from SIM-CP, where both ^{13}C and ^{15}N RF amplitudes (ω) are simultaneously Hartmann-Hahn matched to ^1H RF (ω_{H})^{29, 32}. For a given MAS rate (ω_{r}), the ^{13}C and ^{15}N RF amplitudes satisfy the matching condition $\omega_{\text{H}} = n \cdot \omega_{\text{r}} \pm \omega$, where $n=1$ and 2 are known as 1st and 2nd sideband Hartmann-Hahn matching conditions. Figure 1 shows the systematic comparison of CP and SIM-CP intensities for the membrane protein SatP at fast (65 kHz) and slow (12 kHz) MAS rates using separate ^{13}C - and ^{15}N -detected experiments (Figure 1A) with a single receiver. At 65 kHz MAS rate, heteronuclear ^1H decoupling was obtained by 10 kHz WALTZ16 pulses; whereas at 12 kHz MAS rate the ^1H RF was set to 100 kHz for SPINAL64 proton decoupling. During CP and SIM-CP contact time (t), ^{13}C and ^{15}N RF amplitudes (ω) were set to 40 kHz, and ^1H RF was optimized to satisfy the first or $n=1$ sideband matching condition ($\omega_{\text{r}} + \omega$), which corresponds to 52 kHz and 105 kHz at MAS rates of 12 and 65 kHz, respectively. The ^1H radio frequency (RF) was linearly ramped from 90 to 100%. The integrated intensities for ^{13}C and ^{15}N spectra were calculated for spectral regions spanning from 0 to 80 ppm and 95 to 140 ppm, respectively. As shown in Figure 1B and C, the ^{13}C intensities for SIM-CP follow a trend similar to the conventional CP for both 65 and 12 kHz MAS rates. The average intensity loss with the ^{15}N SIM-CP experiments are approximately 15 and 8% for 12 and 65 kHz spinning rates, respectively, with contact times ranging from 0.3 to 0.8 ms. Note that the data reported in Figure 1 are recorded under identical sample conditions using a 1.3 mm MAS probe. The effective sample temperatures for SatP at 12 and 65 kHz were set to 25 °C and monitored using the ^1H peak position of the water signal. The relative intensities of CP and SIM-CP spectra at 12 kHz MAS are in close agreement with our previous results obtained for crystalline and membrane protein samples using a 3.2 mm probe with MAS rates of 8 and 12 kHz^{29–30}. Interestingly the ^{15}N SIM-CP scheme is more efficient at faster MAS rates. At contact times greater than 1 ms, the performance of SIM-CP at 65 kHz MAS gradually decreases with an average intensity loss of approximately 15% with respect to CP. Due to the complexity of ^1H – ^1H dipolar coupling network, it is difficult to quantify the CP and SIM-CP dynamics. However, it is possible to speculate that the increased efficiency of ^{15}N SIM-CP at lower contact times and higher MAS rates may originate from more localized polarization transfer from the directly bonded amide protons to the ^{15}N atoms. In fact, at 65 kHz MAS rate we observe a more oscillatory buildup for the ^{15}N polarization (Figure 1B). In contrast, at 12 kHz MAS rate the oscillations of the ^{15}N polarization are significantly damped by the strong ^1H – ^1H dipolar couplings (Figure 1C).

3.2 ^1H -detected DUMAS (H-DUMAS): simultaneous acquisition of (H)CH and (H)NH experiments

In the original implementation of DUMAS (DUal acquisition Magic Angle Spinning) experiment²⁹, the ^{13}C and ^{15}N polarization from SIM-CP was used to acquire 2D ^{13}C - and ^{15}N -edited experiments such as DARR and NCA with ^{13}C detection at slow MAS rates (8–12 kHz). Analogously, the H-DUMAS experiment enables the acquisition of 2D ^1H -detected ^{13}C - and ^{15}N -edited experiments (Figure 2A). After SIM-CP transfer from ^1H to ^{13}C and ^{15}N , a pair of 90° pulses on ^{13}C and ^{15}N flips the polarization to the z-direction followed by a water suppression period. A 90° pulse on ^{13}C brings the polarization to the transverse plane, which evolves during t_1 followed by a back CP period (T_{CP}^{C}) from ^{13}C to ^1H . The ^1H magnetization is then detected during the first t_2 acquisition that records a 2D (H)CH experiment (blue). After the first acquisition, a 90° pulse on ^{15}N flips the polarization to the transverse plane, which evolves during ^{15}N t_1 followed by an ^{15}N – ^1H back CP (T_{CP}^{N}) and a second t_2 acquisition period that records a 2D (H)NH spectrum. During the t_1 and t_2 evolution periods, heteronuclear decoupling is achieved by a WALTZ16 pulse sequence⁴⁹.

An important feature of the DUMAS experiments is the distinct t_1 evolution periods for ^{13}C and ^{15}N ²⁹, which enables to optimize ^{13}C and ^{15}N t_1 evolution parameters independently. Therefore, the t_1 evolution time for the ^{13}C -edited (H)CH experiment was set with a maximum evolution time of 10 ms, with a dwell time of 100 μs and 100 increments. On the other hand, the t_1 evolution time for the ^{15}N -edited (H)NH experiment was divided into two identical t_1 evolution periods with 50 increments each corresponding to a maximum t_1 evolution period of 10 ms and a dwell time of 200 μs ($n=2$, Figure 2A). As described previously^{29, 34, 38}, the two data sets for the (H)NH experiment are added post acquisition during the data processing. In this manner, the effective number of scans for the (H)NH is double that of the (H)CH experiment. Figure 2B displays the 1D spectra of SatP obtained from the first increment ($t_1 = 0$) of the H-DUMAS-(H)CH-(H)NH experiment shown in blue and red, respectively. As a comparison, the single-acquisition spectra obtained from the conventional (H)CH and (H)NH pulse sequences are overlaid in black. As expected, the intensity of the H-DUMAS-(H)CH spectrum is identical to the conventional (H)CH. On the other hand, for the H-DUMAS-(H)NH spectrum shows an intensity loss of approximately 5%, which is expected from the lower performance of the SIM-CP scheme. Figure 2C shows the application of the 2D H-DUMAS-(H)CH-(H)NH experiment to SatP acquired simultaneously at 65 kHz for a total experimental time of ~2 hours. Although ^1H resolution at 65 kHz MAS rate is still challenging for larger proteins such as SatP, we anticipate that it is possible to further improve sensitivity and resolution using a combination of 3D experiments, higher MAS rates (100–110 kHz) as well as sparse protein deuteration⁵⁵. Note that the acquisition of 2D (H)NH and (H)CH spectra at fast MAS rates can also be obtained using single acquisition as reported in references^{42, 44} at slow and fast MAS rates. The H-DUMAS experiment is more flexible and enables to choose different T_{CP}^{C} and T_{CP}^{N} back CP periods and optimize sensitivity and resolution of the ^1H spectra. Similar to ^{13}C detected DUMAS experiments, H-DUMAS can easily be extended to the third dimension with homo- or hetero-nuclear (CC, HH or NC) mixing times.

3.3 ^1H detected MEIOSIS (H-MEIOSIS): simultaneous acquisition of (H)CH, (H)N(CA)H, (H)CA(N)H and (H)NH experiments

We previously demonstrated that when a given ^{13}C - or ^{15}N -edited pulse sequence includes NC or CN polarization transfer (SPECIFIC-CP) periods, it is possible to nest into it three additional experiments using the MEIOSIS (Multiple Experiments via Orphan Spin operatorS) strategy³¹. To implement this for proton detection, we first investigated the relative intensities of the polarization pathways using ^{13}C -detected MEIOSIS experiments at fast MAS rates (Figure 3A). In this case, the preparation period (SIM-CP) is followed by SPECIFIC-CP that creates two *bidirectional polarization transfer pathways* from ^{15}N to ^{13}C and vice versa named $\text{NCA}_{\text{trans}}$ and $\text{CAN}_{\text{trans}}$, respectively. The residual ^{13}C and ^{15}N polarization pathways (*i.e.*, CC_{res} and NN_{res}) remain on ^{13}C and ^{15}N at the end of SPECIFIC-CP period³¹. The polarization from the CC_{res} and $\text{NCA}_{\text{trans}}$ pathways is recorded in the first acquisition; whereas the ^{15}N polarization from NN_{res} and $\text{CAN}_{\text{trans}}$ pathways is transferred to ^{13}C via SPECIFIC-CP and is recorded in a second acquisition period. For each acquisition, the decoding of two pathways is obtained by altering the phase ϕ of ^{15}N SPECIFIC-CP pulse between x and $-x$, and the resultant data sets are stored in two separate files. The phase ϕ inverts the transferred polarization (CN_{trans} and NC_{trans}), whereas the CC_{res} and NN_{res} are not affected by flipping ϕ ³¹. In the first acquisition, CC_{res} and $\text{NCA}_{\text{trans}}$ are decoded by adding and subtracting the two data sets recorded with $\phi = x$ and $-x$. Similarly, addition and subtraction of two data sets of the second acquisition decodes both $\text{CAN}_{\text{trans}}$ and NN_{res} pathways.

Figure 3B shows the 1D normalized intensities of SatP protein spectra for all four pathways measured at 65 kHz. SPECIFIC-CP mixing times (τ) were arrayed from 0 to 8 ms. The optimal NCA and CAN transfer is obtained at a mixing time of 6.5 ms. Figure 3C shows the 1D spectra corresponding to four pathways obtained at 0 and 6.5 ms SPECIFIC-CP mixing times. The residual ^{13}C and ^{15}N polarization at optimal NCA or CAN mixing time (~ 6.5 ms) corresponds to approximately 52 and 40% of the normalized signal as previously shown for a 3.2 mm probe at slower MAS rates³¹. Note that the $T_{1\rho}$ relaxation times for ^{13}C and ^{15}N during SPECIFIC-CP are relatively longer at faster MAS rates and the transfer efficiency for NCA and CAN can vary significantly based on the MAS rates. As a result, the extent of residual polarization depends on the net effect of $T_{1\rho}$ and SPECIFIC-CP transfer efficiencies for a given MAS rate.

The spectra of Figure 3 were recorded using 256 scans with ϕ phase switching between x and $-x$, which corresponds to 512 effective scans. If the signal-to-noise achieved with ' n ' scans is ' S_0 ', then $k * S_0$ signal-to-noise requires ' $k^2 * n$ ' scans. Therefore, using conventional single acquisition methods ($\tau=0$) to obtain 52% ($k=0.52$) ^{13}C CP signal it would take 128 scans (k^2*512), which corresponds to approximately one fourth of the experimental time. Similarly, to obtain 40% ($k=0.4$) NCA signal, it would require 82 scans (k^2*512), which corresponds to one sixth of the experimental time. The experimental time for 1D MEIOSIS using 512 scans and $\tau=6.5$ ms is 18 minutes (Figure 3C). Using conventional, single acquisition methods, NCA and CAN 1D spectra require 36 minutes, whereas to obtain 52% ^{13}C CP and 40% NCA signal it would take 4.5 and 3 minutes respectively. In other words, to achieve similar signal intensities, it would require

approximately 43.5 minutes with conventional methods; whereas the MEIOSIS experiment would only take 18 minutes, with a net time saving of ~59%. Note that in Figures 3B and 3C, each of the four pathways are normalized separately to show gains and losses of transferred and residual polarization intensities with respect to the initial value at $\tau=0$. For setting up the 2D MEIOSIS experiment, it is important to consider the relative intensities of the four 1D spectra. Figure 3D shows the relative intensities of four MEIOSIS pathways for a SPECIFIC-CP period of 6.5 ms calculated by integrating the intensity values between 45 to 75 ppm. The residual CC pathway shows the highest intensity, which was normalized to 100%, and the relative intensities of NCA, NN, and CNC spectra, corresponding to 41%, 18%, and 17% respectively.

Similar to ^{13}C -detected MEIOSIS experiments at slow MAS rates, the four polarization pathways can be used to design various combinations of H-MEIOSIS experiments at fast MAS rates³⁸. Figure 4A shows one example of two-dimensional H-MEIOSIS, where ^{13}C and ^{15}N t_1 evolution is followed by SPECIFIC-CP to create CC_{res} , $\text{NCA}_{\text{trans}}$, $\text{CAN}_{\text{trans}}$, and NN_{res} pathways to record (H)CH and (H)N(CA)H spectra in the first acquisition, and (H)CA(N)H and (H)NH spectra in the second acquisition. The ^{13}C and ^{15}N t_1 evolutions of H-MEIOSIS are synchronized by using two loops ($n=2$) for ^{15}N t_1 evolution. By addition and subtraction of two data sets acquired with phase ϕ set to x and $-x$, it is possible to decode the two pathways for the first and second acquisitions. Figure 4B shows four 2D spectra [(H)CH, (H)N(CA)H, (H)CA(N)H, and (H)NH] of SatP acquired simultaneously using H-MEIOSIS. The total experimental time was approximately 10 hours. The 2D spectra (H)CA(N)H and (H)N(CA)H are obtained from transferred polarization pathways (CN_{trans} and NC_{trans}) under optimal SPECIFIC-CP conditions; while the 2D (H)CH and (H)NH spectra are obtained from 40–50% orphan polarization. Thus the relative intensities of (H)CH and (H)NH spectra are 40–50% lower than the corresponding H-DUMAS or single acquisition spectra. Note that the peaks in the 2D (H)N(CA)H and (H)CA(N)H experiments have lower intensity due to NCA (or CAN) transfer period, and thus require higher number of scans. The latter produces sufficient residual polarization that enables the acquisition of the additional (H)CH and (H)NH spectra. In this way, using the H-MEIOSIS strategy we combined a pair of low sensitive experiments [(H)N(CA)H and (H)CA(N)H] with another pair of high sensitive experiments [(H)NH and (H)CH].

3.4 How much residual ^{13}C and ^{15}N polarization remains after back CP transfer periods? Can it be used for additional experiments?

To quantify the residual ^{13}C and ^{15}N polarization at the end of back CP periods (T_{CP}^{C} and T_{CP}^{N}), we modified the H-DUMAS experiment. The 1D pulse sequence reported in Figure 5A consists of four acquisition periods, where the first and third FIDs (orange and green) correspond to H-DUMAS polarization pathways obtained from SIM-CP. After ^{13}C - ^1H and ^{15}N - ^1H back CP periods, a 90° pulse on ^{13}C and ^{15}N stores the residual polarization along the z -direction followed by ^{13}C and ^{15}N WALTZ16 decoupling periods, $T_{\text{WALTZ}}^{\text{C}}$ and $T_{\text{WALTZ}}^{\text{N}}$, respectively. After a WALTZ16 decoupling period, the longitudinal ^{13}C and ^{15}N residual polarization is transferred to ^1H by applying a 90° pulse followed by another ^{13}C - ^1H and ^{15}N - ^1H back CP period that records aliphatic (H^{C}) and amide (H^{N}) proton spectra. In this way, it is possible to quantify the net residual polarization by varying T_{CP} and

T_{WALTZ} decoupling periods. Typically, for fully protonated samples under 40–100 kHz MAS rates the back-CP are set to 100 to 400 μs and the ^{13}C and ^{15}N WALTZ16 decoupling periods are set between 7 and 10 ms. The H-DUMAS and H-MEIOSIS spectra reported in Figures 2 and 4 were obtained using ^{13}C – ^1H and ^{15}N – ^1H back CP periods set to 150 and 200 μs respectively; whereas t_2 WALTZ16 decoupling on ^{13}C and ^{15}N was set to 8.3 ms.

We first evaluated the effect of WALTZ16 decoupling pulses on ^{13}C and ^{15}N residual z-polarization during T_{WALTZ} ($T_{\text{WALTZ}}^{\text{C}}$ and $T_{\text{WALTZ}}^{\text{N}}$). Figure 5B shows H^{C} (blue) and H^{N} (red) 1D spectra of SatP protein acquired at various T_{WALTZ} periods with T_{CP} (T_{CP}^{C} and T_{CP}^{N}) set to zero. Note that the second back-CP periods prior to 3rd and 4th acquisitions were set to a fixed value of 200 μs . The RF amplitudes of both ^{13}C and ^{15}N WALTZ16 pulses were set to 10 kHz, corresponding to a 25 μs 90° pulse. The WALTZ16 decoupling is based on composite rotation and consists of ninety-six 90° pulses with four phase switched Q blocks, namely, $Q\bar{Q}\bar{Q}Q$ ⁴⁹. The total time of WALTZ16 cycle corresponds to 2.4 ms ($= 96 \times 25 \mu\text{s}$). Whereas each Q unit is a spin inversion sequence with twenty-four 90° pulses that corresponds to 600 μs ($= 24 \times 25 \mu\text{s}$). The patterns of the 1D spectra in Figure 5B show a remarkable agreement with the WALTZ16 cycle. The latter demonstrates that the residual ^{13}C and ^{15}N z-polarization can be preserved at the end each Q unit (i.e., at multiple values of 600 μs) as well as 2.4, 4.8, 7.2, and 9.6 ms WALTZ16 periods, which correspond to 1, 2, 3, and 4 WALTZ16 cycles. Therefore, the residual ^{13}C and ^{15}N polarization can be recovered even in the presence of ^{13}C and ^{15}N decoupling pulses by synchronizing the total length of T_{WALTZ} to multiples of the (n/4) of cycle time, where ‘n’ is an integer. As shown in Figure 5B, the loss of ^{15}N signal intensity is only 10% even after a WALTZ16 period of 9.6 ms. On the other hand, the WALTZ16 period on ^{13}C acts as homonuclear TOCSY mixing, which causes ~ 50% of signal loss for $T_{\text{WALTZ}}^{\text{C}}$ of 9.6 ms.

We then evaluated the effect of back-CP or T_{CP} (T_{CP}^{C} and T_{CP}^{N}) periods on the four pathways, namely, ^{13}C – ^1H and ^{15}N – ^1H transferred polarization pathways (orange and green, Figure 5A) detected in the 1st and 3rd acquisitions, and ^{13}C and ^{15}N residual pathways (blue and red) detected in 2nd and 4th acquisitions. The four acquisition periods were set to 8.3 ms. To preserve the residual polarization after 1st and 3rd acquisitions, the WALTZ16 decoupling was set on for another 1.3 ms (τ_1 in Figure 5A) to complete four WALTZ16 cycles for a total duration of $T_{\text{WALTZ}}^{\text{C}}$ and $T_{\text{WALTZ}}^{\text{N}}$ periods of 9.6 ms. When T_{CP} is set to zero, no signal was observed for the 1st and 3rd acquisitions, due to the absence of ^{13}C – ^1H and ^{15}N – ^1H back CP transfer (Figure 5C). On the other hand, when T_{CP} is zero, full ^{13}C and ^{15}N polarization is used for residual pathways, showing maximum signal intensities. At higher T_{CP} values, efficient ^{13}C – ^1H and ^{15}N – ^1H transfers lead to maximum intensities for 1st and 3rd acquisitions, whereas residual polarization (2nd and 4th acquisitions) gradually decreases. Typically, for U- ^{13}C , ^{15}N labeled proteins selective transfer from ^{13}C to ^1H is obtained by using 100 to 200 μs back CP period. Whereas ^{15}N to ^1H back CP transfer is obtained by using the T_{CP} values between 200 to 500 μs ²¹. As shown in Figure 4C, at T_{CP}^{C} value of 150 μs , we observed approximately 10% ^{13}C residual polarization. On the other hand, we observed 54 and 27% ^{15}N residual polarization for T_{CP}^{N} values of 200 and 300 μs , respectively. Figure 6 shows the normalized intensities for the H^{C} and H^{N} spectra illustrated in Figure 5C. The transferred and residual polarization from the CH back-CP are CH_{trans} and

CH_{res} , respectively. Whereas NH back-CP transferred and residual polarization are represented by NH_{trans} and NH_{res} , respectively. Note that the efficiency of transferred and residual polarization pathways of back-CP periods depends on sample conditions as well as the MAS rate. The NH back-CP periods of 400 to 600 μs were reported for crystalline proteins with fast MAS rates and perdeuteration sample conditions. On the other hand, for SatP protein, the back-CP transfer efficiency reached a plateau at 300 μs .

3.5 ^1H detected MAeSTOSO-4 (H-MAeSTOSO-4): simultaneous acquisition of four experiments

The ^{13}C and ^{15}N residual polarization of the back-CP periods can be used to implement the experiments based on the MAeSTOSO (Multiple Acquisitions via Sequential Transfer of Orphan Spin polarizatiOn) strategy³³. The 2D pulse sequence for H-MAESTOSO-4 is obtained by extending the 2D H-DUMAS pulse sequence for four acquisition periods as shown in Figure 7A. As in H-DUMAS, the ^{13}C and ^{15}N polarization from SIM-CP is used for recording ^{13}C - and ^{15}N -edited experiments in the 1st and 2nd acquisitions, respectively, where a ^1H - ^1H RFDR period was incorporated for homonuclear transfer between ^1H nuclei prior to the second acquisition period. The resulting signal in the 1st and 2nd acquisitions corresponds to the (H)CHH and (H)NHH 2D spectra, respectively. The residual ^{15}N polarization after the first ^{15}N - ^1H back-CP (T_{CP}^{N}) is stored along the z-direction by applying a ^{15}N 90° pulse followed by WALTZ16 decoupling. As shown in Figure 5, the total length of the $T_{\text{WALTZ}}^{\text{N}}$ period can be adjusted to 9.6 ms to retrieve the residual ^{15}N polarization. Similarly, in the H-MAESTOSO-4 pulse sequence the $T_{\text{WALTZ}}^{\text{N}}$ period is set to 9.6 ms. After the $T_{\text{WALTZ}}^{\text{N}}$ period, the ^{15}N residual polarization is transferred to ^1H followed by a ^1H - ^1H RFDR transfer⁵³ that records another (H)NHH experiment in the third t_2 acquisition. After the 3rd acquisition, the ^{15}N residual polarization resulting from second ^{15}N - ^1H back CP is transferred to ^1H followed by a 4th acquisition that records a (H)NH 2D experiment. At T_{CP}^{N} of 200 μs , The residual ^{15}N polarization after the first ^{15}N - ^1H CP is approximately 54%, which is utilized in the 3rd acquisition of the H-MAESTOSO-4 sequence. On the other hand, the residual polarization after the second ^{15}N - ^1H CP becomes approximately 29% (54% of 54% of the polarization), which is recorded in the 4th acquisition period. Note that in Figure 7, for T_{CP}^{N} value of 300 μs , the signal intensities of the 3rd and 4th acquisitions become 27 and 7 %, respectively, due to lower residual polarization (Figure 5c). To increase the signal intensities of 3rd and 4th acquisitions, a lower T_{CP}^{N} duration (equal to 200 μs) was used, which reduces the polarization transfer for the 2nd acquisition.

Figure 7B shows the SatP 2D spectra (H)CHH (RFDR=1.47 ms), (H)NHH (RFDR=1.47 ms), (H)NHH (RFDR=0.74 ms), and (H)NH acquired simultaneously using the H-MAESTOSO-4 pulse sequence (Figure 7A) with a total experimental time of 5.3 hours. At 1.47 ms RFDR mixing, intense cross peaks were observed between H^{N} and H^{C} groups, whereas 0.74 ms mixing resulted in more resolved peaks, particularly for the aliphatic proton region (0 to 5 ppm). The HH cross peaks in the ^{15}N -edited spectra recorded at two mixing times can be compared with the reference (H)NH spectrum recorded in the 4th acquisition. During the execution of these pulse sequences, some of the water signal can interfere with the 2D spectra acquisition. Therefore, the spectra in Figure 7B were acquired

with two additional water suppression periods ($0.25 \cdot t_{\text{supp}}$ in Figure 7A) set to 50 ms, prior to second and third ^{15}N - ^1H back CP periods. As shown in Figure 7C, in the absence of second and third water suppression periods ($0.25 \cdot t_{\text{supp}}$ in Figure 7A) we observed a weak water peak signal in (H)NHH (RFDR=0.74 ms) and (H)NH 2D spectra acquired in 3rd and 4th acquisitions respectively. Note that the solvent suppression element (t_{supp}) can also be incorporated prior to T_{CP}^{N} periods, which may improve water suppression as well as lipid signal intensities⁵⁷. Similarly, a constant time version can be used keeping the total number of ^1H pulses constant for all t_1 increments, which will improve water suppression^{19, 21}.

3.6 ^1H detected MAeSTOSO-10 (H-MAeSTOSO-10): simultaneous acquisition of ten experiments

By exploiting the ^{13}C - ^1H and ^{15}N - ^1H back CP residual polarization pathways, we also designed the H-MAeSTOSO-10 sequence for simultaneous acquisition of ten 2D spectra using five acquisition periods. The H-MAeSTOSO-10 (Figure8) experiment records one pair of ^{13}C - and 2D ^{15}N -edited spectra in each of the five acquisition periods by addition and subtraction of data sets recorded with phase $\phi = x$ and $-x$. The 1st and 3rd acquisitions are respectively obtained from H-MEIOSIS pathways (CC_{res} , $\text{NCA}_{\text{trans}}$, $\text{CAN}_{\text{trans}}$, and NN_{res}) followed by HH RFDR mixing periods to record (H)CHH and (H)N(CA)HH spectra in the 1st acquisition, and (H)CA(N)HH and (H)NHH spectra in the 3rd acquisition. During the 1st acquisition, two residual ^{13}C polarization pathways of first ^{13}C - ^1H back CP (T_{CP}^{C}) period are stored along the z-direction by synchronizing the $T_{\text{WALTZ}}^{\text{C}}$ period to 9.6 ms. Unlike ^{15}N , at fast MAS rates ^{13}C z-polarization undergoes TOCSY ^{13}C - ^{13}C homonuclear transfer under ^{13}C WALTZ pulses ($T_{\text{WALTZ}}^{\text{C}}$ period). After a $T_{\text{WALTZ}}^{\text{C}}$ period the ^{13}C polarization is flipped to the transverse plane by a 90° pulse and then transferred to ^1H by another ^{13}C - ^1H back CP followed by the 2nd acquisition. The corresponding ^{13}C - and ^{15}N -edited 2D spectra of 2nd acquisition are (H)CCH and (H)N(CAC)H, where CC TOCSY mixing is achieved during ^{13}C $T_{\text{WALTZ}}^{\text{C}}$ period⁵⁷. The two ^{15}N residual polarization pathways of the first ^{15}N - ^1H back-CP period (T_{CP}^{N}) are recovered after the 3rd acquisition by setting the $T_{\text{WALTZ}}^{\text{N}}$ period to 9.6 ms. A 90° pulse is then applied on ^{15}N followed by a second ^{15}N - ^1H back-CP and HH RFDR mixing that records (H)CA(N)HH and (H)NHH 2D spectra in the 4th acquisition. Similarly, two residual ^{15}N polarization pathways resulting from a second ^{15}N - ^1H back-CP (T_{CP}^{N}) are recovered for acquiring a fifth pair of 2D ^{13}C - and ^{15}N -edited spectra (H)CA(N)H and (H)NH in the 5th acquisition period.

Figure8B shows ten 2D spectra of the NAVL dipeptide acquired simultaneously using H-MAeSTOSO-10 pulse sequence in Figure8A. Depending on the signal intensities of the 1st increment ($t_1 = 0$), one can choose appropriate durations for HH RFDR or CC TOCSY mixing periods. In general, experiments with longer mixing times are less sensitive and require relatively higher spectral intensities compared to lower mixing times experiments. The signal intensities of H-MEIOSIS pathways recorded in the 1st and 3rd acquisition periods are relatively higher, hence a longer RFDR mixing time (1.47 ms) was used prior to 1st and 3rd acquisitions spectra as reported in Figure8B. The 4th acquisition uses ^{15}N residual signal from first ^{15}N - ^1H back CP, hence only 0.74 ms RFDR mixing time was used prior to the 4th acquisition. On the other hand, the 5th acquisition uses weak residual polarization after two ^{15}N - ^1H back CP periods, hence the spectra in the 5th acquisition were

recorded without RFDR mixing that gives (H)NH and (H)CA(N)H 2D data sets. For the spectra acquired in the 2nd acquisition, the TOCSY mixing or WALTZ16 period ($T_{\text{WALTZ}}^{\text{C}}$) was set to 9.6 ms. Note that the TOCSY mixing can also be increased by setting the $T_{\text{WALTZ}}^{\text{C}}$ period to multiples of 600 μs , as shown in Figure 5. The 2D spectra, (H)NH and (H)CA(N)H, recorded in 5th acquisition are used as a reference to identify the cross peaks in the spectra acquired in the first four acquisition periods with RFDR and TOCSY mixing periods. The CC TOCSY transfer is mediated by the J-coupling Hamiltonian that achieves intra-residue CC transfer in the 2D spectra [(H)C(C)H and (H)N(CAC)H] as shown in Figure 8B. At a RFDR mixing time of 1.47 ms, both ^{13}C - and ^{15}N -edited 2D spectra showed both intra- and inter-residue HH cross peaks between Val and Leu protons. On the other hand, more selective intra-residue HH transfer was observed with a RFDR mixing period of 0.74 ms. As expected, the TOCSY transfer only showed intra-residue CC correlations. The ^1H peak at 12.5 corresponds to carboxyl-terminal group of leucine⁵⁸. Note that the HH transfer efficiency can vary depending on the sample conditions and MAS rates. Although we used RFDR mixing periods for HH transfer, H-MAeSTOSO sequences can easily be modified with other mixing schemes⁵⁹. For example, DQ-SQ pulse sequences using BABA-XY16 recoupling is shown to be useful for assigning the proton spectra at 100 kHz MAS rate^{60–61}. The H-MAeSTOSO-10 spectra (Figure 8) on NAVL were acquired using 24 scans per t_1 increment. Although four scans were sufficient to see most of the cross peaks, we used 24 scans to detect the lowest intensity cross-peaks ($V\gamma_1$ and $V\gamma_2$) in the (H)CHH and (H)C(C)H spectra. Of course, the application of H-MAeSTOSO-10 to proteins requires highly sensitive samples and careful optimization of the mixing periods. Note that in this case, long-range correlations might not be feasible due to limited signal-to-noise ratio.

4. DISCUSSION

Solid-state NMR experiments utilize 5–10% of the total experimental time for pulse sequence execution and acquisition of the signal (FID), while for 90–95% of the time the spectrometer is idle (2–4 seconds) waiting for the spin system to return to the equilibrium through longitudinal relaxation, T_1 . Classical ssNMR methods are designed for single acquisition period, where each 2D or 3D experiment is acquired using a single coherence transfer pathway. The introduction of POE with the DUMAS, MEIOSIS, and MAeSTOSO strategies made it possible to recover orphan spin operators and utilize the full potential of nuclear spin polarization generated by CP^{29–34, 38, 46–47}. Using these strategies, we have successfully acquired two to eight 2D and 3D spectra using ^{13}C detected multiple acquisition experiments. The expansion of the POE family to ^1H -detection experiments and ultrafast magic angle spinning is a stepping-stone to advance the potential of ssNMR to study challenging systems such as membrane proteins.

A key element in the POE pulse sequences is the preparation of both ^{13}C and ^{15}N polarization pathways using SIM-CP that satisfies the Hartmann-Hahn condition simultaneously for ^{13}C and ^{15}N with respect to the ^1H spin bath. We have found that the performance of SIM-CP at a 65kHz MAS rate is more efficient compared to slow MAS rates. Other elements of POE involve the recovery of both transferred and residual polarization pathways during NC SPECIFIC-CP (τ), and ^{13}C – ^1H and ^{15}N – ^1H back-CP periods (T_{CH}^{C} and T_{NH}^{N}). MEIOSIS and H-MAeSTOSO 2D pulse sequences were

implemented using optimal parameters for SPECIFIC-CP transfer periods as described in Figure 3. Thus, the sensitivity of transferred polarization pathways is similar to conventional single acquisition experiments. On the other hand, the sensitivity of residual polarization pathways depends on the number of CP or SPECIFIC-CP periods, as well the efficiency of back CP periods. Figures 3 and 5 quantifies the signal intensities of transferred and residual polarization pathways. Both transferred and residual polarization pathways of NC SPECIFIC-CP, NH back-CP, and CH back-CP were successfully used for the simultaneous acquisition of four to ten two-dimensional experiments using H-MAeSTOSO-4 and HMAeSTOSO-10 experiments. Although, in this work the 2D ^{13}C -edited spectra were acquired with T_{CP}^{C} set to 150 μs , one could use lower T_{CP}^{C} values to increase the residual polarization. For instance, as shown by Rienstra and co-workers, spectral overlap in (H)CH spectra can be reduced by using as low as a 50 μs back CP (T_{CP}^{C}) transfer period²¹. Note that the shorter back-CP periods achieve selective transfer at the cost of lower ^1H signal intensities for transfer pathways ^{13}C to ^1H and ^{15}N to ^1H . As shown in Figure 5C, at a 50 μs T_{CP}^{C} period one can retain up to 26% ^{13}C residual polarization even in the presence of WALTZ16 pulses. WALTZ16 decoupling is routinely used for both protonated as well as perdeuterated protein samples at a range of MAS rates of 40 to 110 kHz^{24, 55, 57}. Note that in the absence of ^{13}C and ^{15}N WALTZ decoupling pulses it is possible to retain higher residual polarization, but such an approach leads to lower sensitivity and resolution.

One of the objectives of this work was to investigate the extent of the orphan spin-polarization pathways using optimal experimental conditions. We found that approximately 30 to 50% ^{15}N residual polarization was obtained from ^{15}N - ^1H back CP periods; while ^{13}C residual polarization drops to 10% due to CC TOCSY mixing during WALTZ16 pulses (Figure8). Note that the efficiency of CC TOCSY mixing can be improved under fast MAS rates. In fact, intra-residue CC correlations were obtained by WALTZ pulse sequence on fully protonated membrane protein at 100 kHz MAS rate⁵⁷. Also, the residual polarization from back-CP periods can also be preserved by applying other decoupling schemes in place of WALTZ that use back to back 180° pulses. In fact, we have recently developed a hybrid 2D and 3D TEDOR-NCX experiments in which the ^{15}N z-polarization is stored under XY-4 ^{15}N decoupling with 180° pulses⁴⁷. Similarly, continuous wave (CW) decoupling can also be used to preserve the residual polarization for simultaneous acquisition of multiple experiments^{45, 62–63}. In this work, we used fully protonated samples with ^1H acquisition times set to 8.3 ms. As reported by Pintacuda and co-workers^{57, 64}, even at 100 kHz MAS rate, ^1H acquisition times of 10 ms are sufficient for fully protonated protein samples. However, proton resolution can be further improved by using fast MAS rates together with protein perdeuteration, which increases the ^1H T_2 relaxation times. For samples with longer T_2 relaxation, ^1H acquisition times are typically set to 20 to 30 ms. Note that for longer acquisition times the residual polarization may be lower, decreasing the sensitivity for the subsequent acquisitions. The pulse sequences shown here lay the groundwork for future investigation of transferred and residual polarization pathways under various sample conditions, including ^1H dilution or perdeuteration as well as different decoupling conditions.

In principle, there are multiple combinations of pulse sequences that are possible for each of the H-DUMAS, H-MEIOSIS, and H-MAeSTOSO strategies by incorporating various

homonuclear pulse sequence blocks with different mixing times³⁸. However, to best optimize the experimental time it is recommended to record the first increment ($t_1 = 0$) and then choose the appropriate homonuclear recoupling scheme and mixing times. Of course, as the number of back-CP periods increases, the signal decreases due to the lower amount of residual polarization. A key for the successful application of MAeSTOSO experiments is the use of larger number of scans to accumulate sufficient residual polarization to drive the 4th and 5th acquisitions³³. To this extent, it is important to code the low-sensitivity experiments, which require a larger number of scans and enable the accumulation of residual polarization, during the first two acquisitions of the pulse scheme and the more sensitive experiments for the following acquisitions. In this way, it is possible to accumulate sufficient residual polarization to drive the acquisition of the other experiments. The power of these experimental strategies were tested on the NAVL model peptide, as well as the SatP membrane protein. As shown in Figure 8, for small peptides with resolved peaks, sequential assignment can be obtained from a single H-MAeSTOSO-10 (or H-MAeSTOSO-4) experiment with multiple mixing periods. For larger proteins such as SatP, the ¹H resolution in the 2D spectra is still challenging for resonance assignment. Nevertheless, these 2D fingerprints are useful in setting up the corresponding 3D experiments. This new class of POE are not alternatives to the existing approaches to speed up data acquisition; rather it provides a clever strategy to concatenate multiple experiments into a single pulse sequence to optimize the timing of the spectrometers and speed up data acquisition through the acquisition of multiple 2D and 3D experiments. We also anticipate further applications of POE at fast MAS rates (e.g., 100 kHz) using other sensitivity enhancement approaches such as DNP, PRE, and non-uniform sampling^{65–67}. In fact, recent work by Pintacuda, Reif, and Meier groups demonstrates the feasibility of resonance assignment of large proteins using 100–110 kHz MAS rates^{55, 68–70}.

Pines and Waugh were the first to implement multiple acquisition solid-state NMR, also known as proton enhanced experiments, using a rich ¹H spin bath of unlabeled organic molecules⁶³. POE pulse sequences are built on a similar philosophy with two basic observations in ssNMR, (1) the rich ¹H spin bath enables the simultaneous generation of ¹³C and ¹⁵N polarization pathways, and (2) storage of ¹⁵N z-polarization which is unique for solid samples where the T1 relaxation times are significantly longer²⁹. In the previous POE pulse sequences, we utilized ¹³C and ¹⁵N residual polarization pathways resulting from NC cross polarization periods via MEIOSIS and MAeSTOSO experiments at slow spinning speeds. In this work, we systematically used similar experimental protocols with respect to slow MAS experiments to understand the relative efficiency of SIM-CP and SPECIFIC-CP residual polarization pathways at fast MAS rates. Furthermore, we also designed new strategies for recovering residual polarization from ¹³C–¹H or ¹⁵N–¹H back CP transfer periods. The ‘afterglow’ pulse sequence developed by Traaseth lab also exploits residual ¹⁵N polarization of SPECIFIC-CP and simultaneously records two 2D spectra NCA and NCO using CP as a preparation period⁷¹. Similarly, the residual ¹⁵N polarization resulting from hetero-nuclear NC mixing sequences has also been utilized at fast MAS rates⁴³. Proton detected H-DUMAS and H-MEIOSIS experiments are indeed extensions of its ¹³C-detected counterparts namely, DUMAS and MEIOSIS, and other versions have also been proposed

by other research groups using proton detection experiments at slow and fast MAS rates^{42–44}.

Finally, we would like to point out that POE can also be modified for dual receiver experiments using ^{13}C and ^1H detection. In fact simultaneous acquisition of ^1H and ^{13}C detected dual receiver experiments have already been reported by other research groups⁷². Multi-nuclear acquisition experiments can also be implemented using single receiver systems as demonstrated in UTOPIA experiment⁷³. From a technical point of view, at slow MAS rates POE may cause RF heating due to high power decoupling (~ 100 kHz), hence, it is strongly recommended to use low-E or E-free probes MAS probes³⁴. On the other hand, the low power decoupling (~ 10 kHz) at fast MAS rates make the POE more promising for multi-acquisition pulse sequences.

5. CONCLUSIONS

In this work, we have developed a new set of ^1H detected POE (Polarization Optimized experiments) that simultaneously acquire two to ten two-dimensional experiments. POE represent a general strategy for multiple acquisitions of almost all types of triple-resonance ssNMR experiments at slow and fast MAS rates. Proton detected H-DUMAS and H-MEIOSIS experiments are simple extensions of its ^{13}C -detected counterparts namely, DUMAS and MEIOSIS. On the other hand, MAeSTOSO experiments require synchronized WALTZ decoupling sequences to preserve the residual polarization pathways. The proposed methods can be extended to three and four-dimensional experiments together with multi-receiver technology. These new class of experiments are not alternatives to the existing approaches to speed up data acquisition; rather it provides a clever strategy to concatenate multiple experiments into a single pulse sequence to optimize the timing of the spectrometers and speed up data acquisition through the acquisition of multiple 2D and 3D experiments. When applied in concert with other fast acquisition and sensitivity enhancement techniques (*e.g.*, Dynamic Nuclear Polarization, Paramagnetic Relaxation Enhancements, *etc.*) this approach can further push the boundaries of ssNMR applications to structural biology.

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Highlights

- New polarization optimized experiments for proton detection of biomacromolecules
- Proton detection enables up to 10 experiments acquired with one pulse sequence
- Application to six transmembrane protein transporter SaTP

Figure 1:

(A) CP and SIM-CP pulse sequences used for quantifying relative signal intensities. For SIM-CP, ^{13}C and ^{15}N spectra were acquired in separate experiments with a single receiver. (B) and (C) Comparison of CP and SIM-CP on SatP membrane protein obtained from integrated intensities of ^{13}C and ^{15}N spectra acquired at 65 kHz and 12 kHz MAS rates, respectively.

Figure 2:

(A) Pulse sequence for two-dimensional H-DUMAS (proton detected DUMAS) experiment for simultaneous acquisition of ^{13}C - and ^{15}N -edited experiments, (H)CH and (H)NH. (B) 1D (H)CH and (H)NH spectra of SatP membrane protein obtained from first increment ($t_1=0$) of H-DUMAS (shown in blue and red), and the corresponding spectra (shown in black) obtained from single acquisition methods using 65 kHz MAS rate. For comparison the 1D spectra shown in blue and red are slightly shifted to the right. (C) Two-dimensional (H)CH and (H)NH spectra of SatP protein acquired using H-DUMAS pulse sequence with $n=2$ loops for ^{15}N t_1 evolution.

Figure 3:

MEIOSIS experiment at fast MAS rates. (A) Pulse sequence for 1D MEIOSIS that records four polarization pathways (CC_{res} , $\text{NCA}_{\text{trans}}$, NN_{res} , and $\text{CAN}_{\text{trans}}$) resulting from the first SPECIFIC-CP period τ . (B) Normalized integrated intensities of all four pathways measured for SatP protein by varying the τ mixing times. The polarization in each pathway is normalized with respect to initial value at $\tau=0$. (C) 1D spectra of CC_{res} , $\text{NCA}_{\text{trans}}$, NN_{res} , and $\text{CAN}_{\text{trans}}$ (color coded accordingly) polarization pathways obtained at $\tau=0$ and 6.5 ms. (D) 1D spectra at $\tau=6.5$ ms with corresponding intensities normalized with respect to highest intensity spectrum of CC polarization pathway.

Figure 4:
(A) Two-dimensional H-MEIOSIS (proton detected MEIOSIS) pulse sequence for simultaneous acquisition of two pairs of ^{13}C - and ^{15}N -edited experiments, (H)CH, (H)N(CA)H, (H)NH and (H)CA(N)H. (B) 2D H-MEIOSIS spectra of SatP protein acquired at 65 kHz MAS rate using two loops (n=2) for ^{15}N t1 evolution. A pair of 2D spectra in the 1st and 2nd acquisitions are obtained by adding and subtracting the two data sets recorded with $\phi=x$ and $-x$.

Figure 5:

(A) Pulse sequence for evaluating the residual ^{13}C and ^{15}N polarization that remains on ^{13}C and ^{15}N at the end of back CP periods (T_{CP}^{C} and T_{CP}^{N}). (B) Membrane protein SatP spectra acquired in 2nd and 4th acquisition periods by varying $T_{\text{WALTZ}}^{\text{C}}$ and $T_{\text{WALTZ}}^{\text{N}}$ decoupling periods. (C) Transferred and residual polarization of ^{13}C - ^1H and ^{15}N - ^1H back CP periods measured by varying T_{CP}^{C} and T_{CP}^{N} back CP periods.

Figure 6: Normalized integrated intensities of transferred and residual polarization pathways of ^{13}C - ^1H and ^{15}N - ^1H back CP obtained from the spectra reported in Figure 5C using the pulse sequences of Figure 5A.

Figure 7:

(A) Pulse sequence for the H-MAeSTOSO-4 experiment for simultaneous acquisition of (H)CHH, (H)NHH (RFDR=1.47 ms), (H)NHH (RFDR=0.74ms), and (H)NH experiments. Corresponding 2D spectra on SatP membrane protein are shown in (B). Suppression of ¹H signals from water and lipids is obtained by using t_{supp}=200ms. Prior to 3rd and 4th acquisitions 0.25*t_{supp} (=50 ms) period was used for eliminating waters signals. (C) 2D spectra (H)NHH and (H)NH obtained from H-MAeSTOSO-4 pulse sequence in the absence of 0.25*t_{supp} period, showing the residual water signals.