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Application of Paramagnetic Relaxation Enhancements to Accelerate the Acquisition of 2D and 3D Solid-State NMR Spectra of Oriented Membrane Proteins

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Abstract

Oriented sample solid-state NMR (OS-ssNMR) spectroscopy is uniquely suited to determine membrane protein topology at the atomic resolution in liquid crystalline bilayers under physiological temperature. However, the inherent low sensitivity of this technique has hindered the throughput of multidimensional experiments necessary for resonance assignments and structure determination. In this work, we show that doping membrane protein bicelle preparations with paramagnetic ion chelated lipids and exploiting paramagnetic relaxation effects it is possible to accelerate the acquisition of both 2D and 3D multidimensional experiments with significant saving in time. We demonstrate the efficacy of this method for a small membrane protein, sarcolipin, reconstituted in DMPC/POPC/DHPC oriented bicelles. In particular, using Cu²⁺-DMPE-DTPA as a dopant, we observed a decrease of ¹H T₁ of sarcolipin by 2/3, allowing us to reduce the recycle delay up to 3 times. We anticipate that these new developments will enable the routine acquisition of multidimensional OS-ssNMR experiments.

Keywords

Oriented sample solid-state NMR; paramagnetic relaxation enhancements; separated local fields; sensitivity enhancement

1. Introduction

Solid-state NMR (ssNMR) is the only spectroscopic technique that provides atomic-resolution structural information of membrane proteins in lipids under liquid crystalline state [1–5]. Currently, ssNMR methods come in two different flavors: magic angle spinning (MAS), and oriented sample (OS) ssNMR. While MAS experiments are the primary source

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for isotropic chemical shifts, torsion angles, and dipolar couplings [6–8], OS-ssNMR provides topological information of membrane proteins embedded in fully hydrated lipid membranes [9–12], through anisotropic parameters, such as chemical shift anisotropy (CSA) and dipolar coupling (DC) [13]. These two techniques are being applied synergistically to characterize the secondary and tertiary structures as well as the architecture of the proteins within lipid bilayers [1, 14–16]. OS-ssNMR techniques, however, remain hampered by challenges such as the low protein concentrations (protein to lipids ratios range from 1:100 to 1:200), hardware limitations (RF heating), as well as length of the multidimensional experiments.

The introduction of oriented lipid bicelles [17] and the low-E probes [18] have dramatically improved both sample preparation and spectroscopy. In pursuit of further reducing the acquisition times, our group recently implemented sensitivity enhancement (SE) schemes into the separated local field (SLF) experiments [19], which boosted the sensitivity of multidimensional experiments up to 180%. However, the acquisition of 2D and 3D OS-ssNMR spectra is still time-consuming. During the execution of a pulse sequence, the majority of the experimental time is spent waiting for the spin systems to return to equilibrium via the longitudinal relaxation mechanism (T_1). Therefore, the spectrometers remain idle for ~90–95% of the time, and, as a result, 3D experiments may take up to several weeks.

In recent years, several groups have used paramagnetic agents as means of providing distances as well as to accelerating the acquisition of multidimensional experiments in MAS experiments [20–23]. In fact, paramagnetic centers with unpaired electrons enhance nuclear T_1 relaxation rates of proteins *via* dipolar interaction [24], significantly reducing the experimental time associated with long recycle delays. In particular, Cu^{2+} -chelated complexes have been employed as doping agents to facilitate data collection on microcrystalline protein preparations as well as fibrillary proteins [22, 25–33]. Remarkably, 100-fold saving in time was achieved by combining Cu^{2+} -EDTA doping with ^1H detection and ultra-fast MAS [29]. Other successful applications include chelated complexes of paramagnetic metal ions (*i.e.*, Co^{3+} , Ni^{2+} , and Gd^{3+}) and radicals [34–37].

Ramamoorthy and co-workers first demonstrated the use of paramagnetic ion chelated lipids to reduce longitudinal ^1H relaxation times for experiments with bicelles using OS-ssNMR techniques [38]. Comparing with Cu^{2+} -chelated complexes, paramagnetic ion chelated lipids eliminate the mobilizing of the ions avoiding RF heating that would damage the samples. More recently, Nevzorov's group used PRE arising from free radicals to speed-up the acquisition of 2D OS-ssNMR of pf1 coat protein reconstituted in lipid bicelles [37, 39]. Here, we show that doping lipid bicelles with ~5% of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid copper salt (Cu^{2+} -DMPE-DTPA) reduced ^1H T_1 relaxation rates of a membrane protein significantly, subsequently shortening the recycle delays and speeding up the acquisition of a 3D NMR spectrum used for sequential resonance assignment of a membrane protein amide backbone. As a benchmark, we used sarcoplipin (SLN), a 31-residue single-pass polypeptide that regulates the activity of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) [40–45]. We show a significant reduction in time for both 2D and 3D OS-ssNMR experiments. These

advancements will make it possible to carry out routine multidimensional NMR experiments on membrane proteins.

2. Material and Methods

2.1. Detergent-mediated reconstitution of SLN in oriented lipid bicelles

Recombinant [^{15}N] SLN was produced as previously described [46]. Figure 1 shows a schematic of our detergent-mediated reconstitution protocol for membrane proteins in magnetically aligned bicelles. As previously observed [1], we found that using detergents rather than organic solvents to reconstitute SLN in bicelles improved the amide line widths and enable a better control of pH of the preparation. For the SLN in bicelle samples, we prepared three different lipid samples: the first in which we used CHCl_3 to solubilize 26.6 mg 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, long chain lipids) and 7.5 mg 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, long chain lipids), the second, 6.1 mg 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC, short chain lipids), and the third, 2.4 mg 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-diethylenetriaminepentaacetic acid copper salt (Cu^{2+} -DMPE-DTPA, paramagnetic lipids). To eliminate any trace of organic solvents, we dried all lipids under a flux of N_2 gas and lyophilize overnight. We dissolved DMPC and POPC in C_{12}E_8 (detergent/lipid ratio of 2:1 w/w) to form a clear suspension of mixed micelles. We then solubilized SLN (1.8 mg) separately with 1% C_{12}E_8 solution and then added to the mixed micelle preparation. We adjusted the sample volume to 20 mL using the 'NMR buffer' (20 mM HEPES, 50 mM KCl, 2.5 mM MgCl_2 , 0.25 mM DDT, 0.02% NaN_3). We incubated the mixture containing of SLN, long chain lipids, and C_{12}E_8 with Biobeads® (1:30 w/w ratio for detergent/Biobeads®) for 3h to completely remove the detergent. The cloudiness of the suspension confirmed the formation of the DMPC/POPC vesicles containing SLN. After Biobeads® removal, we adjusted the pH of the sample to 7.0 and centrifuged the DMPC/POPC vesicles containing SLN at a speed of 50,000 g for one hour. We suspended the other pellets containing Cu^{2+} -DMPE-DTPA and DHPC separately in two test tubes with 30 μL of NMR buffer and added them in sequence to the vesicle preparation. To ensure the formation of a homogeneous suspension, we vortexed the protein/lipid mixture for about 2 min and formed the bicelle phase by carrying out 3–5 freeze/thaw cycles. Finally, we concentrated the sample to a volume of 180 μL prior to loading it into a 5 mm flat-bottom glass tube for spectroscopy.

2.2. NMR Methods, and Instrumentation

All the experiments were performed on Varian VNMRS spectrometers operating at a ^1H frequency of either 600 MHz or 700 MHz equipped with low-E static bicelle probe built by the RF program at the National High Magnetic Field Laboratory (NHMFL) in Florida [18]. We used a 90° pulse length of 4 μs (62.5 kHz) for both ^{15}N and ^1H channels. We set the cross-polarization (CP) time at 750 μs on ^1H and ^{15}N channels. During CP, the ^1H amplitude was linearly swept from 38 kHz to 62 kHz, while ^{15}N amplitude was kept constant at 50 kHz. We used a SPINAL64 decoupling sequence [47] during acquisition with 50 kHz ^1H RF amplitude. During the SE-SAMPI4 [48], we implemented a t_1 homonuclear decoupling on ^1H channel using 62.5 kHz RF amplitude with a dwell time of 96 μs . We used phase-switched spin-lock pulses on ^{15}N channel with 62.5 kHz RF amplitude during the t_1

evolution matching the Hartmann-Hahn condition. For sensitivity enhancement, we set τ delay of the SE scheme to 75 μ s, corresponding to three cycles of phase-modulated Lee-Goldberg (PMLG) [49] with effective RF amplitude of 80 kHz. For water-edited experiments, we set the τ value to 1.8 ms, which acts as a T_2 filter for dephasing the ^1H magnetization of both protein and lipids, while retaining water magnetization. We achieved water-protein polarization transfer by varying the τ_{mix} value. For the proton driven spin diffusion (PDSD) experiment [50, 51], we set the mixing time at 3 s. In the absence of paramagnetic lipids, we used a recycle delay of 3 s, whereas we set the recycle delay to 1 s for Cu^{2+} -DMPE-DTPA doped samples.

3. Results

3.1. PRE in OS-ssNMR

The use of paramagnetic ion chelated lipids such as Cu^{2+} -DMPE-DTPA (Figure 2a) is an efficient way to introduce dopants into magnetically aligned bicelles [38]. In fact, paramagnetic ion chelated lipids have two major advantages: 1) the ion centers are located close to the proteins, providing more significant PRE effects, and 2) only 5%–10% doped lipids are typically needed to induce a significant effect on the ^1H T_1 (Figure 2b).

Figure 3a shows the amide region of SLN detected using the ^1H inversion recovery pulse sequence (180° - τ - 90°). As shown by the spectra as a function of τ , the T_1 relaxation is reduced from 0.89 s to 0.29 s using bicelles doped with 5% of Cu^{2+} -chelated lipids. For diamagnetic samples, a recycle delay of approximately $3 \times T_1$ is generally used, which recovers about 95% of the ^1H polarization back to z-direction prior to next scan. As shown in Figure 3b, for the paramagnetic-doped bicelle samples the recycle delay can be reduced from 2.67 to 0.87 s, speeding up the execution of the pulse sequences.

Although the low-E probe technology dramatically reduces RF heating, it is recommended to use 1 to 1.5 s recycle delay to avoid sample overheating [38]. Lowering the RF decoupling field (*i.e.*, 25 to 30 kHz) makes it possible to reduce the recycle delay to less than 1 s. However, under our experimental conditions, we found that low decoupling powers lead to significant resonance broadening, jeopardizing the spectral resolution (Figure 3c).

3.2. Application of paramagnetic ion chelated lipids to speed up 2D SE-SLF experiments

Figure 4a shows the residue-specific ^1H T_1 relaxation for $[\text{U}-^{15}\text{N}]$ -SLN using 2D SE-SAMPI4 experiment preceded by a ^1H inversion recovery sequence (180° - τ - 90°). The distance between the membrane surface and the center of the bicelles is ~ 25 Å, which is beyond the effective distance for Cu^{2+} to influence ^1H longitudinal relaxation mechanism. However, the site-specific ^1H T_1 values (Figure 4b) show the PRE enhancement throughout the transmembrane residues of SLN, probably due to a spin diffusion mechanism [38].

Figures 4c and 4d compare the 2D SE-SAMPI4 spectra of $[\text{U}-^{15}\text{N}]$ -SLN with and without 5% Cu^{2+} -chelated lipids. The total acquisition times were 3 hours using 3.0 s of recycle delay and only 35 minutes using 1.0 s for the samples without and with Cu^{2+} -chelated lipids, respectively. The identical resonance positions (CSA and DC) in these two spectra suggest that no conformational or topological changes were caused by the addition of paramagnetic

lipids. Moreover, no significant line broadening of the resonances was observed for all the residues including those near the membrane surface (see Figure 4). The latter result indicates that the Cu^{2+} -chelated lipids do not change ^{15}N T_2 relaxation significantly, even though the paramagnetic centers are localized close to SLN, as observed for A β (1–42) peptide in membrane mimetic environments [33]. Other MAS ssNMR studies have shown that quenching of the resonances belonging to residues located on the extra-membrane loops using Gd^{3+} -chelated lipids, due to strong effects on both ^1H T_1 and $^{13}\text{C}/^{15}\text{N}$ T_2 relaxation mechanisms for residues nearby the paramagnetic centers [52].

3.3. Speeding up protein sequential assignment using PRE for acquisition of 2D and 3D OS-ssNMR experiments

For single-pass membrane proteins, the regular patterns of the 2D SE-SLF spectra together with selective labeling of amino acids are often sufficient to assign the backbone resonances [53, 54]. However, this approach fails when deviations from ideal helices occur or when multispans membrane proteins are analyzed. To obtain backbone sequential assignments, a successful strategy is combining SLF experiments with through-space correlations [55–57]. These experiments can be accomplished via homonuclear ^{15}N - ^{15}N polarization transfer using proton-driven spin diffusion (PDSD) [58, 59], cross-relaxation driven spin diffusion (CRDSD) [60], or mismatch Hartmann-Hahn (MMHH) polarization transfer methods [61, 62]. In our lab, we successfully implemented these schemes in both 2D and 3D sequential correlation experiments such as *dual acquisition* 2D SLF-PDSD and 3D SE-SLF-PDSD [57]. Usually, we utilize 2D SLF-PDSD experiments as a quick way to confirm resonances sequential assignments, while we use the 3D SE-SLF-PDSD for *de novo* resonance assignments.

Figures 5a and 5b show the 2D SE-SAMPI4-PDSD spectra of $[\text{U-}^{15}\text{N}]$ -SLN reconstituted in bicelles with and without 5% Cu^{2+} -DMPE-DTPA. The overall patterns of the two spectra are essentially identical. This result suggests that the polarization transfer efficiency of the PDSD element is not affected by the paramagnetic centers. We then tested the PRE effects on the 3D SE-SAMPI4-PDSD experiment with $[\text{U-}^{15}\text{N}]$ -SLN using 1.5 s as recycle delay. Figures 5c and 5d show that most of the resonance peaks display sequential correlations. In fact, in the 3D strip plots it is possible to assign all of the resonances from the transmembrane region of SLN unambiguously. This new set of data matches the previous assignment acquired in the absence of paramagnetic centers [57]. In this case, however, the time saving is not as significant as for the 2D SE-SLF experiments, due to the long PDSD mixing time (3 s). Nonetheless, the saving of experimental time is 25% with respect to the experiment acquired with diamagnetic sample. The total experimental time for 3D SE-SAMPI4-PDSD spectra is ~95 hours against ~120 hours for the diamagnetic sample.

3.4. Application of paramagnetic ion chelated lipids for water-edited OS-ssNMR experiments

Water-protein interactions play an important role in driving the structural dynamics and functional properties of proteins [63–67]. Recently, we implemented a new spectral editing technique for oriented membrane proteins to map the residue-specific water-protein interactions (water-edited SE-SLF) [68]. In this experiment, a 90° pulse followed by a spin-

echo period (~1.6 ms) retains ^1H polarization from bound water molecules close to the membrane surface, while dephasing the ^1H coherences of protein and lipid molecules. The water polarization is then transferred to protein protons during the homonuclear transfer period (τ_{mix}), followed by 1D CP or 2D SE-SLF sequence. Figure 6a shows the build-up curve extracted from 1D water-edited CP spectra of SLN bicelle sample doped with 5% of Cu^{2+} -DMPE-DTPA. The mixing times (τ_{mix}) were set to 0, 3, 5, 7, 10, 30, 50, 100, 150, and 400 ms. The integrated peak intensities of the 1D spectra between 0 and 200 ppm are plotted with respect to the square root of mixing time. The polarization builds up monotonously until 100 ms mixing time and is followed by a gradual decay. This expected behavior is due to paramagnetic quenching of T_1 relaxation of bound water molecules that are in close proximity to the Cu^{2+} center on the lipid head groups. Nevertheless, only shorter mixing times (less than 20 ms) are generally useful for spectral editing and thus paramagnetic sample preparations make it possible to acquire these water-edited experiments with shorter recycle delay. As an example, Figures 6b and 6c show the 2D water-edited SE-SAMPI4 spectra of $[\text{U-}^{15}\text{N}]$ -SLN using 1.5 s recycle delay with 7 and 100 ms mixing times. At 7 ms of mixing time, only those residues close to the membrane surface were polarized. Whereas at 100 ms, the entire transmembrane domain residues are polarized via water-protein and ^1H - ^1H spin-diffusion processes.

4. Discussions

The use of PRE is becoming more widespread for both liquid-state and ssNMR spectroscopy, either for measuring long-range distances or for decreasing NMR experimental time. In the latter case, the key is to use paramagnetic agents that act only on the ^1H T_1 mechanism without affecting the $^{13}\text{C}/^{15}\text{N}$ T_2 relaxation times. In the past decade, many studies have been devoted to the study of a wide range of paramagnetic agents such as 5-DOXYL stearic acid, Gd^{3+} and Cu^{2+} ions. Obtaining quantitative information from the use of PRE for membrane protein samples is not straightforward due to the presence of the membrane and complex protein-lipid interactions. However, a careful sample preparation can accelerate the spectroscopy of oriented samples. In agreement with previous studies, our results indicate that using a low concentration of paramagnetic ion chelated lipids (~5 mM Cu^{2+}), the ^1H T_1 values of membrane proteins can be reduced by a factor of three without jeopardizing the spectral resolution. In addition, the incorporation of paramagnetic lipids does not require modification of the target membrane proteins, preventing chemical shift perturbations and/or conformational alteration. Using 2D SE-SLF and 3D-SE-SLF-PDSD experiments we were able to map all of the residues of a small membrane protein, SLN, and its topology in lipid membranes [57]. Note that the normal of the membrane in the bicelle preparations is oriented perpendicularly to the direction of the static field, *i.e.*, ‘unflipped bicelles’. This orientation gives rise to narrower ^{15}N line widths with respect to the ‘flipped bicelles’, with the normal of the bilayer oriented parallel to the static magnetic field [69]. However, both CSA and DC dimensions for unflipped bicelles are scaled by a factor of 0.5, reducing the spectral resolution. For larger, multispan membrane proteins, the oriented bicelles can be flipped by using YbCl_3 , as in the case of bicelles without paramagnetic chelated lipids, doubling the spectral resolution as reported previously [70]. Remarkably, Ramamoorthy and coworkers have shown the application of lanthanide ions such as Yb^{3+} to

change the orientation of macro-nanodiscs [71], a new, versatile membrane mimetic system that promise to further boost the application of OS-ssNMR to larger membrane proteins.

5. Conclusions

In conclusion, we demonstrated that by doping bicelles with Cu^{2+} -DMPE-DTPA it is possible to accelerate the acquisition of 2D and 3D OS-ssNMR experiments on membrane proteins. We found that only a small concentration of Cu^{2+} -DMPE-DTPA (~5%) is required to significantly reduce the ^1H T_1 without inducing any structural changes or affecting T_2 relaxation mechanism. Taken together with the recent developments in low-E probe technology, the 2D and 3D sensitivity enhanced OS-ssNMR methods can be routinely used for fast data collection in the assignment of both chemical shifts and the topology of membrane proteins in liquid crystalline phase. We anticipate that the reduction of the experimental time for the 3D OS-ssNMR and the recent advances (such as high magnetic fields and non-uniform sampling) will propel the OS-ssNMR spectroscopy toward larger and more complex membrane proteins and membrane protein complexes.

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HIGHLIGHTS

- Paramagnetic relaxation enhancement accelerate 3D NMR spectroscopy of oriented sample
- Cu^{2+} chelated lipids decrease ^1H T_1 relaxation mechanism of membrane proteins in bicelles
- Detergent-mediated reconstitution of membrane proteins in micelles improve resolution

Figure 1.
Schematic representation of sample preparation of membrane proteins reconstituted in magnetically aligned bicelles containing paramagnetic lipids.

Figure 2. Paramagnetic chelated lipids[38]. a) Chemical structure of Cu^{2+} -DMPE-DTPA, and b) schematic representation of a hypothetical SLN reconstituted in lipid bicelles containing Cu^{2+} chelated lipid.

Figure 3.

Effects of paramagnetic chelated lipids on the spin relaxation properties of SLN reconstituted in lipid bicelles. a) Measurement of the bulk ^1H T_1 relaxation time by ^{15}N -detected inversion recovery experiment for $[\text{U}-^{15}\text{N}]$ -SLN reconstituted in oriented bicelles with (blue) and without (green) 5% Cu^{2+} -chelated lipids. b) Experimental data and fitting curves for the inversion recovery experiments. The bulk ^1H T_1 for $[\text{U}-^{15}\text{N}]$ -SLN in bicelles are 0.29 s and 0.89 s with and without 5% Cu^{2+} -chelated lipids, respectively. c) 1D ^{15}N spectra of $[\text{U}-^{15}\text{N}]$ -SLN reconstituted in oriented bicelles acquired using different ^1H decoupling RF amplitudes.

Figure 4.

Site-specific ^1H T_1 relaxation times of the backbone resonances of $[\text{U-}^{15}\text{N}]$ -SLN oriented in lipid bicelles with 5% Cu^{2+} -chelated lipids measured by the 2D inversion recovery SAMPI4 experiment. b) Mapping of the site-specific T_1 relaxation times on SLN structure (blue), where unobservable/overlapped residues in the SAMPI4 spectrum are shown in white. c) 2D SE-SAMPI4 spectra of $[\text{U-}^{15}\text{N}]$ -SLN without paramagnetic and d) with 5% Cu^{2+} -chelated lipids. The average ^{15}N linewidth for isolated peaks are 82.9 Hz and 86.7 Hz without and with 5% Cu^{2+} -chelated lipids, respectively.

Figure 5.

Comparison of 2D SE-SAMPI4-PDSD spectra of [U-¹⁵N]-SLN with a) and without b) 5% Cu²⁺-chelated lipids. The PDSD mixing time was set to 3.0 s for both experiments. c) 3D SE-SAMPI4-PDSD spectrum of [U-¹⁵N]-SLN in bicelles doped with 5% Cu²⁺-chelated lipids. d) 3D strip plots at specific dipolar coupling values from the 3D SE-SAMPI4-PDSD spectrum. The solid blue lines indicate [i, i+1] cross peaks.

Figure 6.

Water-edited experiments using PRE. a) 1D water-edited CP spectra of $[\text{U-}^{15}\text{N}]$ -SLN oriented in lipid bicelle using 5% Cu^{2+} -chelated lipids (b) and (c) 2D water-edited SE-SAMPI4 spectra acquired with 7 and 100 ms mixing times, respectively. The residues that are mapped at 7 ms mixing time, are close to the water-lipid interface. All of the experiments were acquired using 1.5 s recycle delay