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Dynamic Nuclear Polarization as an Enabling Technology for Solid State Nuclear Magnetic Resonance Spectroscopy

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Graphical Abstract

Magic angle spinning (MAS) solid state nuclear magnetic resonance spectroscopy (ssNMR) can yield unique and insightful information for complex systems; most notably structural and dynamical information can be obtained at atomic resolution.^{1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23} A particular strength of MAS ssNMR is it can be applied to heterogeneous systems, which are not amenable to study by other high-resolution experimental techniques due to sample characteristics. For example biomolecular assemblies, such as membrane proteins, are often not tractable for solubilization, a requisite for standard solution state NMR experiments, or crystallization, as needed for diffraction studies.^{1, 24} Solid state materials with limited global order are often difficult to characterize by diffraction or other spectroscopic methods (e.g. UV-Vis, IR, etc.), and information on reactive moieties, of particular interest for chemical and functional insight, can be difficult to differentiate from bulk signals.

Unfortunately, conventional ssNMR is fundamentally an insensitive technique and the aforementioned types of systems are further afflicted by the often inherently low concentrations of the molecules or nuclear spins of interest. The insensitivity of NMR stems

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from the low Boltzmann population difference (i.e. polarization) of nuclear spin states in accessible magnetic fields. For example, at 14.1 T (600 MHz ^1H resonant frequency) and 100 K only $\sim 0.0045\%$ of ^1H nuclei are polarized (Figure 1). The polarization is even less for low gamma nuclei important in biological or materials NMR experiments, such as ^{13}C and ^{15}N or ^{29}Si and ^{27}Al , respectively. Low polarization leads to extremely long data acquisition times for the types of experiments, often multidimensional, required for atomic resolution studies, and, depending on the type of sample, can require weeks of signal averaging. Dynamic nuclear polarization (DNP), an emerging magnetic resonance technique to enhance polarization, has quickly gained traction in the NMR community as a means to overcome the sensitivity bottleneck.

The primary aim of DNP is to enhance the sensitivity of NMR by combining electron paramagnetic resonance (EPR) phenomena with NMR experiments. Unpaired electrons have significantly higher spin polarization than NMR active nuclei in a given magnetic field, due to an electron's much higher gyromagnetic ratio relative to nuclear gyromagnetic ratios (e.g. $\gamma_e \approx 28,000 \text{ MHz/T}$ and $\gamma_e/\gamma_{1\text{H}} \approx 660$). In a typical DNP MAS ssNMR experiment, the relatively large polarizations of paramagnetic electrons are transferred to NMR active nuclei of interest by excitation of electron spin transitions via microwave (MW) irradiation, resulting in an increased nuclear polarization (Figure 1). The traditional depiction of polarization transfer in DNP enhanced ssNMR experiments is shown in Figure 2. Theoretically, to first order the signal enhancements obtained from transferring electron polarization to ^1H nuclei could lead to an experimental time saving of $\approx 435,000$, as experimental time scales inversely with the square of signal enhancement.²⁵

This review will focus on developments over the past three years in implementing DNP MAS ssNMR with an emphasis on bimolecular and materials applications. In addition, an overview of DNP mechanisms, hardware requirements, and the types of paramagnetic polarization agents used and their introduction into the sample will be given. Lastly, future prospects for the development of DNP as a more robust, broadly applicable spectroscopy technique will be discussed.

Polarization Transfer Mechanisms

In DNP MAS ssNMR, polarization transfer from a paramagnetic species (i.e. polarization agent) to NMR active nuclei, in the high temperature limit, occurs by primarily two mechanisms: (1) the two spin (electron, nuclear) mediated solid effect and (2) the three spin (electron, electron, nuclear) mediated cross effect.²⁶ Other polarization transfer mechanisms, such as the Overhauser effect and thermal mixing, have been observed, the former in some high field DNP MAS ssNMR experiments^{26, 27} and via low field DNP solution state NMR experiments^{28,29} and the latter in dissolution DNP experiments with polarization at ultralow temperatures^{30,31,32} but are beyond the scope of this review and we refer the reader to the referenced literature.^{26, 27}

The choice of paramagnetic species primarily dictates which polarization transfer mechanism dominates. If the homogenous (δ) and inhomogeneous (γ) linewidths of the electron spin resonances are smaller than the nuclear resonant frequency ($\omega_{0\text{I}}$), such that $\omega_{0\text{I}}$

$\omega_{0I} \sim \delta$, then at intermediate fields (5–14 T) the solid effect dominates. Optimal polarization transfer will then occur when $\omega_{MW} = \omega_{0S} \pm \omega_{0I}$, where ω_{MW} is the microwave excitation frequency and ω_{0S} is the electron resonant frequency. When this condition is met polarization transfer from an electron to nearby nuclei occurs and is distributed to the bulk nuclei throughout the sample via nuclear spin diffusion.³³ Polarization transfer occurs because the non-secular terms of the hyperfine interaction Hamiltonian allow for a mixing of nuclear spin states, which partially allow normally forbidden zero and double quantum electron-nuclear transitions.²⁶ Upon MW irradiation either the single or double quantum transitions are driven and result in enhanced nuclear polarization.

Alternatively, a three spin (electron, electron, nuclear) cross effect can take place if $\omega_{0I} > \delta$ and the matching condition $\omega_{0I} = \omega_{0S2} - \omega_{0S1}$ is met (Figures 3 and 4).^{26, 34} The dipolar coupling between the two electrons results in a mixing of states and upon inducing an electron spin transition by MW irradiation at one electron resonance the other dipolar coupled electron undergoes a spin transition while simultaneously inducing a nuclear spin transition in a hyperfine coupled nucleus. This again results in enhanced nuclear polarization, which is distributed throughout the nuclei in the sample by nuclear spin diffusion.^{26, 34}

Recent Advances Using DNP Enhanced ssNMR

Over the last few years MAS ssNMR has been able to take aim at key biomolecular and materials based research queries that without the enhanced sensitivity provided by DNP would not be achievable. Here we summarize a few studies that highlight the potential of DNP and the types of questions that can now be answered, using MAS ssNMR, due to improvements in sensitivity, specificity, and experiment timescales.

Biomolecular Applications

High-resolution protein structures have been solved by MAS ssNMR for numerous systems, such as microcrystalline, membrane, and amyloidogenic proteins.³⁶³⁷³⁸³⁹ Protein structures require resonance assignment of individual amino acid residues and distance constraints, which necessitates numerous multidimensional and dipolar recoupling experiments. A major application area of MAS ssNMR is characterizing membrane proteins in their native lipid environments. Fundamental studies via traditional ssNMR on membrane-embedded peptides and small proteins have established the relevance and need of this approach for understanding membrane protein function and the roles of lipids.⁴⁰⁴¹⁴²⁴³⁴⁴ Nonetheless, studying intact larger membrane proteins, via MAS ssNMR, has long been hampered by the insensitivity of the technique. It is particularly challenging for membrane proteins where the protein concentration is limited by its requisite membrane environment. With the advent of DNP, membrane protein studies via MAS ssNMR have made significant strides in sample throughput and the complexity of membrane protein assemblies which can be characterized to address questions of structure-function-dynamics relationships that were previously unattainable through the use of traditional structural biology techniques.

The translocon is a membrane embedded complex of proteins that regulates transport of nascent polypeptides into the membrane to form mature, membrane-embedded proteins or

across the membrane to form mature cytosolic and extracellular proteins. During translation, proteins destined for the translocon protein assembly are recognized via a peptide signaling sequence. To date only low resolution structures of the translocon assembly, acquired via Cryo-EM, have been achieved. However, there is much interest in understanding the translocon structural basis underlying recognition of signaling sequences for translocon targeted proteins and subsequent steps in translocon function. M. Lorch et al were able to utilize DNP MAS ssNMR to identify the contacts between a peptide signaling sequence and a bacterial Sec Translocon.⁴⁵ Due to the abundance of lipids and the large size of the Sec Translocon, the amount of signaling peptide present was inherently small, on the order of ~40 nmols, and ssNMR experiments necessitated the use of double quantum filtering (DQF) to distinguish signals of interest in the signal sequence from the relatively large ¹³C natural abundance background signals of the intact translocon. While DQF enables exquisite signal selection, it typically results in the loss of nearly 60% of the desired signal intensity. Through the use of DNP a ¹³C signal enhancement of 32 was obtained (at 9.4 T) which resulted in ~1000-fold data acquisition time savings. In addition, due to the need for DQF experiments and the low concentration of signaling peptide, detecting the signaling peptide resonances was only possible via DNP; their detection enabled the identification of amino acid residues in the signaling peptide that were in contact with the Sec Translocon (Figure 5).

Another membrane protein study made feasible with the added sensitivity of DNP was the verification that a specific, highly conserved, glutamate residue in EmrE, an efflux pump responsible for multi-drug resistance in bacteria, was critical to its functional mechanism.⁴⁶ The substrate TPP+ was ¹³C labeled on one of its phenyl rings and bound to sparsely ¹³C labeled EmrE (using 2-¹³C glycerol as the sole carbon source for EmrE expression and resulting in C8 of glutamate residues to be isotopically enriched). A 2D ¹³C-¹³C DARR correlation experiment, which detects spins in close spatial proximity, determined that the residue E14 of EmrE was in direct interaction with the TPP+ substrate, thus confirming its functional importance (Figure 6).⁴⁶ A ¹³C signal enhancement of 19 (at 9.4 T) was obtained and resulted in ~360 fold time savings.

In addition to enabling difficult *in-vitro* studies of membrane proteins reconstituted into lipid bilayers after purification, DNP has also enabled the use of MAS ssNMR to structurally probe membrane proteins embedded in intact cellular membranes. For example, M. Baldus et al were able to test and validate structural models of the Type IV secretion system, a megadalton protein assembly embedded in an E. coli membrane.⁴⁷ DNP MAS ssNMR experiments were carried out at 9.4 and 18.8 T resulting in enhancements of ~60 and ~15 respectively. These enhancements permitted multidimensional ¹⁵N-¹³C correlation experiments to be carried out which allowed for validation of a Type IV secretion system structural model. Ramamoorthy et al were also able to collect preliminary structural data for the electron transport protein cytochrome-b5 in its native E. coli membrane with DNP providing a ~16 fold enhancement in ¹³C signals at 14.1 T.⁴⁸

The structural characterization of cell walls and their associated proteins in gram-positive bacteria and plants have similarly made significant strides in recent years with the advent of commercial DNP MAS ssNMR instrumentation. S. Hediger et al demonstrated that the

signal enhancements afforded by DNP allow for the study of bacterial cell walls with a time savings of ~600 relative to conventional ssNMR, based on preliminary experiments.⁴⁹ In their studies they also found that the peptidoglycan cell wall provides an ideal matrix for capture of radical agents and subsequent gains in signals of sugars specific to the peptidoglycan coat. M. Hong et al demonstrated large DNP enhancements ($\epsilon \approx 27$) of plant cell walls and the associated expansin protein. The expansin binding site on the cell wall was determined by using a ^{13}C - ^{15}N dipolar filter whereby ^{13}C spins of ^{13}C , ^{15}N enriched expansin were specifically selected to transfer their magnetization to nearby ^{13}C spins of the plant cell wall.⁵⁰ Thus, the cell wall sites nearby the bound expansin protein were determined. Due to the low concentration of expansin protein (~0.23 mg per sample) the dipolar-filtered experiments were only made possible by the signal enhancements afforded by DNP.

Materials Applications

Much of the interest in modern materials science research is in characterizing amorphous materials. Materials functions frequently depend upon their atomic structure, particularly the surfaces of materials with advantageous chemical properties that are distinct from the bulk material. The low concentration of protons in many materials and the inability to specifically isotopically enrich chemical moieties of interest make the use of conventional ssNMR to characterize materials challenging. A unique advantage of DNP is that it can be used to specifically enhance signals at surfaces, through introduction of radical polarization agents in a matrix infiltrating the materials of interest. Since DNP relies on spin diffusion to polarize nuclei beyond a few angstroms from the radical, NMR experimental parameters and sample characteristics can be matched to only transfer polarization to nuclei in close proximity to the matrix.⁶

Silica nanoparticles have numerous applications in catalysis, as imaging contrast agents, and potentially as membranes in fuel cells.⁵¹ In order to create high surface area materials, silica nanoparticles can be grafted with siloxanes and characterization of the siloxane grafts is important to the understanding and further development of these nanoparticles for specific applications. G. De Paëpe and coworkers were able, through the use of DNP enhanced MAS ssNMR, to characterize the interconnectivities of the functionalized surface of the nanoparticles by 2D ^{29}Si - ^{29}Si correlation experiments utilizing natural abundance ^{29}Si (Figure 7).⁵¹ In addition, they were able to show that the siloxanes undergo self-condensation resulting in aggregates of reacted and non-reacted siloxanes sites on the surface of the nanoparticles. This type of natural abundance characterization would not be possible without the sensitivity gains from DNP.

Heterogeneous catalysts can display unparalleled activity, and in particular Sn- β -zeolites have been demonstrated to be efficient catalysts. Despite their effectiveness in industrial processes, the distribution and structure of Sn^{IV} active sites in these catalysts are not well understood. Through the use of DNP MAS ssNMR, Hermans and coworkers were able to characterize the Sn^{IV} active sites as having an octahedral coordination, with both open and closed states depending on the binding of the surrounding siloxy framework.⁵² Mesoporous alumina (Al_2O_3) is another important heterogeneous catalyst that has critical industrial

applications. The ability to rationally design alumina-based catalysts is hampered by the lack of atomic resolution information on possible structures. The quadrupolar nature of ^{27}Al makes it particularly insensitive and difficult to study via MAS ssNMR. De Paëpe and coworkers were able to show that through the use of DNP enhancements of ^{27}Al signals ($\epsilon \approx 15$) it is possible to study the ^{27}Al interfaces in mesoporous alumina via standard 1D techniques and ^{27}Al homonuclear dipolar recoupling experiments (Figure 8).⁵³

Another quadrupolar nucleus of critical importance to chemical reactions yet notoriously difficult to use in NMR studies is ^{17}O , which has the additional challenge of extremely low natural abundance (0.37 %). Thus, the use of ^{17}O is hampered by extremely time consuming and expensive experiments (due to the necessity of isotopic enrichment). C.P. Grey *et al* demonstrated that through the use of DNP enhanced MAS ssNMR it was possible to obtain natural abundance ^{17}O ($\epsilon \approx 21$) spectra in a matter of minutes.⁵⁴ Specifically, they were able to show direct DNP polarization transfer to ^{17}O or indirect transfer to ^{17}O via ^1H of $\text{Mg}(\text{OH})_2$ and marked signal enhancements, which opens up opportunities to probe relevant materials containing oxygen and ^{17}O at natural abundance.

Hardware Requirements for MAS DNP

While DNP is compatible with commercial MAS ssNMR systems, it has unique microwave and cryogenic requirements entailing the addition of major hardware components. The primary upgrades needed are a source of high power (~ 10 W) MW, a MAS probe capable of operating at cryogenic temperatures, and a means of efficiently transporting the MW from their source to the sample in the MAS probe with minimal losses. Figure 9 shows a schematic of DNP MAS ssNMR instrumentation.

Gyrotron

For the 100 K temperature regime obtainable with commercial MAS ssNMR instrumentation, efficient DNP requires the use of high power (~ 10 W) MW to drive the necessary electron-nuclear transitions to attain polarization transfer. In addition, at magnetic fields where high resolution NMR becomes possible (i.e. ~ 9.4 T), the electron resonant frequencies approach the terahertz regime. To date, the only way to generate continuous, reproducible microwave irradiation at these frequencies with sufficient power is to use a gyrotron.⁵⁵ Currently, commercial gyrotrons generating MW at 263, 395, and 527 GHz are available for use with 400, 600, or 800 MHz NMR instruments, respectively.

The two major components of a gyrotron are the gyrotron high vacuum tube and a magnet in which the gyrotron tube sits. Briefly, an annular electron beam is generated by a high voltage cathode and is directed up through the magnet.⁵⁶ When the beam enters the magnetic field the electrons start to coherently bunch together and eventually emit MW via cyclotron resonance. The MW are emitted perpendicular to the electron beam and are guided to the gyrotron output window via a series of quasioptical mirrors. The electron beam continues to travel up through the magnet and terminates at the collector. Gyrotrons operating using the fundamental cyclotron resonance mode require a separate magnet operating near the field of the NMR magnet, while gyrotrons utilizing a second harmonic mode require a persistent magnet operating at approximately half the field of the NMR

magnet. Traditionally, gyrotrons operate a fixed MW frequency; therefore to meet the matching condition for different polarization agents the field of the NMR magnet is varied.

It should be noted that if a DNP experiment is conducted at lower temperatures, for instance if cold He gas is used to cool the sample rather than N₂, much lower power MW can be used to drive the necessary electron-nuclear transitions. In such a case a gyrotron is not necessary and other solid-state MW sources can be used. However, this approach has not been commercialized and is beyond the scope of this review.

MW Transmission

Once high power MW are generated, they must be transmitted from the gyrotron to the NMR sample in the MAS probe in the NMR magnet. Corrugated waveguides are the most utilized method of high power MW transmission, primarily because they provide a mechanically reliable, safe, low loss means of MW transmission. Typically, a corrugated waveguide's radius is larger than the MW wavelength (λ), and the corrugation depth is optimized at $\lambda/4$, with a periodic groove of $\lambda/3$.⁵⁷ While corrugated waveguides provide an efficient means of MW transmission, at the frequencies where DNP MAS ssNMR instruments currently operate the required high precision machining of the corrugated waveguide is difficult and therefore quite costly.

An alternative to corrugated waveguides is to use a quasioptical system. Quasioptics refer to a system where the electromagnetic radiation wavelength approaches the size of the optical components and the MW are transmitted by more traditional methods with mirrors.^{57,58} An added benefit to a quasioptical system is that the MW can now be easily manipulated, as opposed to using a corrugated waveguide, which only transmits the MW. This allows power attenuation, MW beam splitting, gating of the MW, and coherent phase selection of the MW. The ability to split and independently attenuate the MW beams allows for one gyrotron to supply MW to more than one instrument. A mechanical shutter which can gate MW irradiation on a msec timescale allows synchronization of MW with NMR pulse sequences, enabling polarization transfer during the traditional relaxation delay period while mitigating cross relaxation effects of the electron spins on nuclear coherences during quantitative multidimensional NMR experiments.

Cryogenic MAS ssNMR Probe

The ability to conduct DNP experiments at low temperatures is imperative, especially if large DNP enhancements are desired. This adds the complication of stably spinning a sample filled rotor at high frequencies and low temperatures. For DNP MAS ssNMR at 100 K, this is accomplished by cooling N₂ gas to just above its dew point by passing it through heat exchangers submerged in liquid N₂. The cold N₂ gas is then used as the bearing and drive gases to spin the sample rotor. In addition, a third gas line directly cools the sample.^{59,60} In addition to traditional mechanical and RF requirements for a MAS ssNMR probe, a waveguide needs to be incorporated into the probe. Specifically, once the MW are transmitted from the gyrotron to the probe, an additional waveguide which provides focused MW irradiation of the spinning rotor is required.

Factors Affecting DNP Enhancement

Magnetic Field

The mechanisms of polarization transfer and their dependence on magnetic field, relaxation properties of the spins, and other parameters can lead to significant variations in attainable DNP enhancements. For instance, DNP enhancement scales as B_0^{-2} and B_0^{-1} for the solid effect and cross effect respectively, where B_0 is the external magnetic field.²⁶ Despite the inverse scaling of enhancement with magnetic field it is still desirable to do DNP MAS ssNMR experiments at high magnetic fields (i.e. 9.4 T) to obtain the needed spectral resolution to resolve resonances from individual chemical moieties. Most DNP MAS ssNMR experiments are conducted at 100 K to maximize enhancement, but at this temperature there is a marked loss in chemical shift resolution, particularly for biomolecular samples, further necessitating high magnetic fields. This loss in resolution can be attributed to freezing of multiple structural conformations, dipolar couplings that are no longer attenuated by molecular motions, and cross-relaxation effects.

Temperature

At experimentally obtainable temperatures, polarization behavior is in the high temperature limit and increases with the inverse natural log of the temperature (Figure 1). Thus, reaching the lowest feasible cryogenic temperatures increases NMR sensitivity. Equally important to DNP are the increase in electron polarization and the lengthening of electron and nuclear T_1 s, which assures effecting maximal enhancements by DNP. As mentioned above, most MAS DNP experiments are performed at 100 K. This temperature regime provides a compromise between maximal achievable polarization, experiment times, and hardware feasibility. At 100 K a marked electron polarization ($\sim 2 - 4\%$ at 9.4 – 18.8 T) exists at the fields typically used and the construction of a NMR probe capable of MAS and high power decoupling is feasible using inexpensive and easily sustainable cold nitrogen (N_2). Lower temperatures would yield higher electron polarization, but would require the use of helium or hydrogen gas as the cooling gas. The use of hydrogen poses significant safety risks. Using He can be prohibitively expensive and requires a recirculating system if it is to be sustained. Additionally, gaseous He has breakdown voltage characteristics that are less favorable with high power decoupling. Despite these barriers there has been recently demonstrated development of a He-cooled DNP MAS ssNMR probe with He recirculation. D. Lee, G. De Paëpe, and coworkers have developed a DNP MAS ssNMR probe capable of stably operating at 30 K with MAS frequencies up to 25 kHz.⁶¹ At a temperature of 55 K and MAS of 10 kHz, they were able to obtain a DNP enhancement of 677 ± 34 on ^{13}C enriched urea. (The enhancement factor calculation does not include depolarization effects of the paramagnetic dopant. With those effects taken into account the DNP enhancement would not exceed the theoretical limit of $\gamma_e/\gamma_{1H} \approx 660$.⁶¹) The results obtained with the He cooled DNP MAS ssNMR probe are compelling and warrant further development.

Sample Types

A key factor in determining attainable DNP enhancements are the types of sample being studied, as the efficiency of polarization transfer is highly dependent on electron and nuclear

spin characteristics. To date maximal DNP enhancements of ~ 515 on ^1H , which is near the theoretical maximum of ~ 660 , have been obtained using commercial instrumentation on tetrachloroethylene at 9.4 T.⁶² However, DNP enhancements for more complex samples, such as membrane proteins, have yielded much more modest enhancements, on the order of 20–30 at similar field strengths (9.4 T).⁶³ The most likely reasons for the lower observed enhancements are absorption of the microwaves by the rotor and/or solvent and the complex nature of large biomolecular assemblies, which leads to multiple relaxation pathways for the observed nuclei, the nuclear spin bath enabling bulk polarization, and the electrons of the polarization agents. These relaxation pathways lead to more opportunities for the spin populations to return to equilibrium before polarization can be transferred or observed. Despite these competing processes there are still opportunities to improve upon the levels of observed DNP enhancements.

Polarization Agents

Equally important to the development of DNP MAS ssNMR as a generalized NMR technique are the polarization agents, which can be added to NMR samples as part of a glassy matrix. These agents provide electron polarization which can be efficiently transferred to the NMR active nuclei of interest and are broadly utilized across biomolecular and materials science applications. To date, a major focus of DNP MAS ssNMR methodology development has been on the design of efficient polarization agents which can simply be added to NMR samples. These agents capitalize on the cross effect as it has more favorable scaling with respect to magnetic field ($\epsilon \propto B_0^{-1}$). Stable nitroxide radicals have EPR spectral properties particularly well suited to meeting the requirements of the cross effect, in particular a large g -anisotropy, which scales with magnetic field, that ensures $\omega_{0I} > \omega_{0I}$ due to inhomogeneous broadening (Figure 4).

The cross effect also depends upon state mixing induced by dipolar coupled electrons.^{35, 64} One way to create a strongly dipolar coupled network of electrons is to use sufficiently high concentrations of nitroxide monoradicals, such as (2,2,6,6-Tetramethylpiperidin-1-yl)oxyl (TEMPO). However, at such high concentrations the radicals can have paramagnetic relaxation effects (PRE) on the nuclei in the sample.⁶⁵ Typically these effects are manifested as broadened resonances of the affected nuclei resulting in a complete loss of observable signal or at the very least a loss of chemical shift resolution.⁶⁵ In order to avoid counterproductive PRE, it was realized that tethering two radicals together could create a sufficiently strong electron-electron dipolar coupling for the cross effect to occur efficiently.⁶⁶ Development of DNP agents has focused on tethering nitroxides to ensure an optimal dipolar coupling of the electron spins as well as g -tensor orientations which favor the matching condition $\omega_{0I} = \omega_{0S2} - \omega_{0S1}$. The most efficient demonstrated cross effect polarization agents are TOTAPOL, AMUPol, and TEKPol biradicals with dipolar couplings on the order of ~ 20 – 30 MHz (~ 15 – 12 Å) and roughly perpendicular g -tensor orientations (60 – 90° relative orientations of the nitroxide-containing moieties).^{60, 62–64}

Physical Introduction of Polarization Agents in Conventional ssNMR Samples

Biradical polarizing agents are most effective when they are evenly and reproducibly doped into the NMR sample of interest at a concentration of 1 – 20 mM. Nitroxide biradicals have limited solubility in aqueous buffers; if ice crystals form on sample freezing, concentrated microdomains of polarizing agents can form which are phase separated from the molecules interrogated by NMR and thus substandard DNP enhancements are observed. Additionally, non-productive nuclear cross-relaxation processes need to be minimized so solvents are typically 80–90% deuterated to minimize proton-mediated relaxation while preserving a high enough level of proton-mediated spin diffusion. Typically glycerol- d_8 or DMSO- d_6 is used as a glassing agent at fairly high levels. This can be problematic for many samples as they affect both osmotic pressure and lipid bilayer morphologies.⁶⁷

Biradicals commonly used in biomolecular samples, namely AMUPol and TOTAPOL, are designed to be water soluble, which is desirable for ease of introduction into aqueous biomolecular assemblies. However, membrane proteins are largely embedded in the hydrophobic core of a lipid bilayer. Water soluble biradicals are excluded from the bilayer interior, and it has been observed that in heterogeneous samples, such as membrane proteins, mesoporous materials, or nanocrystals, the exclusion of polarization agents from parts of the sample results in a polarization gradient.^{68, 69} The highest DNP enhancement is observed at the hydrophilic periphery near the biradicals and the lowest enhancement is observed in the hydrophobic core. Oftentimes, for membrane proteins, the hydrophobic portion of the protein is the region of most interest. Therefore, it is desirable to obtain large DNP enhancements at those sites. Hydrophobic biradicals might overcome this problem but their resulting intercalation into lipid bilayers might adversely affect membrane morphologies and affect protein structure at high concentrations.

Optimization of biradical containing solvent systems has also been carried out for materials based studies. Specifically, it was found that the most enhancement was obtained when tetrachloroethane (TCE) was used as a matrix for DNP MAS ssNMR studies of materials.⁷⁰ Overall, halogenated solvents seem to give rise to better DNP enhancement than protonated solvents, which is thought to arise from more efficient nuclear spin diffusion processes. Specifically, the diminished proton bath leads to directed polarization transfer to NMR active nuclei on or near the regions of experimental interest. Similar reasoning can be used to explain the efficiency of deuterated biradical solutions used in aqueous samples. In addition, TCE has a relatively low vapor pressure at room temperature, allowing for easier sample preparation, and is chemically inert with respect to the radical polarization agent.

Endogenous or Chemically Introduced Polarization Agents

An alternative to adding solutions of biradicals, which have been the most efficient polarization agents to date, to conventional NMR samples is to use stable electron spins that are endogenous to the system of study or to covalently attach nitroxide radicals to or near the molecular species of interest. It has been demonstrated that through the use of radical labeled lipids, fatty acid functionalized biradicals, or site-directed biradical labeling more efficient DNP enhancements of membrane proteins can be obtained than with aqueous phase

biradicals.^{69, 71–73} It has also been demonstrated that paramagnetic metals and cofactors endogenous to a protein or ribozyme can be used as polarization agents.^{74, 75}

Recently, multiple approaches have been taken in the development of polarization agents optimal for membrane protein assemblies. The use of nitroxide radical labeled lipids markedly improved DNP enhancements for a membrane embedded peptide (Figure 10). In this approach, since the polarization agent is part of the lipid milieu, the polarization gradient observed with traditional water soluble biradicals is diminished.⁶⁹ In a similar fashion De Paëpe and coworkers synthesized an analog of TOTAPOL where the linker connecting the two nitroxide moieties was palmitylated (resulting in N-Propyl-Palmipol).⁷¹ In this case the TOTAPOL biradical partitioned into the lipid bilayer and demonstrated improved DNP enhancements of a lipid bilayer. These two approaches (utilizing nitroxide labeled lipids or N-Propyl-Palmipol) enable generalized sample preparations strategies that do not require mutation or any other special processing of the protein of interest. In two separate studies, an alternative strategy specific to membrane proteins of interest was pursued. Namely, the linker between the two nitroxide moieties was functionalized with methanethiosulfonate group to enable site-directed biradical labeling of cysteine residues in membrane proteins.^{72, 73} This approach was demonstrated to give efficient DNP enhancement using either TOTAPOL or AMUPol (resulting in ToSMTSL and AMUPol-MTSSL respectively) attached to either *Anabaena* sensory rhodopsin or a bacterial potassium channel (KcsA), respectively.^{72,73}

The aforementioned selectively introduced or endogenous polarization agents and relevant DNP MAS ssNMR details are summarized in Table 1. As stated above, DNP enhancements should scale as B_0^{-1} for the cross effect. With this scaling factor taken into account, the observed DNP enhancements for these strategies are scaled to what would be expected at 14.1 T (600 MHz ^1H resonant frequency) to allow comparison of the approaches. Interestingly, when compared, most of the re-calculated enhancements are similar to one another and significantly outperform traditional methods of introducing aqueous phase TOTAPOL into membrane protein samples. In general, MAS ssNMR studies of membrane proteins suffer from low sensitivity compared to other biomolecular systems, such as biomolecules in a microcrystalline or fibril state. This insensitivity stems from the requisite dilution of a membrane protein with lipids. In DNP MAS ssNMR, standard approaches using biradical solutions pose a further challenge as they can further dilute the protein, disrupt lipid assemblies if using a high concentration of glassing agent, and make pelleting of lipid assemblies problematic. For these reasons the development of direct polarization agents for membrane proteins has been of particular interest to the biomolecular DNP MAS ssNMR community and will continue.

Biocompatible, endogenous polarization agents have also been successfully utilized in other biomolecular systems. Specifically, Corzilius and coworkers substituted Mg^{2+} with paramagnetic Mn^{2+} in a hammerhead ribozyme and observed DNP enhancements with the endogenous metal acting as the polarization agent.⁷⁴ In addition, A.F. Miller in collaboration R.G. Griffin and coworkers utilized a naturally occurring flavin mononucleotide semiquinone radical of flavodoxin to generate DNP enhancements of the protein.⁷⁵ The results of the latter two studies are also summarized in Table 1. In contrast to the broad EPR

spectra of nitroxide radicals, the endogenous polarization agents used in the latter studies have relatively narrow EPR spectra, when compared to the ^1H nuclear Larmor frequency. As a result, polarization transfer was mediated by the solid effect and required an adjustment of the external magnetic field to meet the solid effect matching condition, $\omega_{\text{MW}} = \omega_{\text{OS}} \pm \omega_{\text{OI}}$.

Current DNP MAS ssNMR Challenges

As discussed above, DNP MAS ssNMR experiments are typically conducted at cryogenic temperatures (~ 100 K) to maximize attainable polarization. Conducting MAS experiments in this temperature regime provides its own set of technical hurdles. Specifically, the gas used to spin the rotor must not liquefy at the desired operating temperatures and must not be too viscous or it will impede rotor spinning. In addition, the breakdown voltage of the gas used should be able to withstand high power proton decoupling, which is necessary for modern high-resolution ssNMR experiments. For these reasons N_2 has been the most utilized cryogen for DNP MAS ssNMR experiments.

The need to conduct DNP MAS ssNMR experiments at cryogenic temperatures also presents spectroscopic challenges, due to the loss of spectral resolution at cryogenic temperatures. In biomolecular systems at physiological temperatures, chemical moieties such as amino acid side chains exhibit a range of rotational and vibrational motions that partially average chemical shift anisotropies and dipolar-coupling interactions thus narrowing observed resonances. Proteins typically undergo a phase transition in which most motions are frozen out at $170 - 230$ K.³⁶⁷⁶ This can lead to significant broadening of observed resonances, making the discernment of resonances of specific residues in universally isotopically labeled macromolecules difficult or impossible. Therefore, methods to regain the loss of spectral resolution are desired when conducting DNP MAS ssNMR experiments. To enable this, studies are needed on measuring specific sources of homogenous versus inhomogeneous broadening and to develop more efficient freezing strategies. In addition, improvements to hardware, such as faster MAS and more efficient decoupling, would yield better spectral resolution.

Future Directions

There has been tremendous progress made in DNP enhanced MAS ssNMR over the last decade, specifically in the commercialization of the requisite hardware and the development of efficient biradical polarization agents. Progress continues to be made in both of these arenas, but there are also other areas where innovative improvements should be pursued.

Polarization Agent Refinement

The development of nitroxide biradicals has led to tremendous gains in efficient DNP transfers. However, commercially available polarization agents have largely been developed with the use of homogenous aqueous samples in mind. There is still a need for the refinement of polarization approaches which use endogenous or chemically introduced polarization agents to effectively use matrix free sample preparations strategies. This would obviate the need for glassing agents, preserve sample integrity, and allow higher concentrations of sample to be used. Several demonstrations of this strategy have already

been discussed, but the observed DNP enhancements have been below 20, far below the theoretical maximum of ~ 660 for ^1H nuclei. In the development of the most efficient nitroxide biradicals, namely AMUPol and TEKPol, the methyl protecting groups of the nitroxide radicals have been replaced with bulky substituents such as tetrahydropyran.⁷⁷ The replacement of the methyl protecting groups leads to a lengthening of the T_{1e} and T_{2e} relaxation times by removing the relaxation pathways created by the fluctuating dipolar fields created by methyl rotation. It would be advantageous to follow suit in the further development of chemically introduced polarization agents, for instance replacing the nitroxide methyl protecting groups of nitroxide labeled lipids may result in more efficient DNP transfers. Alternatively, if there is concern over adverse morphological effects by adding bulky radical protecting groups, another option is to deuterate the methyls.

In addition to lengthening T_{1e} and T_{2e} relaxation times of tailored radical agents it would be beneficial to design efficient lipophilic biradicals which could simply be added as a minor component of lipid bilayers. Work to date with nitroxide labeled lipids used as monoradicals gave an order of magnitude in DNP enhancement. Based on the evidence supporting the efficacy of biradicals, it would be advantageous to develop lipophilic biradicals. For example, designing biradicals based on a cholesterol scaffold could be an efficient design strategy for procuring homogenous DNP enhancements throughout a lipid bilayer and membrane-embedded proteins while preserving the integrity of the lipid bilayer.

Nitroxide radicals have dominated the DNP enhanced MAS ssNMR landscape and there have only been a few studies utilizing narrow line radicals. Specifically, little has been done with paramagnetic metals as polarization agents. However, the studies that have used paramagnetic metals have shown promise.^{74, 78} Given the abundance of paramagnetic metals present in metalloproteins and materials it would be advantageous to further explore metals as polarization agents. With the recent advent of commercial swept field DNP MAS ssNMR systems, the selection of accessible polarization agents has significantly expanded and will enable significant growth in this area.

Instrumentation and DNP Methodology Development

Development and commercialization of gyrotrons capable of stable fixed-frequency, CW operation have aided in the adoption of DNP MAS ssNMR as an enabling technology and further hardware refinement will add to its utility. A.B. Barnes *et al.* have recently demonstrated the feasibility of frequency tunable gyrotrons, which would be capable of hyperfine decoupling via adiabatic inversion of electron spins by sweeping the MW frequency.⁷⁹ Similar to the advantages afforded by proton decoupling in MAS ssNMR experiments, hyperfine decoupling in DNP MAS ssNMR could lead to improved spectral resolution, longer electron relaxation times, and larger DNP enhancements.

R.G. Griffin *et al.* have shown the viability of pulsed DNP through the use of the NOVEL method (nuclear orientation via electron spin locking), where polarization is transferred from the electrons to nuclei through a Hartman-Hahn match condition ($\omega_{1S} = \omega_{0I}$, where ω_{1S} is the electron nutation frequency and ω_{0I} is the nuclear larmor frequency) in a method similar to traditional cross polarization in MAS ssNMR experiments.⁸⁰ This method has the advantage of providing efficient DNP transfer that is independent of the strength of the

magnetic field. NOVEL, for DNP applications, was demonstrated at a low magnetic field (0.35 T) where current MW sources (i.e. travelling wave tube amplifiers) have the pulse capability required by the NOVEL method. Further application of this method at high magnetic fields requires the development of compatible MW sources at the requisite frequencies.

Vitrification and Fast Freezing Techniques

It has been discussed that the cryogenic temperatures at which DNP enhanced MAS ssNMR experiments are conducted lead to a loss of resolution, specifically in biological macromolecular samples. This is partially due to the fact that cold N₂ gas streams that are used in most MAS probes freeze samples at a relatively slow rate. Slow freezing can result in conformational heterogeneity and loss of resolution. It would be beneficial to explore fast freezing or sample vitrification approaches. Several techniques successfully applied in cryoelectron microscopy (Cryo-EM), X-ray crystallography, and EPR spectroscopy could be used to approach this problem. For example liquid ethane is known to freeze samples at a much faster rate than liquid nitrogen.⁸¹ Alternatively, application of sample to a metal surface that is pre-cooled to liquid nitrogen temperatures can freeze samples on a relatively fast timescale.⁸²

Conclusion

DNP aims to alleviate the inherent insensitivity associated with NMR by transferring the much larger polarization of a paramagnetic dopant to NMR active nuclei of interest. Dramatic advances have been made over the last 15 years in DNP MAS ssNMR, specifically in hardware and polarization agent development, and the advent of commercial DNP MAS ssNMR instrumentation has allowed the technique to take hold in the broader NMR community. Applications of DNP MAS ssNMR to biomolecular and materials studies have yielded insightful information that would otherwise be unattainable without DNP and the technique has nearly matured to the point of generalized usage for non-specialists. Further refinement of polarization agents and sample preparations will lead to even larger DNP enhancements and new frontiers in the application of MAS ssNMR techniques.

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Biographies

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Joanna R. Long earned her Ph.D. (1997) in Physical Chemistry from the Massachusetts Institute of Technology. After postdoctoral studies at the University of Washington, she joined the faculty of the University of Florida. Presently, she is a professor of biochemistry as well as an associate laboratory director at the National High Magnetic Field Laboratory, where she has been developing a DNP user facility as well as new hardware and polarizing strategies for DNP enhanced MAS ssNMR. Her current research interests include membrane protein folding, lipid trafficking, biofilm formation, and characterization of DNP mechanisms.

Figure 1. Percent electron and nuclear polarization at the indicated resonant frequencies and magnetic fields. The approximate percent polarization of an electron and ^1H at 100 K and 14.1 T are indicated. The general scheme of DNP is also shown: MW irradiation of an unpaired electron resonance results in the transfer of polarization to nuclei, thereby giving DNP enhancement.

Figure 2. Molecular representation of polarization transfer in cross effect DNP enhanced ssNMR (left) and the resulting ¹³C signal enhancement after cross polarization under magic angle spinning (CPMAS; right). The polarization transfer scheme shows MW irradiation of AMUPol biradical and polarization transfer via ¹H-¹H spin diffusion to alanine dissolved in a glycerol and water matrix. The resulting DNP enhanced ¹³C CPMAS spectrum (red) has an enhancement of 128 relative to the control, MW off spectrum (black). Labeled resonances result from alanine and the glycerol matrix; unlabeled resonances are spinning sidebands.

Figure 3.

Energy level diagrams depicting polarization transfer via the cross effect (upper) for a nitroxide biradical with the relevant spectral properties and MW irradiation (lower). S and I refer to electron and nuclear spin states, respectively. α and β refer to the two available spin states of an $m_s = 1/2$ electron or nuclear spin. The grey shaded regions show where a mixing of states can occur with sufficient electron-electron dipolar coupling. The orange spheres represent relative amounts of nuclear polarization. The blue and red double arrows represent single quantum nuclear and electron transitions, respectively, and the purple double arrows represent the partially allowed zero and double quantum electron-nuclear transitions.

Figure 4.

Echo detected EPR spectra collected at 95 GHz e^- / 3.35 T for A) a nitroxide biradical, and B) BDPA, a symmetric carbon centered radical. The chemical structures of the nitroxide biradical TOTAPOL and BDPA are shown above their respective EPR spectra; the radical is depicted in orange. The broad EPR linewidth of the nitroxide biradical makes it ideal for cross effect DNP. The narrow EPR linewidth of BDPA is ideal for solid effect DNP.³⁵ Representative DNP enhancement profiles collected at 212 MHz ^1H / 140 GHz e^- / 5 T for C) the cross effect and D) the solid effect. C) and D) reproduced from Ni, Q.; Daviso, E.; Can, T.; Markhasin, E.; Jawa, S.; Swager, T.; Temkin, R.; Herzfeld, J.; Griffin, R., *Accounts of Chemical Research* **2013**, 46 (9), 1933–1941 (ref 26). Copyright 2013 American Chemical Society.

Figure 5.

^{13}C spectra of the Sec translocon signaling sequence bound to the Sec translocon. Standard ^1H to ^{13}C cross polarization spectra with and without MW irradiation are shown along with the double quantum filtered spectra that are necessary to remove the ^{13}C background signal. It is evident that DNP is necessary to observe the double quantum filtered spectra. Reproduced from Reggie, L.; Lopez, J. J.; Collinson, I.; Glaubitz, C.; Lorch, M., *Journal of the American Chemical Society* **2011**, *133* (47), 19084–19086 (ref 45). Copyright 2011 American Chemical Society.

Figure 6.

DNP enhanced ^{13}C - ^{13}C DARR correlation spectra of sparsely ^{13}C labeled EmrE in complex with TPP substrate. The observed correlation between the TPP substrate and E14 are evident in the spectra on the left. When glutamate ^{13}C labeling is suppressed the correlations are no longer observed (right), indicating E14 directly interacts with TPP. Reproduced from Ong, Y. S.; Lakatos, A.; Becker-Baldus, J.; Pos, K. M.; Glaubitz, C., *Journal of the American Chemical Society* **2013**, *135* (42), 15754–15762 (ref 46). Copyright 2013 American Chemical Society.

Figure 7.

MW on and off ^{29}Si NMR spectra of silica nanoparticles at natural abundance. The observed DNP enhancements enabled collection of natural abundance 2D ^{29}Si - ^{29}Si correlation spectra. Reproduced from Lee, D.; Monin, G.; Nghia Tuan, D.; Lopez, I. Z.; Bardet, M.; Mareau, V.; Gonon, L.; De Paepe, G., *Journal of the American Chemical Society* **2014**, *136* (39), 13781–13788 (ref 51). Copyright 2014 American Chemical Society.

Figure 8.

DNP enhanced ^{27}Al spectra of different Al coordination states in mesoporous alumina. MW off and on cross polarization (e.g. ^1H to ^{27}Al) and MW on direct ^{27}Al spectra are shown.

Reproduced from Lee, D.; Takahashi, H.; Thankamony, A. S. L.; Dacquin, J.-P.; Bardet, M.; Lafon, O.; De Paepe, G., *Journal of the American Chemical Society* **2012**, *134* (45), 18491–18494 (ref 53). Copyright 2012 American Chemical Society.

Figure 9.

Schematic of a commercial DNP MAS ssNMR system. A cryogenic DNP MAS ssNMR probe is inserted into a standard wide bore NMR magnet. The gyrotron generates high power MW that are transmitted to the sample in the probe via a corrugated waveguide. The probe is actively cooled to ~100 K by N₂ gas cooled passing through a heat exchanger submerged in liquid N₂.

Figure 10. Sample preparation strategy used for A) nitroxide labeled lipids and B) a traditional preparation with an aqueous suspension of TOTAPOL biradical.⁶⁹

Table 1

DNP enhancements for various endogenous or selectively introduced polarization agents.

Polarization Agent	Sample	Magnetic Field	Observed ϵ	ϵ Normalized to 14.1 T ^a
SL-lipids	Membrane Peptide (KL ₄)	14.1 T	8.9	8.9
N-Propyl-Palmipol	Liposome	9.4 T	8.1	5.4
ToSMTSL	Membrane Protein (ASR)	9.4 T	15	10
AMUPol-MTSSL	Membrane Protein (KcSA)	9.4 T	15	10
AMUPol-MTSSL	Membrane Protein (KcSA)	18.8 T	4.0	5.3
TOTAPOL	Membrane Peptide (KL ₄)	14.1 T	3.7	3.7
Mn ²⁺	Ribozyme	9.4 T	8.0	-
Semiquinone	Flavodoxin	5 T	15	-

^aThe reported DNP enhancements were normalized to a DNP enhancement at 14.1 T based on the assumption ϵ scales as B_0^{-1} for the cross effect.