Solid-State NMR Spectroscopy

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A Method for Dynamic Nuclear Polarization Enhancement of Membrane Proteins**

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Abstract: *Dynamic nuclear polarization (DNP) magic-angle spinning (MAS) solid-state NMR (ssNMR) spectroscopy has the potential to enhance NMR signals by orders of magnitude and to enable NMR characterization of proteins which are inherently dilute, such as membrane proteins. In this work spin-labeled lipid molecules (SL-lipids), when used as polarizing agents, lead to large and relatively homogeneous DNP enhancements throughout the lipid bilayer and to an embedded lung surfactant mimetic peptide, KL_4. Specifically, DNP MAS ssNMR experiments at 600 MHz/395 GHz on KL_4 reconstituted in liposomes containing SL-lipids reveal DNP enhancement values over two times larger for KL_4 compared to liposome suspensions containing the biradical TOTAPOL. These findings suggest an alternative sample preparation strategy for DNP MAS ssNMR studies of lipid membranes and integral membrane proteins.*

Dynamic nuclear polarization (DNP) coupled with solid-state NMR spectroscopy (ssNMR) can dramatically impact research efforts aimed at membrane protein structure determination in native lipid bilayers. DNP increases the sensitivity of NMR by exploiting the increased polarization, at a given magnetic field strength, of a paramagnetic dopant and transferring that polarization to NMR-active nuclei by microwave (μW) irradiation.[1–5] In recent years, magic-angle spinning (MAS) ssNMR (ssNMR) has become a powerful technique to characterize membrane protein structure and dynamics in situ. However, the relatively low sensitivity of MAS ssNMR remains an obstacle for many structural studies of membrane proteins. In particular, the challenges of producing sufficient quantities of isotopically enriched protein and subsequent dilution of the protein in the NMR sample by the requisite lipid environment increase the complexity of membrane protein characterization relative to other biomolecular samples, such as microcrystalline or amyloidogenic proteins, which have been successfully characterized by ssNMR spectroscopy. The advent of low-temperature (100 K) DNP MAS ssNMR spectroscopy, with sensitivity gains of up to two orders of magnitude, shows promise for overcoming this sensitivity bottleneck.[6,7]

Currently, the most commonly used polarizing reagents for DNP MAS ssNMR experiments of membranes and membrane proteins have been nitroxide biradicals, such as TOTAPOL.[8–16] The strength of the dipolar coupling between the tethered nitroxides, the relative perpendicular orientation of their two g-tensors, and the protonation of nearby substituents have all been cited as important factors impacting signal enhancements in DNP experiments.[17,18] These properties have been further exploited in the design of next-generation polarizing agents AMUPol and TEKPol, resulting in additional enhancements.[19,20]

The work presented here demonstrates an alternative strategy for introducing polarizing reagents into membrane protein samples; specifically, one that enables increased DNP enhancement within the lipid bilayer and does not require the use of small molecule cryoprotectants, which can further dilute a sample and potentially introduce artifacts in the membrane mimetic environment.[21] Instead of a water-soluble biradical, mixtures of 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycero-3-phosphocholine (5-Doxyl PC) and 1,2-dipalmitoyl-sn-glycero-3-phospho(tempo)choline (TEMPO-PC) are used as polarizing agents. Results at 14.1 T demonstrate that appreciable DNP enhancements are achieved throughout the lipid bilayer and that a DNP enhancement factor of 8.9 is achieved for KL_4, a 21 residue amphipathic peptide which deeply partitions into the hydrophobic core region of lipid bilayers. Figure 1 shows a schematic of the sample preparation strategy using SL-lipids and the structure and depth of membrane partitioning of KL_4, which has been previously characterized by NMR and EPR investigations[22–25] This particular combination of SL-lipids was chosen because the chemical connectivity and physical location of the radicals in each lipid species provides for close spatial proximity of the radicals while also enabling a perpendicular orientation between the g-tensors of the nitroxides. These are characteristics that emulate the properties of nitroxide biradicals, such as TOTAPOL, used in DNP experiments to date.

DNP enhancements of the lipophilic peptide KL_4, resulting from polarization by SL-lipid or TOTAPOL, are com-

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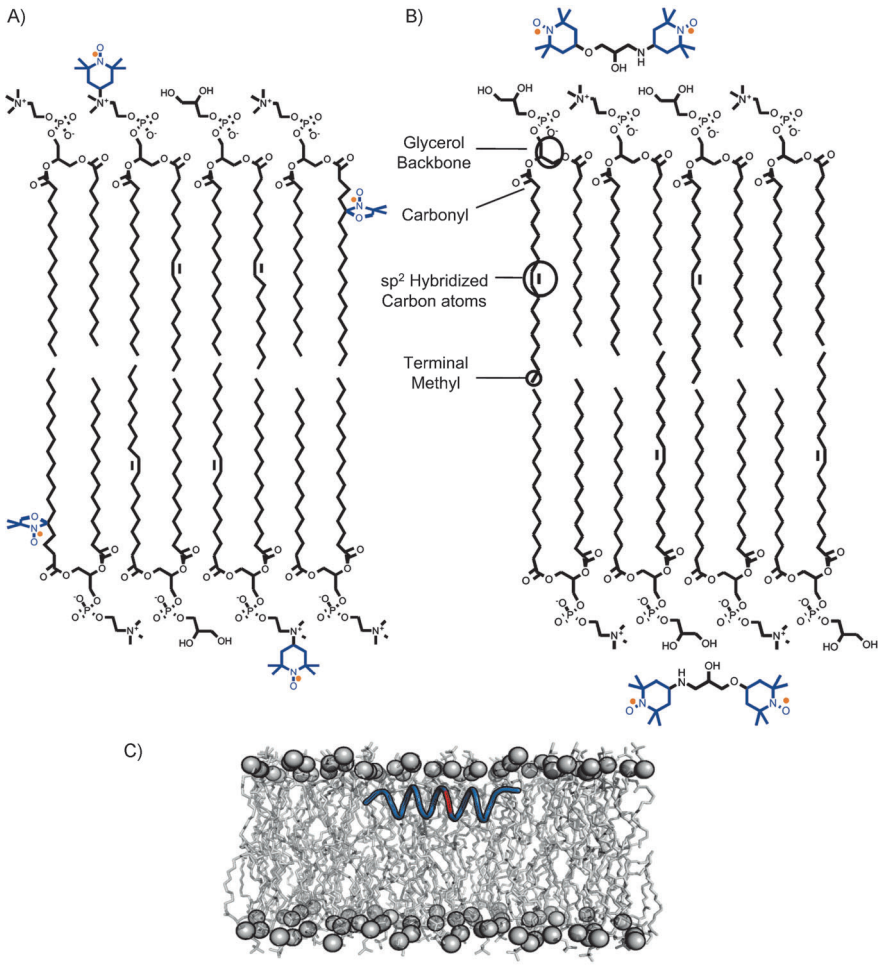

Figure 1. Schematic cartoon depicting sample preparation strategies for DNP investigations of KL_4 peptide and lipid bilayers. A) A DPPC:POPG lipid bilayer with SL-lipids TEMPO-PC and 5-Doxyl PC (1:1) as the source of electron polarization. B) A DPPC:POPG lipid bilayer with TOTAPOL biradical as the source of electron polarization. Nitroxide moieties are shown in blue with the radical distributed over the N—O bond depicted in orange. C) Model depicting KL_4 peptide backbone (blue) partitioned in a membrane; the structure and partitioning were modeled from ssNMR distance restraints and EPR power saturation data, respectively.[22–30] For quantification of KL_4 DNP enhancement, leucine 12 (red) was $^{13}C'$-enriched.

pared in Figure 2. KL_4 was $^{13}C'$-enriched at leucine 12, a position known to partition deeply within the lipid interior; because of spectral overlap of the $^{13}C'$-enriched position and natural abundance lipid, difference spectra in which the lipid signals are subtracted are shown in Figure 2.[25] Full details of spectra and subtractions are provided in the Supporting Information. When the μW on vs. off spectra in Figure 2 are compared, two major variations because of the use of different polarizing agents are apparent. First, the SL-lipid system leads to a much higher peptide $^{13}C'$ signal enhancement ($\varepsilon = 8.9$) compared to the enhancement observed in the TOTAPOL system ($\varepsilon = 3.7$). Second, two resonances (172 and 176 ppm) are observed in the SL-lipid system whereas only one resonance (176 ppm) is seen in the TOTAPOL system. The partitioning and structure of KL_4 is sensitive to both the degree of lipid acyl chain packing in proteoliposomes as well as pH.[22–25,31] The standard protocol for DNP sample preparation with water-soluble biradicals uses 60% glycerol as a glassing agent in the solvent; however, it has been shown that small molecule cryoprotectants may alter the membrane environment.[7] Given that the SL-lipid system does not require or contain any glycerol, the inclusion of glycerol in the TOTAPOL system is likely changing the lipid acyl chain packing within the lipid bilayers and affecting the conformational equilibrium KL_4 adopts; alternatively differences in the pH or dielectric gradient at the lipid bilayer interface could be affecting the partitioning and ultimately the structure of the peptide.

Not only does the SL-lipid system result in higher DNP enhancements for a membrane-embedded peptide but it also alleviates the enhancement gradient that we and others observe in lipid bilayers and other nonsolvated phases of heterogeneous systems when water-soluble polarizing agents are used. Specifically, when using TOTAPOL in heterogeneous systems, such as protein nanocrystals,[33] mesoporous silica nanoparticles,[34] or hydrated lipid bilayers, an enhancement gradient across the nonsolvated phase of the sample is observed; the largest enhancement is seen at the periphery of the crystal, nanoparticle, or bilayer whereas less enhancement is observed in the interior. TOTAPOL's water solubility prevents it from entering deeply into the hydrophobic lipid bilayer interior. Any DNP enhancements from TOTAPOL within the nonsolvated interior are thought to arise from transfer of enhancement from the membrane periphery to the interior via ^1H-^1H spin diffusion.[35,36] ^{13}C cross polarization (CP) MAS μW on vs. off proteoliposome spectra are shown in Figure 3 for the different sample preparations. A few striking observations can be made from comparison of the μW on spectra for TOTAPOL vs. SL-lipid sample. First, the maximum DNP enhancement in the SL-lipid sample occurs for the acyl chain resonances, whereas the maximum enhancement in the sample containing TOTAPOL is seen for the bulk glycerol glassing agent. Second, for the TOTAPOL containing sample, the acyl chain resonances are only enhanced by a factor of 1.7 compared to a factor of 7.9 in the SL-lipid preparation. Third, resonances for the terminal methyl groups, glycerol backbone, point of unsaturation, and carbonyl carbon atoms in the SL-lipid sample are clearly visible, indicating that strong DNP enhancements occur throughout the bilayer interior; no appreciable enhancement of the resonance resulting from the point of unsaturation in POPG is observed in the TOTAPOL sample. Fourth, the greatly enhanced bulk

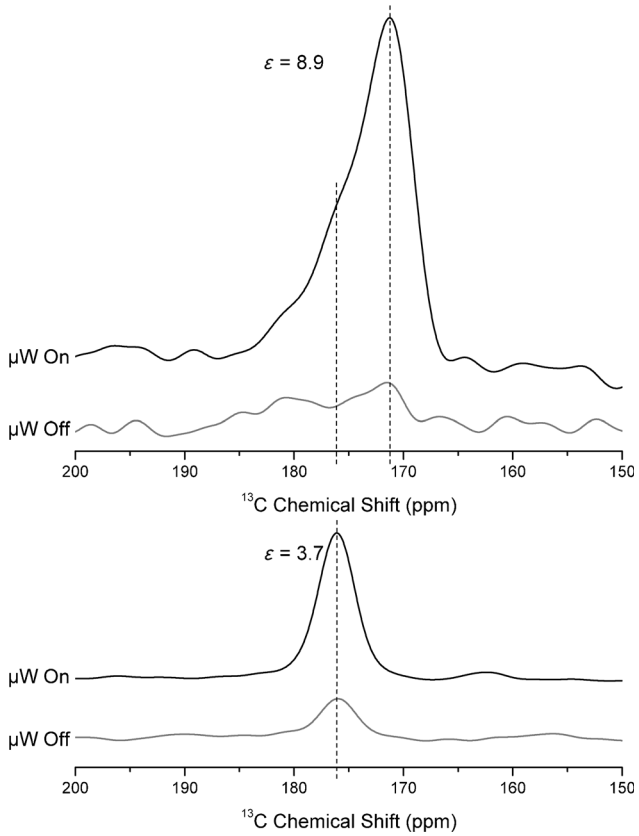

Figure 2. ^{13}C CP MAS difference spectra of $^{13}C'$-L12 KL_4 in 1:1 $[D_{62}]$DPPC:POPG liposomes with the μW on (black) and μW off (gray) for samples containing paramagnetic dopants of either (top) SL-lipids TEMPO-PC/5-Doxyl PC or (bottom) TOTAPOL are shown. The membrane-embedded $^{13}C'$ signal was enhanced by a factor of 8.9 in the SL-lipid system and by a factor of 3.7 in the TOTAPOL system. KL_4 can adopt two different conformations, with $^{13}C'$ resonances at 172 and 176 ppm (dashed lines); see the Supporting Information for discussion.[33] Proteoliposomes were prepared with about 40 mM total nitroxide spin and a final quaternary molar lipid mixture of 47:47:3:3 $[D_{62}]$DPPC:POPG:5-doxyl PC:TEMPO-PC for the SL-lipid sample.

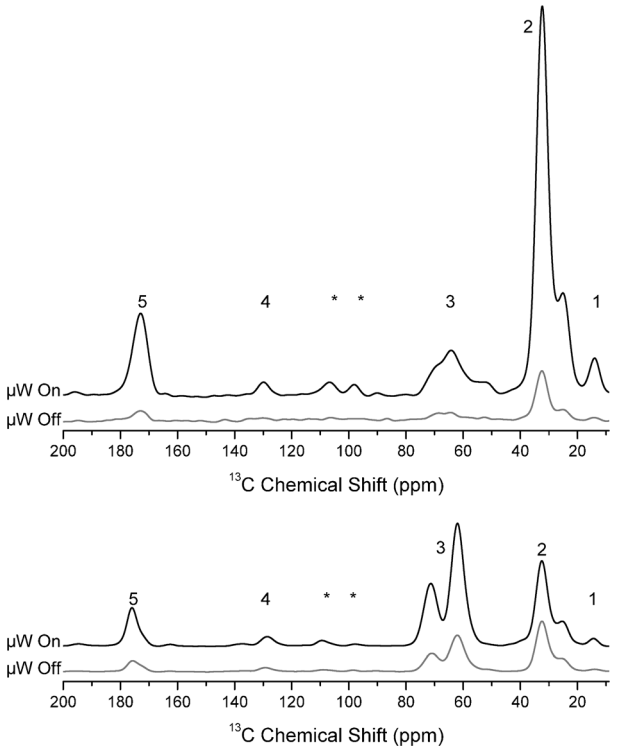

Figure 3. ^{13}C CP MAS μW on (black) and off (gray) spectra of proteoliposomes with SL-lipids (top) or TOTAPOL (bottom) as the polarizing agent. All proteoliposomes were composed of 1:1 $[D_{62}]$DPPC/POPG with 1:50 peptide/lipid. The SL-lipid system was hydrated with BisTris buffer pH 7.4 in D_2O/H_2O (90:10 v/v). The TOTAPOL system was suspended in $[D_8]$glycerol/D_2O/H_2O (60:30:10). A total of about 40 mM SL-lipid or TOTAPOL spin was used. The observed resonances are labeled as follows: 1 lipid terminal methyl groups, 2 sp^3 carbon atoms of lipid acyl chain, 3 lipid glycerol backbone, 4 sp^2 carbon atoms of unsaturation point in lipid acyl chain, 5 lipid carbonyl groups and $^{13}C'$-L12 enriched KL_4, * resonances from detection of material in the rotor end cap. The lipid glycerol backbone resonances were masked by the glycerol in the bulk solvent of the TOTAPOL system. Enhancements for the various resonances are given in Table 1. Resonance assignments were based on Ref. [32].

glycerol signals in the TOTAPOL sample diminishes the ability to clearly observe underlying lipid resonances at those frequencies; additionally, for high fields and currently achievable MAS rates at 100 K with a 3.2 mm rotor, glycerol spinning sidebands can further obscure other sample resonances.

Table 1 summarizes the enhancement values determined for SL-lipid and TOTAPOL systems investigated here. We observe the development of an enhancement gradient for the TOTAPOL-containing sample; L12-$^{13}C'$-enriched KL_4 is partitioned into the hydrophobic region of the bilayer and thus only has $\varepsilon = 3.7$. However, when SL-lipids are used as the polarization reagent the enhancement increases to 8.9, demonstrating that the introduction of a polarization reagent into the hydrophobic core of the lipid bilayer diminishes the enhancement gradient typically observed when water-soluble polarization reagents are used. Interestingly, the terminal methyl resonances are enhanced by about two times more than the lipid acyl chain resonances in both systems. Experimental evidence and MD simulations have shown the propensity for transient motions of the lipid acyl chain, in unsaturated lipids, to bring the methyl terminus toward the membrane periphery;[37,38] the rate of sample freezing is fast

Table 1: Measured DNP enhancement values for different moieties in the lipid membrane using either SL-lipids or TOTAPOL as the polarizing reagent.

Resonance	Measured enhancement (ε)	
	SL-lipids	TOTAPOL
$^{13}C'$-L12 KL_4 peptide	8.9	3.7
glycerol backbone	6.5	3.4[a]
Carbonyl groups	8.9	3.6
sp^3 carbon atoms of lipid acyl chains	7.9	1.7
sp^2 carbon atoms of unsaturation point	6.4	2.4
terminal methyl groups	15	3.5

[a] The glycerol backbone resonances were masked by the glycerol in the bulk solvent of the TOTAPOL sample, therefore enhancement reported is for both.

enough that the lipids would be unable to anneal in an all trans configuration.[39] Hence, the propensity for the terminal methyl groups to kink up towards the membrane periphery can explain the relatively large DNP enhancements for the terminal methyl resonances in the TOTAPOL system compared to the other enhancements observed for this system. Alternatively, the residual dynamics at 100 K for the terminal methyl could account for the larger enhancement observed for this moiety in both the SL-lipid and TOTAPOL preparations.[40] Overall, these results demonstrate that the SL-lipid DNP sample preparation provides for more consistent and substantive DNP enhancements across the membrane and provides a more native membrane environment without the need for small molecule glassing agents. This method of DNP sample preparation with nitroxides attached to the lipids may facilitate ssNMR studies of integral membrane proteins, where transmembrane protein segments are buried within the membrane interior.

In conclusion, structural characterization of membrane proteins remains an important and prolific area of research, particularly by ssNMR spectroscopy. The advent of high-field DNP in conjunction with MAS ssNMR techniques in recent years has provided an attractive avenue to increase throughput. We demonstrate that SL-lipids can be used as polarization reagents for membrane proteins that lie within the lipid bilayer with enhancement factors that are comparable to or better than those achieved with traditional DNP sample preparation. This method also provides a straight forward sample preparation strategy that should be amenable to the panoply of membrane protein preparations and topologies. It also obviates the use of glassing agents, such as glycerol, to enhance radical solubility which can obscure important NMR resonances, dilute sample concentrations, and cause changes in lipid morphology and protein structure.[21] For MAS DNP of membrane protein samples at cryogenic temperatures, the lipids themselves can act as a glassing agent to stabilize protein structure and the addition of trehalose can provide further cryoprotection.[41] Although appreciable DNP enhancements are observed when TEMPO-PC and 5-Doxyl PC act as the source of electron polarization, further optimization of sample preparation techniques for membrane protein structure determination utilizing DNP MAS ssNMR remains; this includes optimizing SL-lipid concentration, the ratio of deuterated to protonated lipid used, and the placement of the radical moieties. We plan to extend this strategy by using biradical moieties attached to lipophilic molecular scaffolds as polarizing agents. Nonetheless, our method has immediate applications in DNP MAS ssNMR studies of membrane protein systems.

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