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New NMR tools for protein structure and function: Spin tags for dynamic nuclear polarization solid state NMR

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Abstract

Magic angle spinning solid state NMR studies of biological macromolecules [1–3] have enabled exciting studies of membrane proteins [4,5], amyloid fibrils [6], viruses, and large macromolecular assemblies [7]. Dynamic nuclear polarization (DNP) provides a means to enhance detection sensitivity for NMR, particularly for solid state NMR, with many recent biological applications and considerable contemporary efforts towards elaboration and optimization of the DNP experiment. This review explores precedents and innovations in biological DNP experiments, especially highlighting novel chemical biology approaches to introduce the radicals that serve as a source of polarization in DNP experiments.

Keywords

Dynamic nuclear polarization; Solid state NMR; Nitroxide biradical; Paramagnetic tags; In cell NMR

1. Introduction: DNP broadens the horizons of solid state NMR

Magic angle spinning solid state NMR (MAS SSNMR) continues to grow in importance for structural biology and mechanistic studies of biological macromolecules [1–7]. Some recent notable achievements of solid state NMR include complete structures of amyloid fibrils [8,9] and membrane proteins [10] derived from solid state NMR restraints.

However, in comparison with other methods for studying structure and dynamics of ever-larger macromolecules, detection sensitivity remains an important challenge. Dynamic nuclear polarization (DNP) provides a means to sensitize solid state NMR, wherein a paramagnetic species serves as a source of polarization. Couplings between unpaired electrons and nearby nuclei facilitate transfer of polarization from the electron to the nuclei when the electron spin transitions are irradiated. Under favorable conditions, the nuclei are polarized, and under idealized conditions the nonequilibrium polarization for protons could be enhanced to a theoretical maximum of 660-fold. Highly encouraging developments over the past two decades, including the introduction of high-power gyrotron microwave sources and radicals optimized for DNP [11] have led to significant interest in these methods [12–14]. The improved sensitivity afforded by DNP can be combined with other advanced NMR

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techniques, such as higher dimensional experiments to ease spectral assignment [15], experiments for measuring structural restraints [16], and non uniform sampling methods.

DNP has been successfully applied to a number of biological systems, including rhodopsin membrane proteins [17–19], the Pf1 bacteriophage [20], transporter-bound peptides [21] and amyloid fibrils [22–24]. This has allowed observation of otherwise invisible NMR signals [18,20,25] from dilute components such as DNA in the Pf1 bacteriophage or peptides bound to membrane transporters [21,26] (see Fig. 1). Inspired by these remarkable successes, there is currently considerable effort towards optimization of the DNP experiment.

Polarization transfer in DNP proceeds via a variety of mechanisms (see Ref. [27] for a detailed discussion). Consequently, there are a variety of radicals with very different spectroscopic properties that have been successfully used in DNP experiments. The Overhauser effect [28], a cross relaxation effect, was one of the first polarization mechanisms demonstrated in metals, and today still holds promise, particularly for solution NMR [29]. The solid effect relies on zero and double quantum transitions for a single narrow-line radical (or the narrow central transition of a high spin metal) coupled to one or more nuclei [30]. Thus, irradiation is most effective at frequencies $\omega_{MW} = \omega_e \pm \omega_n$. This mechanism has been demonstrated in a number of cases [31–34] and has potential for future applications, especially for high field NMR [35]. The mechanism most widely used in high field NMR thus far is the cross effect [36,37]. Here, the nuclear polarization originates from *two* coupled electrons whose difference in Larmor frequency matches the electron frequency: $\omega_{1e} - \omega_{2e} = \omega_n$. Effective experimental conditions for the cross effect have been discussed in many reports [38–40]. Since successful cross effect DNP requires two paramagnetic centers, the development of DNP optimized biradicals has greatly extended the applicability of this mechanism. Starting with the linked bi-nitroxide TOTAPOL [11], several generations of synthesis have been reported, guided both by empirical results and by rational design that improves upon the relaxation properties and dipolar properties of the electrons, finally yielding a variety of designer biradicals that provide excellent enhancements [41–44].

While DNP has now been carried out in many laboratories on many different samples, there is a broad range in the success of these studies. Enhancements, typically defined as the intensity of the NMR signal with microwaves on vs. microwaves off (I_{on}/I_{off}), can practically range from minimal (i.e., no enhancement) to 515 on model compounds [41,45,46]. Counteracting this enhancement are detrimental effects of the sample conditions typically used for the DNP experiment, and thus this definition of enhancement is not a complete figure of merit. Firstly, the paramagnetic center that serves as the source of polarization can cause depolarization as well as paramagnetic relaxation enhancement of the nearby nuclear spins. The resultant decrease in the overall nuclear signal counteracts the enhancements gained by the DNP experiment [47,48]. Some researchers advocate reporting an overall or absolute sensitivity gain, rather reporting raw enhancements [49–52]. These metrics incorporate other factors such as bleaching (relaxation driven signal losses), depolarization, and changes in sample concentration versus optimized traditional solid-state NMR experiments. Additionally, since the most successful DNP experiments are carried out at cryogenic temperatures, the additional NMR line broadening observed at these temperatures

poses a vexing challenge. Experiments have found this line broadening to be largely inhomogeneous in nature [53,54] and correlated with solvent exposure [55] and conformational inhomogeneity [56,158]. The poor resolution in many DNP experiments prevents the practical use of 1D or 2D experiments, necessitating the collection of multidimensional experiments or the use of selective isotope enrichment to resolve individual sites.

Some of the variability in success is likely to be related to the nature of the radical, its spectroscopic properties, chemical stability, solubility, and its coupling to the nuclei of interest. This review focuses on chemical biology approaches for producing biradicals that offer new opportunities and improvements for DNP experiments.

Typically, radicals used in DNP experiments are introduced by co-dissolving with samples (see Fig. 2). The assumption is that the immediate nuclei that have strong hyperfine couplings to the electron are those of the solvent [32,39,58]. These couplings enable electron-nuclear polarization transfer, and therefore polarization is transferred first from the electrons to the solvent nuclei (typically ^1H). Spin diffusion then relays polarization throughout the nuclear matrix, which includes the solutes of interest. To prepare these samples, the paramagnetic polarizing source is simply mixed into the solvent matrix, usually a glassy mixture of d_8 -glycerol and $\text{D}_2\text{O}/\text{H}_2\text{O}$. Early experiments on Pfl bacteriophages [59] and amyloidogenic nanocrystals [22], in which nuclei on the interior of these assemblies were readily detected, suggested that spin diffusion is sufficiently fast so as to transfer polarization several nanometers. Therefore, the radical does not need to be proximal to the nuclei of interest. This experimental design has been successful for a number of different materials science and structural biology problems.

However, experiments in systems with multiple phases, such as proteoliposomes with membrane proteins [21,60] or highly crystalline substances [49], suggest that poor radical distribution leads to ineffective enhancements. These examples point to a broader concern in generalizing DNP, namely that there may be sample-dependent factors that prevent effective electron-nuclear polarization transfer. This can be related either to the magnitude of the electron-nuclear couplings or poor interactions of the analyte with the solvent, due to hydrophobicity mismatch or the formation of ice crystals.

Moreover, since polarization spreads through proton-proton spin diffusion, the proton/deuterium composition of both the solvent and analyte become key experimental variables. The typical DNP matrix, 60/30/10 d_8 -glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$, has 10% protons, which is thought to yield both an optimized proton relaxation rate and a spin diffusion rate sufficiently fast to spread polarization through the sample. Initial experiments indicate that deuteration of the protein itself can lead to larger enhancements [61]. For large or inhomogeneous samples, however, the ideal deuteration level of the matrix may differ from the 90/10 ratio typically used, and tuning the deuteration level of the analyte itself may be key to efficient hyperpolarization. Moreover, as is discussed below, experiments indicate that biradical/protein geometry can influence the optimal solvent deuteration level.

Additionally, in typical sample preparation methods, high concentrations of paramagnetic compounds are used, which can reduce the overall NMR signal through paramagnetic relaxation effects (see Fig. 2C). Therefore, when choosing a concentration of biradical for DNP, one has to balance the need for sufficient radicals to polarize the system while minimizing the paramagnetic bleaching [51,62].

Here we consider systems in which radicals are “tagged” onto the protein of interest. These could conceivably have strategic advantages, such as control over radical location, selectivity in enhancement, and potentially a reduced perturbation of the system by avoiding high concentrations of radicals and nonspecific binding effects. Although this is still a new area of investigation, with much work remaining to be done, the closely related literature from EPR and NMR define some of the opportunities, and some of these benefits have been illustrated already in the DNP studies outlined below.

2. Spin tagging in magnetic resonance

Selective paramagnetic tags have been developed and demonstrated to be generally useful for magnetic resonance studies of proteins and macromolecules. Studies of numerous proteins containing endogenous metals or radicals have illustrated the wealth of information that can be obtained through direct observation of the unpaired electron by EPR spectroscopy, observation of the paramagnetic tag's effect on the NMR spectrum of the bound protein [63], and hybrid methods studying hyperfine couplings in detail [64]. Labeling proteins with an exogenous radical tag was a natural extension of these methods to diamagnetic systems. Spin labeling is an important tool for biological EPR [65,66], with applications ranging from structure determination [67] and solvent exposure to protein dynamics and ligand binding [68–70].

Paramagnetic tags are also powerful tools for solution NMR studies of proteins [71–75]. Paramagnetic compounds can induce a variety of effects on the spectra of nearby nuclei, for example the broadly used pseudocontact shifts and paramagnetic relaxation effects. These effects are dependent on the distance of the paramagnetic tag from the nuclei, which offers a powerful restraint for structural characterization via solution NMR.

The analogous experiments using paramagnetic tags for solid state NMR are highly exciting. Although the body of work is far less mature, in the past 10 years the field has developed quickly [76–78]. Similar to solution NMR, the field began with solid state studies of paramagnetic proteins [79–81], but the work of Jaroniec *et al* and others has extended this work to proteins tagged with nitroxides [82,83] or Cu(II) and Mn(II) –chelated EDTA tags [84,85]. Using these methods, solid state structures of GB1 [85] and SH3 [83] domains have been solved, the latter in combination with state of the art fast MAS technologies that permit proton detection. Paramagnetic tagging can also determine oligomerization interfaces [86] and map the interactions of membrane proteins [87] by observing reductions in signal intensity when members of an assembly are tagged with nitroxides. For solid state NMR studies of lipid-associated membrane proteins or sterols, spin labeled lipids can be used to study the membrane associations of proteins and sterols [88,89]. Other studies illustrate the value of the pseudocontact shift as a solid state structural restraint, for metalloproteins

[90,91] and for proteins labeled with the metal chelators [92], and these tools have been combined with proton detection [93] for rapid determination of protein structure. In these applications, paramagnetic relaxation effects offer powerful structural restraints. In solid state NMR experiments the relaxation effect can have the additional benefit of improved signal averaging by reducing the nuclear proton T_1 and by extension the recycle delay [94–96].

All of the aforementioned studies involve the introduction of a paramagnetic center to the biomolecule of interest. To incorporate nitroxides, the most widely used probe is the methanethiosulfonate spin label (MTSL), which introduces a nitroxide via a thiosulfate ester reaction with a free cysteine [97]. This cysteine can be native to the protein, or introduced via mutagenesis, enabling most positions in the protein to be labeled. MTSL was the first probe used for *in vitro* structural determination of a protein using paramagnetic NMR restraints [98], and MTSL tags with tailored properties, such as greater rigidity, have been synthesized [99]. Other cysteine attachment chemistries, such as maleimide [100], have been explored as well. *N*-hydroxy succinimide ester derivatized nitroxides can be used to label primary amines such as lysine sidechains [101]. For spin-labeled studies based on peptide synthesis, the nitroxide derivatized unnatural amino acid 1,1,6,6, - tetramethyl-*N*-oxyl-4-amino-4-carboxylic acid (TOAC) is a useful way to incorporate a nitroxide center [102]. *In vivo* incorporation of nitroxides using unnatural amino acid technology based on amber codon suppression has also been reported, both in *Xenopus* oocytes [103] and in *E. coli* [104,105]. Unnatural amino acid technologies can also enable two step spin labeling by incorporating amino acids with bioorthogonal reactive side chains, with the nitroxide subsequently coupled via click chemistry [106] [107].

Similarly, a variety of incorporation methods exist for studies using metal centers [74,108]. Peptide-based lanthanide binding domains can be attached via fusions with the parent protein or via cysteine attachment chemistries. His-tags were recently demonstrated to chelate Mn(II) ions suitable for EPR distance measurements [109]. Cysteine attachment chemistries can again be used to attach organic metal chelators such as EDTA. Efforts towards immobilizing the metals to remove rotational degrees of freedom include using tags that attach to two sites in the protein [73] or the use of more rigid tags [110].

For EPR applications, trityl organic radicals have also been tagged onto proteins [111,112] via the MTS cysteine chemistry. DNP results with trityl radicals [38,40], combined with their resistance to chemical reduction [113], make these spin labels intriguing for DNP applications.

3. DNP *in cell* applications: challenges and opportunities

The very high sensitivity of DNP can theoretically enable in-cell detection of species of moderate to low concentration (nanomolar). Such experiments have the potential to probe events such as binding, catalysis and conformational changes on an atomistic level in native tissue, cells, or cell-like conditions. In-cell solution NMR spectroscopy has already been well established [114–117], with notable applications including the maturation of human superoxide dismutase [118,119], in cell characterization of alpha synuclein [120], and

investigations of the relationship between molecular crowding and protein interactions [121,122]. In-cell solid state NMR [123], while a much less mature field, has been demonstrated to have promise on systems such as membrane proteins [124] or proteins in large complexes [125]. Many proteins of interest are dilute in cell, and have concentrations below the limit of detection for traditional solid-state NMR methods. Therefore, the signal enhancement provided by DNP is key to enabling the detection of many in cell components. DNP experiments have already been performed on yeast prion proteins fibrilized *in situ* in cellular extracts at endogenous concentrations [126], as well as on the membrane anchored cytochrome *b5* protein in whole *E. coli* cells [127]. Baldus and coworkers have used DNP to study membrane proteins in whole cells and cell envelopes extracted without further purification [128,129].

For these in cell applications, selective detection of one protein in the cellular milieu has been achieved by differential isotope labeling of the protein of interest over the background. In *E. coli* this can be achieved using timed control of expression and isotopes in the medium [115,122,128,130,131]. Isotopically enriched proteins can be injected [120,132], electroporated, or tagged into mammalian cells using cell-penetrating peptides [133], as their membranes are easier to penetrate. Banci and co-workers have also demonstrated that isotopically enriched proteins can be prepared in mammalian cells and analyzed *in situ* by using isotopically enriched growth media, in analogy to established procedures in *E. coli*.

However, in applying DNP strategies to cells and cell-derived samples, reduction of the radicals in the reducing cellular environment is a significant challenge that has already been documented in *in cell* EPR studies. Proteins and DNA molecules that are spin-labeled with nitroxides and then injected into *Xenopus* oocytes exhibit reduction, with a half life of approximately one hour [134–136]. Similar challenges occur for *E. coli* cells. For example, in one study [137,138], cysteines in an outer membrane protein were targeted with a spin probe. EPR signals from inside the cell were invisible, while those on the exterior of the cell were detected, suggesting reduction upon transport into the cell. Similarly, in cells expressing proteins with the unnatural amino acid SLK-1, reduction of the EPR probe in cells and in lysate was reported, and prevented successful measurements *in vivo* [104,105,139]. It has been shown that 5-membered piperidine rings have improved stability in biological systems in comparison with the six membered pyrrolidines typically used in DNP applications [140,141]. Substitution of the positions flanking the nitroxides radical with bulky moieties has been shown to increase resistance to reduction [142,143], and nitroxide spin tags based on these bulky tags may be promising for in cell DNP. It is noteworthy that the use of *biradicals* in DNP raises a related specific consideration. Double electron resonance spectroscopy, DEER [144], and other electron-electron coupling measurements, also utilize two spin labels to obtain structural constraints, in the form of inter spin distances, for large macromolecules. These EPR experiments, and cross-effect DNP experiments, fail when *either* of the two electrons is reduced, and consequently the requirement for minimal reduction is much more stringent than for a mono-radical based experiment.

A promising tactic to circumvent the problem of reduction of the radical is use of paramagnetic metal-based tags, such as gadolinium, which is relatively inert in the cellular

environment. This approach has allowed DEER EPR spectroscopy of peptides in *Xenopus* oocytes [145] and of ubiquitin in HeLa mammalian cells [146]. It was recently shown that lanthanide binding peptides can be genetically encoded and used for DEER measurements in *E. coli* cells grown on Gd(III) supplemented media, without the need for introduction of exogenous proteins or tags [147]. Promising DNP experiments using lanthanides [148] suggest that these tags may prove key to the success of *in cell* DNP.

4. Tagging for selective DNP applications

Using DNP tagging strategies, one can envision tagging proteins in complex environments to achieve selective NMR enhancement by differential DNP enhancement of the target protein over background proteins [149,150]. As mentioned above, this selective detection is crucial for NMR studies in the cellular context, to prevent signals of other proteins in the environment from obscuring those of the protein of interest. This was demonstrated in cell lysate for the protein Bcl-X_L using a TOTAPOL derivatized peptide [150]. By subtracting the background signal (from a sample without the selective Bak peptide), researchers were able to collect DNP enhanced 2D spectra of Bcl-X_L in cellular extracts prepared from cells grown in U (¹³C, ¹⁵N) media.

The recent past has seen significant interest in localizing the polarizing agent to the biomolecule of interest to see how DNP parameters such as enhancements and buildup times are affected. These studies are expected to impact both the technology and the theory of DNP. Many details remain to be explored, for example the optimum tag-nucleus geometry and isotopes, but initial results have been very encouraging.

Implementation of tagging has many open choices, and the strategies can generally be divided into two different approaches: those that tag the protein directly, often using chemistries borrowed from the PRE literature referenced above, and those that use either non-specific interactions or that tag neighboring molecules. The choice of paramagnetic tagging strategy depends on the type of DNP mechanism being used. For the solid effect, a single radical is needed, making the simple adoption of tagging strategies from the EPR and conventional NMR literature feasible. For the cross effect, two radicals are required. This can be done by tagging the protein with two paramagnetic tags, where the relative distance and orientation of the two tags will affect on the overall enhancement (see Fig. 3). Alternatively, the protein can be tagged with a biradical such as TOTAPOL.

In comparing different tagging approaches in terms of their enhancement factors, is important to note that enhancements are field dependent, and that the field dependence differs for different DNP mechanisms. For the solid effect and cross effect, enhancements scales as B_0^{-2} and B_0^{-1} , respectively. In Table 1 we normalize cross effect enhancements to 14.1 T, as in Ref. [14]. Moreover, enhancements are strongly temperature dependent in part due to properties of the radical such as its spin lattice relaxation and the ability to saturate the transition; the detailed temperature dependence can vary depending on the polarization agent used. Reported temperatures for the studies discussed here are noted in Table 1, and no correction for temperature differences was made.

An early example of using a proximal radical capitalized on the native flavin mononucleotide semiquinone radical of the protein flavodoxin, which binds noncovalently to the protein [151]. In the protonated protein the observed ^1H signal enhancement was 7, and upon deuteration of the protein, encouraging enhancements of 15 were reached (see Fig. 4).

Another strategy involved synthesizing a peptide labeled with TOTAPOL [50] by coupling TOTAPOL to a peptide produced via solid phase synthesis and studied as a dry powder. Signal enhancements of 4 at 9.4 T were reported for the protonated peptide with very short DNP buildup times.

A simple means to control the location of TOTAPOL in the sample is to rely on the natural affinity of TOTAPOL for sugar-like moieties, such as peptidoglycan [152] or cellulose [153]. Takahashi *et al* [154] have generalized this approach through the use of gluing agents, where the protein of interest is suspended with trehalose or glucose and TOTAPOL. The solvent is then partially removed, and the glucose/trehalose acts as an adhesive to “stick” the TOTAPOL to the protein surface, preventing its aggregation. Notably, they observe no enhancement on crystalline adenosine without the addition of glucose as a gluing agent, implying that localization of the biradical to the crystalline solid is important to enhancements. Since most protein crystals remain hydrated, and often have large water rich channels, there should be room in between individual proteins to introduce TOTAPOL into the crystallization matrix along with the gluing agent.

TOTAPOL can also be co-localized with proteins through sedimented solute NMR (SedNMR), in which the protein is ultracentrifuged from an aqueous, 90% deuterated TOTAPOL-containing solution. The protein enters a highly dense phase segregated from the bulk solvent that is thought to form a glassy state upon freezing. Initial results with apoferritin [155] sedimented from a 2 mM TOTAPOL solution showed enhancements of 42 in the sedimented samples, but only enhancements of 2 in an identical frozen solution (without cryoprotectant). In a follow up paper, the same authors showed that bovine serum albumin (BSA) likely has a binding affinity for TOTAPOL, as optimal enhancements of 64 for BSA sedimented from a TOTAPOL containing solution are dependent not on absolute concentrations but on the biradical:protein doping ratio [156].

TOTAPOL was recently shown by ITC to bind to two different amyloid fibrils, CsgA from *E. coli* and the Het-S domain from *Fusarium graminearum*, with affinities of approximately 9 μM [157]. This binding site appeared to compete with that of the hydrophobic fluorescent dye thioflavin-T. Due to this interaction, optimal enhancements from CsgA were found with biradical concentrations of 1 mM, and enhancements retained even at 50 μM TOTAPOL. These concentrations are 1–3 orders of magnitude lower than typically used for protein DNP spectroscopy, indicating the benefit of the specific binding interactions.

When monoradicals introduced through spin tagging are co-localized or aggregated, the cross effect mechanism can be effective (see Fig. 3). Such a scheme can be used to detect binding or dimerization, as illustrated in experiments involving the intrinsic membrane dimeric protein gramicidin. Gramicidin was covalently tagged with a TEMPO at the N-

terminus. Upon insertion into the membrane, the dimer forms a rigid biradical pair with an interspin distance of 7.5 Å that can polarize the membrane [158,159]. van der Crujisen *et al* [160] tagged cysteine mutants of the intrinsic membrane protein KcsA with MTSL. KcsA forms a tetramer in its native state, thereby bringing together spin labels attached to monomers. One cysteine mutant exhibited enhancements of 14.5. Another interesting example of this strategy used proteoliposomes prepared with monoradical labeled lipids and the membrane-intercalating peptide KL₄. Notably, the enhancement was higher than that seen for soluble TOTAPOL, and there was no need for exogenous glassing agents. In follow-up work [161], Smith *et al* showed that for lipid resonances, flexible monoradicals tagged to the membrane surface provided the best enhancements. In all of these examples, monoradicals are used to provide a source of polarization for cross effect DNP due to intermolecular radical interactions.

Proteins or lipids can also be tagged covalently with biradical compounds. Fernandez-de-Alba *et al* introduced a lipid tagged TOTAPOL, which gave enhancements of 8.1 on the lipid resonances [162]. KcsA has been derivatized with a biradical tag based on AMUPOL using MTS attachment chemistry [160], and enhancements of 14.5 at 9.4 T were achieved. These enhancements are lower than those for 25mM soluble AMUPOL with the same system [56], but interestingly, the same as those observed in KcsA monomers tagged with the monoradical MTSL (see discussion above). Voinov *et al* [163] also reported enhancements of 15 for the Anabana sensory rhodopsin protein when derivatized with an MTS-TOTAPOL moiety, similar to those observed with 17 mM exogenous TOTAPOL. Although the enhancements with the tagged biradicals in these studies are generally comparable to those observed for exogenous biradicals, the lower overall radical concentration of the spin tags may be less perturbative for some situations. Moreover, for proteoliposome preparations, attaching the biradical directly to the protein eliminates the need for a glassy glycerol/water matrix and allows the optimization of cryoprotectants for the sample of choice. In this regard, Hong and coworkers [164] reported that DMSO, polyethylene glycol, and DMF are better cryoprotectants than glycerol for lipid membranes at cryogenic temperatures. These studies in total demonstrate great promise for covalent attachment to reactive cysteines.

Most of the tagging approaches outlined above utilized nitroxide-based tags. Other radical species may also be attractive, particularly in light of their reductive properties in cell (see discussion above). Corzilius and coworkers demonstrated the use of paramagnetic metals as DNP polarizing agents. These metals are effective polarization donors in the solid effect mechanism, and for direct polarization of ¹³C and ¹⁵N. They demonstrated ¹³C enhancement of a ribozyme via a bound manganese [31] and also tagged ubiquitin with a metal chelating tag for ¹³C enhancement [148].

A current theme in chemical biology is high affinity, noncovalent tags, such as His-tags or the trimethoprim tagging systems [165]. Unlike cysteine covalent attachment chemistry, these strategies offer a high degree of selectivity for a particular target in a mixture or even in cellular contexts, and can be “bioorthogonal”, meaning that they could take place in cells without interfering with native processes.

In this vein, Etzkorn and coworkers modified the Bak peptide with TOTAPOL [150] which binds with a K_D of 340 nM to the protein Bcl-X_L. They showed an enhancement of 18 for the bound protein. In buffer-exchanged cell lysate containing only 10% Bcl-X_L, 160 μ M TOTAPOL-tagged Bak peptide can selectively enhance Bcl-X_L. McDermott and coworkers developed a generalizable strategy, by synthesizing a TOTAPOL derivatized trimethoprim polarizing ligand, which binds to DHFR with a K_D of 165 nM [149]. In related optical studies, DHFR can act as a tag for a fused protein of interest [166,167]. These systems allowed for detailed studies of the protein-ligand complexes at mid-micromolar concentrations.

For both of these studies, DHFR and Bcl-X_L, selectivity and good enhancements were achieved when the matrix was fully deuterated (see Fig. 4). The authors speculated that if the solvent contains even 10% protons, spin diffusion to the solvent competes with polarization transfer to the protein, decreasing the observed protein enhancements. This finding highlights the importance of controlling the proton composition for tagged DNP experiments relying on polarization transfer to ¹H. When applying DNP tagging methods to in cell studies, this adds another layer of complexity; in the studies with Bcl-X_L, lysate was exchanged into deuterated solvent for selective detection. Thus, direct polarization transfer to ¹³C or ¹⁵N, as demonstrated for paramagnetic metal tagging agents mentioned above, may prove to be easier to implement in-cell.

5. Future perspectives

In light of the highly encouraging DNP experiments reported to date, many fundamental aspects of the DNP experiment are under study with the broader goal of optimization of the experiment. New discoveries regarding the underlying mechanisms are expected to lead to additional transformative improvements in implementation, including new hardware, pulse sequences and interpretation strategies. For example, it is likely in the near future that DNP enhanced experiments at much higher magnetic fields will become successful, and polarizing agents efficient at magnetic fields of 800 MHz have already been introduced [171].

The broad linewidths of many DNP experiments remain an important challenge. To tackle this problem, judicious sample choices and preparation, as evidenced by the good linewidths observed in highly rigid fibrils [57] or carefully prepared tubular assemblies of the HIV-capsid protein CA [172], will be key to successful DNP implementation. The introduction of multidimensional pulse sequences tailored for DNP samples [15] can also overcome resolution challenges. Preliminary results combining DNP with fast magic angle spinning has led to linewidth reduction in biological samples [15] and offers a promising field of investigation.

Room temperature DNP has been demonstrated in very encouraging experiments [173,174], and further successes in DNP at room temperature would allow experiments that are compatible with biologically relevant conditions and studies of conformational dynamics. Combining DNP with proton detection, another promising emerging solid-state NMR technology, can lead to even higher sensitivity.

Among the solutions to these challenges, strategic choice and placement of the radical, control and stabilization of its properties are expected to continue to be important elements, and we can expect to see more exciting developments on this aspect of the DNP experiment going forward.

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Abbreviations

DNP	Dynamic Nuclear Polarization
DEER	Double Electron Electron Resonance
EPR	Electron Paramagnetic Resonance
MAS	Magic Angle Spinning
PELDOR	Pulsed EElectron electron DOuble Resonance
SSNMR	Solid State Nuclear Magnetic Resonance
TOTAPOL	1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol

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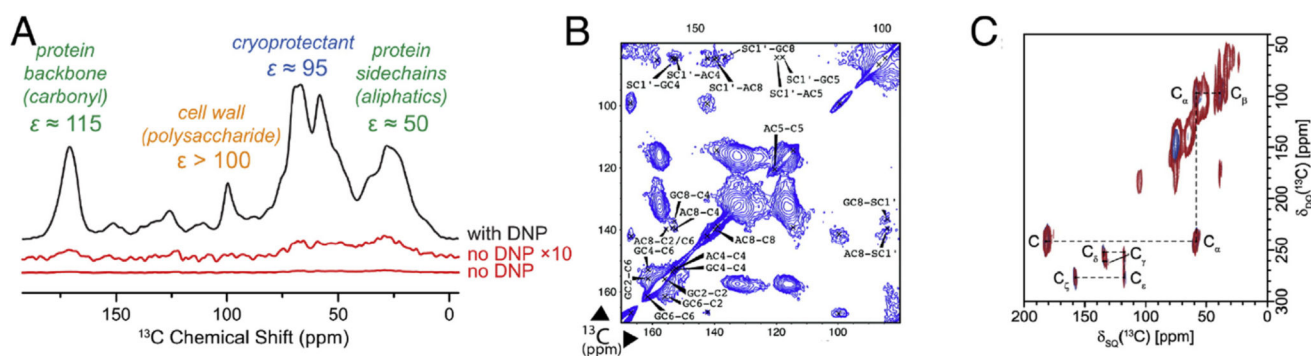
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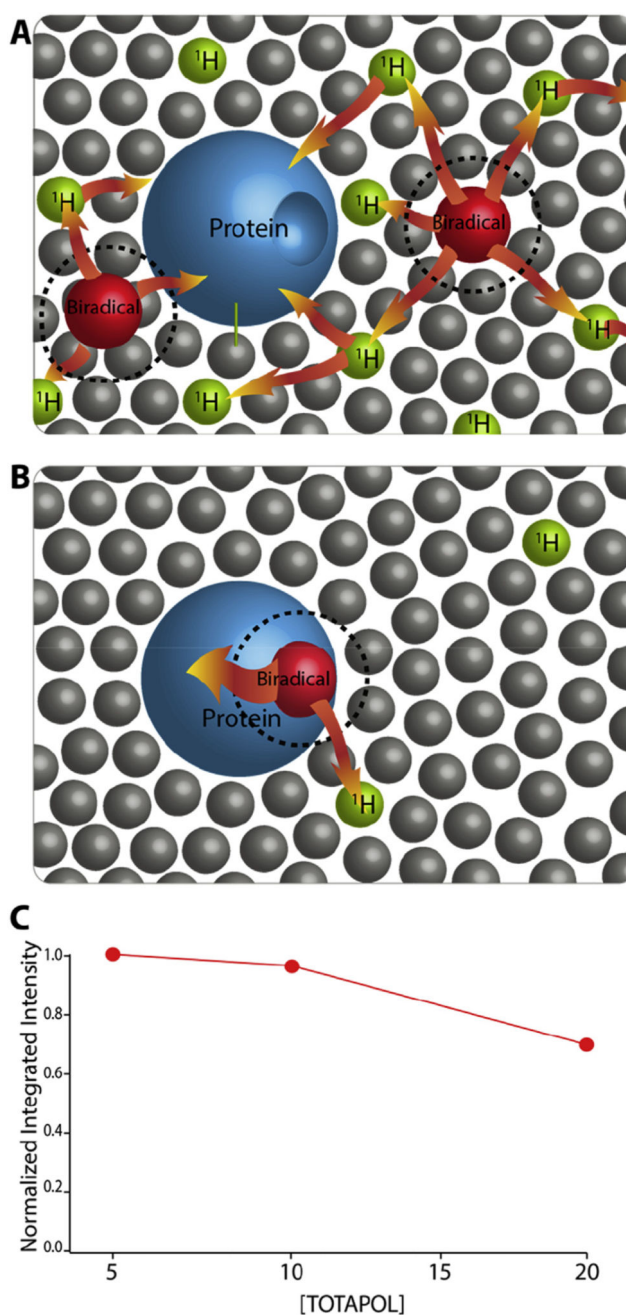
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**Fig. 1.**

DNP permits NMR detection of species too low in concentration to be otherwise detectable without intractable signal averaging. **Panel A**- DNP signal enhancement of 1 μg of the yeast prion protein Sup3 fibrillized *in situ* and then packed into a rotor with 10 mM TOTAPOL.

Panel B- 2D DNP enhanced ^{13}C - ^{13}C correlation map of the Pf1 virion illustrating assignments of the DNA resonances, which are only 6% of the virion by weight. Enhancements of 22 were obtained.

Panel C- 2D ^{13}C - ^{13}C SQ/DQ correlation map of an antigenic peptide bound (9 nmol, concentration 150 μM) to a human ABC transporter protein (4.5 nmol); with 10 mM AMUPOL added enhancements of 37 were reported. A is reproduced from Ref. [126]; B from Ref. [20], and C from Ref. [21].

**Fig. 2.**

Contrast between distribution of biradical polarizing agents and spin polarization transfer in traditional DNP experiment (A) and a targeted approach (B). In the traditional experiment, the polarizing agent (red) is suspended with the protein in a solvent with approximately 10% ^1H (green). Polarization is transferred from the biradical to nearby protons, most likely solvent, with polarization assumed to equilibrate throughout the bath. This mechanism is reliant on either spatial proximity of the biradical to the analyte of interest, or efficient spin polarization transfer across boundaries; if either of these conditions fail, then enhancement is poor. In the targeted experiment, by co-localizing the biradical and the analyte, polarization

is transferred directly to the analyte. However, polarization transfer to the solvent may compete with transfer to the protein (see text for more discussion).

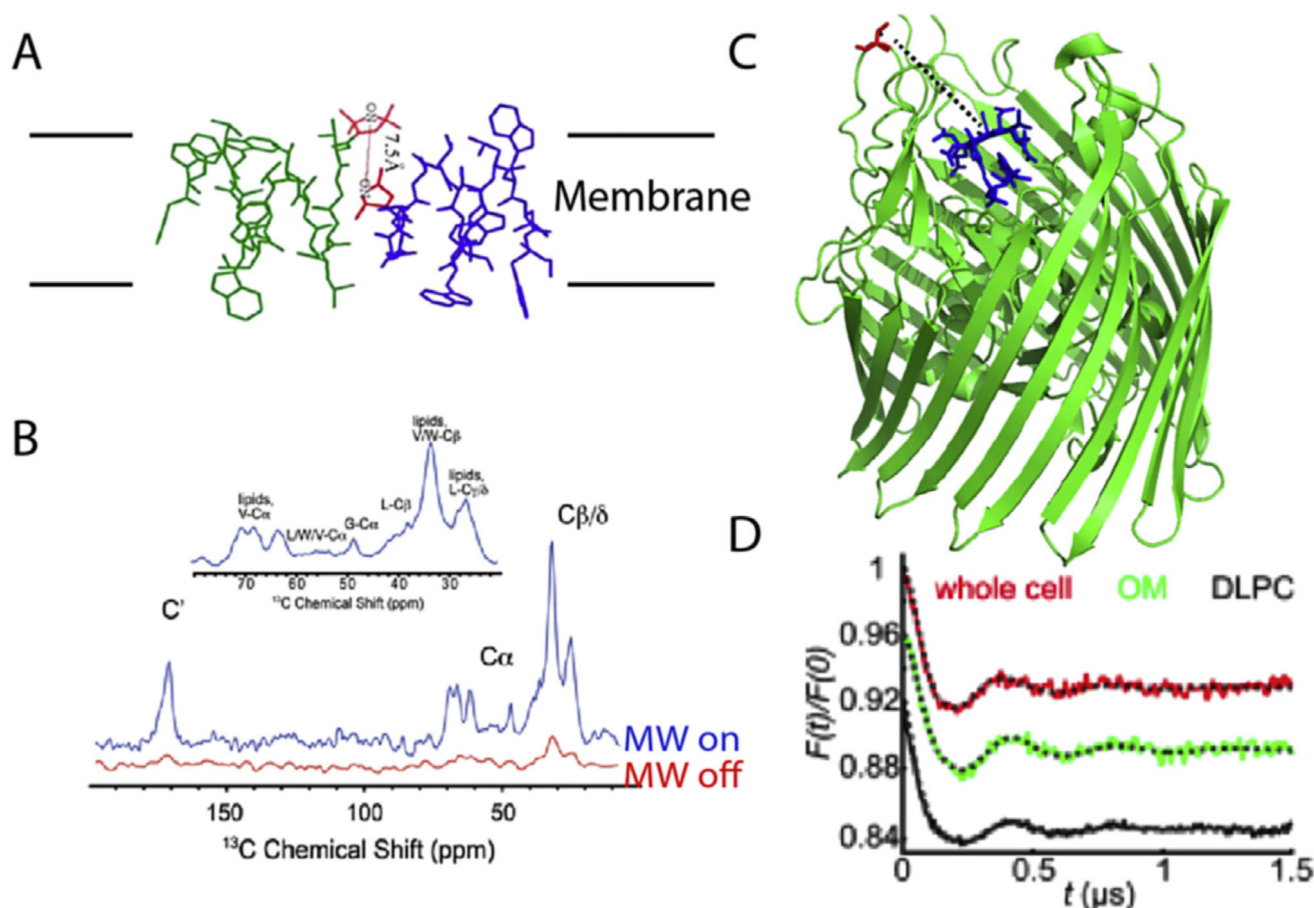
The black dashed line indicates a radius where bleaching is expected to occur, and these NMR signals are therefore NMR silent. The bottom image depicts the experimental manifestation of paramagnetic relaxation effects as signal bleaching in samples of DHFR suspended with TOTAPOL. The integrated signal intensity (without DNP enhancement) as a function of TOTAPOL concentration (white squares) is plotted. The decrease in absolute signal as a function of TOTAPOL concentration indicates bleaching of protein signals by TOTAPOL. In Refs. [62] and [51] estimates for the bleaching radius from TOTAPOL are given based on experiments with model compounds.

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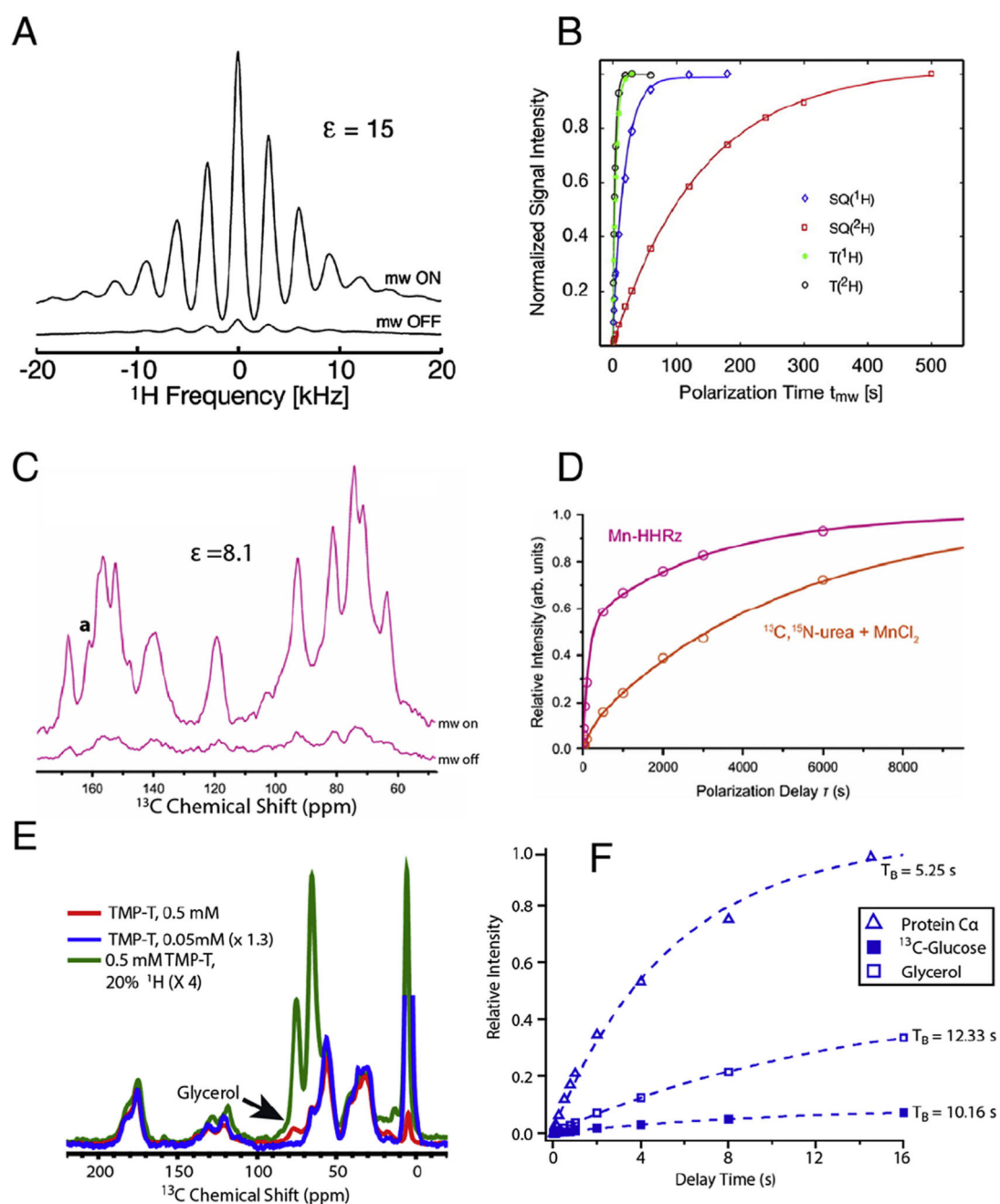
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**Fig. 3.**

Examples of experiments in which radical pairs are formed at binding interfaces, thereby selecting for a particular functional state of the system through DNP or PELDOR spectroscopy. In **A**, dimerization of gramicidin in the membrane positions two nitroxides 7.5 Å apart to serve as a biradical for DNP polarization. In **B**, the DNP enhanced spectrum of the system is shown, with the inset highlighting enhancements of the lipid resonances. **C** depicts the outer membrane protein Btub, which was spin labeled at position 188 (shown in red) on the extracellular face. The ligand (shown in blue) was spin labeled as well. **D** shows traces from pulsed electron double resonance (PELDOR) experiments with the spin-labeled protein and ligand in both whole cells, outer membrane preparations, and purified proteoliposomes, illustrating that this technique is accurate in whole cells. Protein concentration is estimated to be 30 μM . The PELDOR signal only comes from radicals, by analogy with the cross effect, and both approaches illustrate promising ways in which cross-effect DNP can report on dimerization or binding of proteins while also providing information on nuclear chemical shifts in these conformations. A and B are reproduced from Ref. [158]; C and D are reproduced from Ref. [138].

**Fig. 4.**

Potential for selectivity in DNP sample preparations potentially suitable for in cell measurements. A, B, C and D are developed via the solid effect, using endogenous paramagnetic cofactors that are suitable for in cell measurements due to their resistance to physiologic reduction. E and F illustrate the TMP-T methodology, which uses a TOTAPOL tagged trimethoprim ligand highly selective for DHFR in an in cell context. In **A and B** the polarizing agent is a semiquinone radical directly bound a flavodoxin. In **A** an enhancement of 15 is shown for direct transfer to the ^1H . In **B**, polarization buildup times for the semiquinone are compared to those for exogenous TOTAPOL (abbreviated SQ and T,

respectively), for ^1H and ^2H flavodoxin, respectively. The sensitivity of the semiquinone polarization buildup time to protonation of the protein as compared to TOTAPOL suggests that transfer from semiquinone occurs preferentially to protein protons. **C** depicts direct ^{13}C enhancement of a hammerhead ribozyme from bound manganese (II). In **D**, polarization buildup times for the bound manganese are compared to those for exogenously added manganese chloride, illustrating faster buildup times in the presence of the bound manganese. In these studies the polarization buildup times suggested that the experiments achieved selective sensitization of the bound protein. In the studies involving Mn(II), transfer occurs directly to ^{13}C , and selective enhancements were achieved without deuteration of the matrix [149,170]. In **E**, conditions for selective enhancement for the tagged biradical TMP-T are illustrated, namely a well-deuterated matrix; when 20% protons are added to the solvent, the glycerol signals are enhanced greater than the background. Lowering the concentration also increases the selectivity, as demonstrated by the absence of glycerol signals in 0.05 mM samples. In **F**, polarization buildup times for protein are faster than those for the cosolutes glycerol and glucose in the ^2H matrix. However, in ^1H solvent buildup times for both lengthen (see Ref. [149]), indicating that solvent deuteration can confine polarization to the tagged protein. A and B are reproduced from Ref. [151]; B and C are reproduced from Ref. [31].

Table 1

Summary of tagging strategies, both covalent and non-covalent, for DNP enhanced solid state NMR. In the final column, enhancements for cross-effect samples have been scaled to 14.1 T, assuming that enhancement scales as Bo. DNP enhancements can be temperature dependent; therefore temperatures are reported.

Radical	Target	e	Tagging strategy	Field (T)	Temp (°K)	e(at 14.1 T)	Ref
Semiquinone	Flavodoxin	15	Endogenous radical	5	~90	N/A	[151]
Nitroxide tag	Gramicidin	6	Covalent	9.4	115–110	4	[158]
TOTAPOL	Peptide	4	Covalent	9.4	100*	2.4	[50]
TOTAPOL	Cellulose	20	Noncovalent	9.4	100*	13	[153]
TOTAPOL	Apo ferritin	42	Sedimented	5	<90	15	[155]
TOTAPOL	BSA	64	Sedimented	5	<90	23	[156]
TOTAPOL	CsgA fibrils	30	Noncovalent	9.4	Not mentioned	20	[157]
TEMPO	KL ₄	8.9	Spin labeled lipids	14.1	100*	8.9	[168]
MTSL	Kcsa	14.5	MTS chemistry	9.4	100*	9.6	[160]
AMUPOL	KcsA	14.5	MTS tagged	9.4	100*	9.6	[160]
TOTAPOL	Rhodopsin	15	MTS tagged	9.4	102	10	[169]
TOTAPOL	Lipid	8.1	Lipid tagged	9.4	105*	5.4	[162]
Mn (II)	Ribozyme	8	Native complex	9.4	114	N/A	[31]
Gd (II)	Ubiquitin	~3	Metal chelating tag	9.4	114	N/A	[148]
TOTAPOL	Bcl-X _L	18	Protein-binding peptide	14.1	108	18	[170]
TOTAPOL	DHFR	22	Selective ligand	14.1	110–115	22	[149]

For experiments in which the set temperature, without accounting for the effects of sample rotation, microwave irradiation, and NMR pulses, is reported, a * indicates that actual sample temperatures may be higher.