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Communication

Selective Excitation for Spectral Editing and Assignment in Separated Local Field Experiments of Oriented Membrane Proteins

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Abstract

Spectroscopic assignment of NMR spectra for oriented uniformly labeled membrane proteins embedded in their native-like bilayer environment is essential for their structure determination. However, sequence-specific assignment in oriented-sample (OS) NMR is often complicated by insufficient resolution and spectral crowding. Therefore, the assignment process is usually done by a laborious and expensive “shotgun” method involving multiple selective labeling of amino acid residues. Presented here is a strategy to overcome poor spectral resolution in crowded regions of 2D spectra by selecting resolved “seed” residues via soft Gaussian pulses inserted into spin-exchange separated local-field experiments. The Gaussian pulse places the selected polarization along the Z-axis while dephasing the other signals before the evolution of the ^1H - ^{15}N dipolar couplings. The transfer of magnetization is accomplished via mismatched Hartmann-Hann conditions to the nearest-neighbor peaks via the proton bath. By optimizing the length and amplitude of the Gaussian pulse, one can also achieve the phase inversion of the closest peaks, thus providing an additional phase contrast. From the superposition of the selective spin-exchanged SAMPI4 onto the fully excited SAMPI4 spectrum, the ^{15}N sites that are directly adjacent to the selectively excited residues can be easily identified, thereby providing a straightforward method for initiating the assignment process in oriented membrane proteins.

1. Introduction

Solid-state NMR is highly suitable for determining structures of membrane proteins (MPs) in their native-like environments. In particular, oriented-sample (OS) solid-state NMR (ssNMR) is a useful technique for the structural and functional studies of MPs. However, sequence-specific NMR assignment in OS NMR mainly relies on the “shotgun” method [1,2], which consists of isotopic labeling of one or a few specific amino acids, and which is generally limited to regular secondary structures such as α -helices [3,4] or β -sheets [5]. To address this issue, various computational methods have been developed for assigning non-helical resonances [6,7]. While the “shotgun” method can be effective in many cases, it is also quite time-consuming and expensive, as it requires preparation of multiple samples involving selectively labeled aminoacids. An outstanding problem in OS NMR is, therefore, being able to achieve a complete assignment of the NMR resonances for a single, uniformly labeled sample by purely spectroscopic means.

Proton-driven spin diffusion (PDS) [8] can be used to correlate the neighboring spins, and several methods based on proton-driven dilute-spin exchange [9,10] have emerged with the purpose of assigning sequence-specific resonances in OS NMR. A three-dimensional pulse sequence utilizing PDS has been recently developed [11]. Despite the fact that those sequences are useful for detecting cross-correlations between the nearby spins, they have a disadvantage of requiring long mixing times (up to several seconds) in order to establish the cross-peaks, especially between the weakly coupled spins. In addition, routine acquisition of three-dimensional spectra in OS NMR still remains a challenge due to relatively low sensitivity. Alternative methods that are similar to proton-assisted recoupling developed for MAS [12], which speed up magnetization transfer between the nearby spins by nearly three orders of magnitude, have been independently developed and applied for aligned samples [13-16]. One such method [14] is based on the proton-mediated polarization transfer between the low-gamma spins under mismatched Hartmann-Hahn conditions (MMHH). Correlations between the weakly coupled ^{15}N spins separated by up to 6.7 Å have been detected in a N-Acetyl Leucine (NAL) crystal [14]. The MMHH scheme can be combined with the SAMPI4 sequence [17], which correlates the ^{15}N chemical shifts with the ^1H - ^{15}N dipolar couplings, yielding additional internuclear cross-correlations [18,19]. The magnetization transfer under MMHH conditions has the advantage of being much faster than spin diffusion, (a few milliseconds vs. a few seconds)

which shortens the overall acquisition time. Nevertheless, the direct superposition of the SAMPI4 and the spin-exchanged SAMPI4 spectra is not always sufficient to unambiguously assign all the peaks since the cross-peaks and main peaks often overlap with each other. Acquisition of a third spectrum, such as the ^{15}N - ^{15}N chemical shift spin exchange, assists in the disambiguation of peak correlations in the crowded spectral regions [20]. However, some ambiguities associated with unavoidable spectral crowding in two dimensions may still remain even after acquiring the three independent spectra (i.e. SAMPI4, the spin-exchanged SAMPI4 and the homonuclear ^{15}N - ^{15}N exchange). This calls for a spectroscopic method that would allow one to eliminate these ambiguities in the presence of spectral overlap in the uniformly labeled sample.

Highly selective composite pulses such as Gaussian [21], DANTE [22], Sinc [23], phase and/or amplitude modulated pulses, self-refocusing 270° Gaussian [24], Gaussian cascade [25], and others have been previously developed for manipulating select spectroscopic regions. OS NMR exhibits an exceptionally broad range of anisotropic chemical shifts (100's of ppm or several kHz), which makes selective excitation especially attractive. In the present paper, we have implemented selective excitation in the spin-exchanged SAMPI4 sequence [17,18]. The following goals have been addressed here i) excitation of a selected region in a uniformly labeled sample spectrum during the preparation period and ii) evolution of cross peaks at the locations of main peaks in the separated local field (SLF) spectrum by storing the magnetization of the excited spin site followed by nearest-neighbor magnetization transfer. Only the peaks that are spatially proximal to the excited site are subsequently evolved in the 2D SLF spectrum, thereby simplifying crowded regions in the spectra that would otherwise arise in fully excited spectra. It has also been found that a 90° Gaussian pulse truncated at 6% (relative to its maximum height) yields an excitation profile with both positive and negative phases, which provides an additional contrast for the sequential assignment of resonances in 2D SLF experiments.

NMR samples. A single ^{15}N -labeled Acetyl-Leucine (NAL) crystal (18 mg, prepared in-house), exhibiting four sharp resonances corresponding to four different ^1H - ^{15}N bond orientations in a unit cell, was utilized. The protein sample consisted of ca. 4 mg of uniformly ^{15}N -labelled Pf1 coat protein (expressed as previously described in ref. [26]) reconstituted in 170 μL solution of DMPC/DHPC bicelles at a molar ratio of $q = 3.2$. The sample was prepared at the 28% w/v ratio of lipids in a 0.2 M HEPES buffer ($\text{pH} = 7.0$) and the lipid-to-protein ratio was kept at around 55:1.

Fig 1. Pulse sequences employed in the present study: **A.** REP-CP enhanced SAMPI4, **B.** SAMPI4 with internuclear correlations established by the mismatched Hartmann-Hahn conditions, **C.** Selective SAMPI4 sequence, which can be used either with or without the MMHH block.

NMR spectroscopy. The experiments on the NAL crystal were performed on a Bruker Avance II spectrometer operating at the ^1H Larmor frequency of 500 MHz and using TopSpinTM 2.0 software. The crystal spectra were measured in a static Bruker 5 mm round-coil E-freeTM probe. The spectra were acquired at 25°C. Experiments on the Pf1 bicelle samples were performed on a Bruker Avance III spectrometer at the National High Magnetic Field Lab (Tallahassee, FL) operating at the ^1H Larmor frequency of 900 MHz and using TopSpinTM 3.2 software. The spectra were acquired at 37°C on a 5 mm static probe [27]. All the spectra in this paper were processed using either TopSpin or the nmrPipe [28] software.

Pulse sequences. The pulse sequences utilized in this article were: the 2D SLF SAMPI4 sequence [17] (Fig. 1.A), a spin-exchanged SAMPI4 sequence [18,19] (Fig. 1.B), a selectively edited SAMPI4 (Fig. 1.C without the dilute spin-exchange MMHH block) and a selective spin-exchanged SAMPI4 sequence (Fig 1.C with the MMHH block). The REP-CP pulse sequence [29,30] was utilized to acquire Pf1 spectra, which was proven to greatly enhance sensitivity for protein samples exhibiting high degree of rotational dynamics.

3. Results and discussion

Static NAL Crystal. SLF SAMPI4 and spin-exchanged SAMPI4 spectra (Fig. 2A) were acquired for the NAL crystal at an arbitrary orientation. The relevant pulse sequences are shown in Figs. 1A and 1B, respectively. During the spin-exchanged SAMPI4 experiment, the magnetization between the dilute ^{15}N spins is transferred under the mismatched Hartmann-Hahn (MMHH) conditions using the proton dipolar bath by applying rf irradiation on the two channels with ca. 10 kHz mismatch (relative to the Hartmann Hahn match) for up to 10 ms. Here, the Zeeman order of the ^{15}N spins is first transferred to the dipolar order of the ^1H spins, and then transferred back to the Zeeman order of the neighboring ^{15}N spins, thus resulting in cross-peaks in the spin-exchanged spectrum even between ^{15}N spins having extremely small homonuclear dipolar couplings [14]. In the spin-exchange SAMPI4 experiment (Fig. 2A, gray contours), the cross-peaks appear with the same dipolar coupling as the sites that the magnetization has originated from, but with the chemical shift of the observed ^{15}N sites. For example, the polarization from peak #1 (at ca. 175 ppm) is transferred to the three neighboring ^{15}N sites, and the cross-peaks all appear with the dipolar coupling of peak #1 (ca. 6.5 kHz) but with different chemical shift frequencies corresponding to the three adjacent sites. Since the spectrum contains

16 well-resolved peaks (4 main peaks and 12 cross-peaks), the NAL crystal can be used as a model system for sequence optimization. However, in proteins and macromolecules, many more ^{15}N sites are involved, thus potentially resulting in crowded spin-exchanged spectra.

Fig. 2. A. SAMPI4 spectrum (red, pulse sequence shown in Fig. 1A) obtained for the NAL crystal superimposed with a spin-exchanged SAMPI4 spectrum under mismatched conditions (MMHH) (gray, pulse sequence shown in Fig. 1B). A mismatch of 8.4 kHz during 8 ms MMHH contact was applied. The arrows indicate the direction of the magnetization transfer between the coupled ^{15}N spins, #1 and #2.

B. Selective-SAMPI4 spectrum (red, pulse sequence in Fig. 1C without the MMHH block) overlaid with the selective SAMPI4-MMHH spectrum (gray, pulse sequence in Fig. 1C) acquired for the same sample. The carrier frequency was set for the leftmost peak at 50.702187 MHz (or 176 ppm). A 6%-truncated Gaussian was experimentally optimized at a 960 Hz B_1 field amplitude and 1.6 ms duration, thus allowing for a 80%-efficient selection of the peak of interest. A Hartmann-Hahn mismatch of 9.5 kHz was applied, and 10 ms contact time was used. The arrows show the correlations between the first (leftmost) peak and the remaining three ^{15}N sites now appearing at the spectroscopic locations of main peaks.

Selective excitation was then applied to the spin-exchanged SAMPI4 spectrum in order to evolve “cross peaks” as “main peaks”, thus greatly reducing the number of peaks in the correlation spectrum. For this purpose, a selective Gaussian pulse (with 6% truncation) was included in the SAMPI4 sequence immediately after the polarization build-up on the ^{15}N sites, but *before* the ^1H - ^{15}N dipolar coupling evolution. This idea of utilizing selective pulses during the preparation period is somewhat similar to that of Freeman et al. for increasing digitization in the indirect dimension in homonuclear solution NMR experiments [31]. The amplitude and duration for the Gaussian pulse (placed at the carrier frequency corresponding to peak #1, i.e. 50.702187 MHz or 176 ppm) was optimized at 960 Hz and 1.6 ms, respectively. During the Gaussian pulse on the ^{15}N spins the ^1H spins are being decoupled. The resulting pulse is highly selective and allows one to bring back about 80% of the magnetization for peak #1 to the z-axis, while the other three peaks are being dephased. Without the MMHH mixing period, when the spectroscopically selected ^{15}N spins (peak #1) are brought back to the (xy) plane by a hard pulse, only the ^1H - ^{15}N dipolar coupling corresponding to peak #1 is evolved. Thus, a single peak (Fig. 2B, red contours) is observed, thereby confirming the high selectivity of the Gaussian-shaped pulse.

The MMHH mixing period was then included immediately after the selective excitation scheme, followed by evolution of the ^1H - ^{15}N dipolar couplings in the indirect dimension (Fig. 1C). A short (0.25 s) z-filter was used to re-equilibrate the ^1H spins and destroy any residual ^{15}N magnetization. Therefore, the selectively excited ^{15}N site provides the sole source for the polarization that is transferred to the neighboring ^{15}N sites during the MMHH block. Finally, the heteronuclear dipolar couplings of the polarized sites are evolved during the SAMPI4 block. By overlapping the selective spin-exchanged spectrum (grey, Fig. 2-B) with the fully excited SAMPI4 (red, Fig. 2-A), the connectivity between the selectively excited ^{15}N site and its coupled sites can be easily established. It should be noted that in the NAL crystal case all four spins sites are coupled to each other (thus, yielding three cross peaks for one main peak); whereas for the nearest-neighbor interactions between adjacent aminoacid residues in proteins only two cross peaks are expected per residue (assuming only the $i, i\pm 1$ exchange).

The example of the NAL crystal illustrates the method of establishing spatial proximity between the spin sites based on transferring the polarization originating from peak #1 to the coupled spins #2, #3 and #4 in the locations of main peaks in the SLF spectrum (albeit with one-half of the intensity for the cross peaks shown in Fig. 2A, or about 5% of the fully excited peak).

It is entirely possible to selectively excite a different peak by moving the carrier frequency for the Gaussian pulse to the corresponding frequency in order to obtain a selective spin-exchanged SAMPI4 spectrum with the magnetization originating from that peak (results not shown). However, since all the four ^{15}N sites of the NAL crystal are coupled to each other, the selectively evolved spin-exchanged SAMPI4 spectra are all expected to be similar in appearance (except for their relative intensities).

Pf1 Coat protein reconstituted in magnetically aligned bicelles. For membrane proteins reconstituted in macroscopically aligned bilayers, the spectroscopic assignment via spin-spin correlations represents a complex and laborious process [2,20]. To facilitate the initial assignment of the spin-exchanged SAMPI4 spectrum, which often exhibits overlapping of the cross and main peaks, the same selective excitation technique (with the pulse sequence shown in Fig. 1.C) was implemented.

First, fully excited ^{15}N - ^{15}N spin exchange and spin-exchanged SAMPI4 spectra were acquired on the Pf1 sample to illustrate the potential ambiguity in the assignment process for the representative G37 and S41 peaks. As can be seen, misassignments of cross peaks could indeed arise, especially when resonances have very close chemical shifts and/or dipolar couplings. For example, as can be seen from Figs. 3A and 3B, the cross-peak arising from the magnetization transfer from G37 to A36 is expected to be in the spectral region of around 95 ppm and 2.7 kHz (according to the assignment taken from refs. [2,20] and shown in Fig. 3B). However, a clustering of the main peaks and cross-peaks in that region results in poor spectral resolution and, thus, the relevant cross-peaks cannot be unambiguously established. This phenomenon arises for the cross-peaks between S41 to M42 and S41 to Y40, in the region at 90-95 ppm and 3.3 kHz, and many others. Even though the cross-peaks for the opposite pathway of the magnetization transfer (i.e. from A36 to G37, M42 to S41 and Y40 to S41) are resolved, it is always desirable to be able to identify both cross-peaks between the coupled residues to avoid assignment ambiguities. For example, it is unclear from the spin-exchanged SAMPI4 spectrum of Fig 3B (gray contours), which resonance (i.e. at 100, 95, or 84 ppm) is responsible for the lower cross peak to the G37 resonance.

Fig. 3. A. REP-CP enhanced ^{15}N - ^{15}N spin-exchange spectrum obtained for 4.2 mg of Pf1 reconstituted in DMPC/DHPC oriented bilayers at 900 MHz ^1H frequency. Five CP contacts of 120 μs each and a 150 ms z-filter were applied during the REP-CP block. A 90° pulse of 5.53 μs , 64 scans and 128 complex t_1 points in the indirect dimension were utilized. A 5 ms mismatch of 5 kHz was applied by irradiating the ^1H and ^{15}N channels at the B_1 rf amplitudes of 25 and 20 kHz, respectively. Rectangles (black lines) show the correlations between residue G37 and its nearest and next-to-nearest neighbors: V35, A36, L38 and I39.

B. REP-CP enhanced MMHH spectrum (gray) overlapped with a control SAMPI4 (red) for the same Pf1 sample using 512 and 16 scans, respectively and 64 t_1 points. A 6 ms mismatch of 5 kHz was applied by using rf irradiations on the ^1H and ^{15}N channels with the B_1 amplitudes of 25 and 20 kHz, respectively. The rectangular dashed boxes show the correlations between G37 and its nearest coupled spins, L38 and A36, via cross-peaks.

Selectively evolving the spectral peaks based on their spatial proximities would greatly simplify the spectrum, thus assisting in the NMR assignment process. For this purpose, an optimized Gaussian pulse with the duration of 1,500 μs having the maximum B_1 rf field of ca. 680 Hz was utilized with its carrier frequency positioned exactly on G37, i.e. at 90.768843 MHz (or 65 ppm). As a result, the selective pulse yields a positive phase for the selectively excited G37 peak (blue contours) and a negative phase for its closest peak, S41, (red contours) while eliminating all other ^{15}N magnetization (cf. Figs. 4A and B). Such phase contrast can be achieved by varying the B_1 rf field, the pulse length and applying an amplitude truncation to the Gaussian

pulse (with respect to the beginning and the end of the pulse). As was demonstrated previously [21], the excitation profile of a Gaussian pulse has a decaying sinc-like shape with alternating positive and negative intensities. It is possible to further attenuate those oscillations by truncating the Gaussian pulse [31]. As for the NAL crystal, we chose a 6% truncation level to achieve an additional phase contrast in selectivity, since too high a selectivity would require extremely low rf powers (tens of mW or less), which is not always feasible with a typical solid-state NMR preamplifier. It should also be noted that a loss of up to 25% intensity for the selectively excited peak was observed (as compared to a hard-pulse excitation). The relatively short $T_{1\rho}$ relaxation time for the ^{15}N spins (of several ms) [29], together with the Gaussian pulse imperfections, could be responsible for such a decrease in the intensity due to a dephasing during the soft pulses.

The inclusion of the MMHH block right after the Gaussian pulse in the sequence of Fig. 1C allows one to transfer the magnetization from the polarized ^{15}N spins (originating from G37, having a positive intensity and S41, having a negative intensity) to their strongest coupled ^{15}N spin sites. In passing, we should note that other mixing techniques such as PDSO could be employed for the magnetization transfer as well. Slices through two representative peaks (L38 and M42) correlated to the “seed” residues G37 and S41 are shown in Fig 4C and the expected transfer pathways from G37 to V35, A36, and L38 and from S41 to Y40 and M42 are shown by arrows in the corresponding two-dimensional spectrum of Fig. 4D. As can be seen from Fig. 4D, application of the selective MMHH-SAMPI4 sequence (Fig. 1C) leads to a much simpler spectrum and also alleviates some assignment problems arising in the much more crowded fully excited spin-exchange spectra. Moreover, the ‘main peaks’ (being ‘cross-peaks’ in nature) also conserve the phase of the originating signal, thus providing an additional contrast. Indeed, the peaks with a positive phase (blue) originate from G37 and the negative (red) come from S41. This facilitates the interpretation of connectivities between the spectral peaks. For instance, the peaks for L38 and A36 that are closest to G37 clearly show up in the selective MMHH-SAMPI4 spectrum, in accord with the previous assignment [20,32]. Two overlapped longer-distance ($i, i\pm 2$) peaks, V35 and I39, also appear (as in the fully excited ^{15}N - ^{15}N spectrum of Fig 3A). The peak at ca. 138 ppm, corresponding to the highly mobile A46 residue, and the mobile side chains R44 at 75 ppm show up as well in the spectrum, which represents a complication. For the negatively phased S41 peak, the situation is less complicated: only the nearest-neighbor Y40 and M42 peaks arise (however, they are somewhat weaker in amplitude).

Fig. 4. **A.** Selectively excited peaks for residues G37 (blue, positive intensity) and S41 (red, negative intensity) taken as slices from the two-dimensional SAMPI4 spectrum. **B.** Selectively-excited SAMPI4 spectrum (pulse sequence of Fig. 1C but without the MMHH block) obtained for Pf1 coat protein in oriented DMPC/DHPC bilayers at 900 MHz ^1H frequency. The carrier frequency was shifted on residue G37 at 90.768843 MHz (or 65 ppm) and the 6%-truncated Gaussian pulse was experimentally optimized at 1,500 μs and 680 Hz rf amplitude, which allows for a double selectivity: positive phase on G37 (blue) and negative phase on S41 (red). The contour levels were lowered to illustrate the high selectivity of the Gaussian pulse. **C.** Peaks for residues L38 and M42 taken as slices from two-dimensional selective MMHH-SAMPI4 spectrum. **D.** Selective MMHH-SAMPI4 spectrum (blue contours for positive intensities, red for negative intensities, pulse sequence shown in Fig. 1C) overlapped with a control SAMPI4 (gray contours) for the same Pf1 sample. 2048 and 16 scans were used for the selective MMHH-SAMPI4 and the control SAMPI4, respectively. The Gaussian pulse parameters were the same as in Fig. 4A and 6 ms MMHH block was applied with 20 and 26 kHz rf amplitudes on the ^{15}N and ^1H channels, respectively. The arrows indicate magnetization transfer from the ‘seed’ resonances to their nearest neighbors, thus allowing for initiation of the assignment process.

In addition, in the selective MMHH-SAMPI4 sequence about one-half of the cross-peak intensity is lost during the dipolar evolution since the proton magnetization is not employed (as is normally done for SAMPI4), but is kept on the z-axis at the beginning of the dipolar evolution. While this is necessary to avoid any cross-polarization transferring back from the ^1H to ^{15}N sites during the SAMPI4 block, it also results in a zero-frequency artifact in the dipolar dimension (which can be partially removed by using a baseline correction in the indirect dimension). As can be seen from Fig. 4C, the signal-to-noise ratio for the correlated peaks is about 2:1, thereby making sensitivity the most important issue for this technique. For instance, the additional weak peaks arising at 85 ppm/1.8 kHz and 56 ppm/2.6 kHz are clearly noise as they do not appear in the control SAMPI4 spectrum. By monitoring the shape of the Gaussian pulse by an oscilloscope with and without the pre-amplifier connected, we have also observed an appreciable distortion of the Gaussian profile in the former case, which undoubtedly affects selectivity and efficiency of the shaped pulse, especially when relatively low rf amplitudes are used. As a result, the extra peak observed at 80 ppm/2.9 kHz (at the location of L30), as well as phase distortions around the main excited peaks, were attributed to the imperfections of the Gaussian pulse that result in a somewhat broader and irregular excitation profile.

4. Conclusion

The selectively edited 2D Separated Local Field sequence introduced in this paper (a soft Gaussian pulse followed by spin-exchange under mismatched Hartmann-Hahn conditions and a SAMPI4 block) allows for an easier establishment of connectivities between specific ^{15}N “seed” resonances in the spectra of membrane proteins reconstituted in magnetically aligned bicelles. As an illustration of the method, we have chosen resonances G37 and S41 of Pf1 coat protein spectra to establish through-space connectivities to their nearest neighbors. These well-resolved residues also represent outliers from the helical wheel, or PISA, region that often could not be assigned in a straightforward manner. This strategy can also help in initiating the spectroscopic assignment process for the crowded spectral regions utilizing a single, uniformly labeled sample. By taking advantage of the phase contrast of the truncated Gaussian pulse, the coupled ^{15}N sites can appear either with a positive intensity (e.g. V35-I39) or negative intensity (Y40 and M42), thus further assisting the disambiguation process. Since those correlations appear with the chemical shift and dipolar coupling of the corresponding spin sites, it is now possible to start disentangling spectroscopic assignment by directly comparing the selective spin-exchange

spectrum with a fully excited SAMPI4. This strategy of spectral editing during the preparation period as demonstrated here for OS NMR, is also potentially applicable for heteronuclear solution NMR and MAS experiments when selective excitation is practical.

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