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Arnab Chakraborty
Louisiana State University

Fabien Deligey
Louisiana State University

Jenny Quach
Louisiana State University

Frederic Mentink-Vigier
National High Magnetic Field Laboratory

Ping Wang
LSU Health Sciences Center - New Orleans

See next page for additional authors

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Authors

Arnab Chakraborty, Fabien Deligey, Jenny Quach, Frederic Mentink-Vigier, Ping Wang, and Tuo Wang



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Biomolecular Complex Viewed by Dynamic Nuclear Polarization Solid-State NMR Spectroscopy

Arnab Chakraborty¹, Fabien Deligey¹, Jenny Quach¹, Frederic Mentink-Vigier², Ping Wang³, Tuo Wang^{1,*}

¹Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA

²National High Magnetic Field Laboratory, Tallahassee, FL 32310, USA

³Department of Microbiology, Immunology, and Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

Abstract

Solid-state NMR (ssNMR) is an indispensable tool for elucidating the structure and dynamics of insoluble and non-crystalline biomolecules. The recent advances in the sensitivity-enhancing technique Magic-Angle Spinning Dynamic Nuclear Polarization (MAS-DNP) have substantially expanded the territory of ssNMR investigations and enabled the detection of polymer interfaces in a cellular environment. This article highlights the emerging MAS-DNP approaches and their applications to the analysis of biomolecular composites and intact cells to determine the folding pathway and ligand binding of proteins, the structural polymorphism of low-populated biopolymers, as well as the physical interactions between carbohydrates, proteins, and lignin. These structural features provide an atomic-level understanding of many cellular processes, promoting the development of better biomaterials and inhibitors. It is anticipated that the capabilities of MAS-DNP in biomolecular and biomaterial research will be further enlarged by the rapid development of instrumentation and methodology.

Keywords

solid-state NMR; DNP; cell walls; plants; pathogenic fungi; human cell; polysaccharides; membrane proteins

Introduction

Solid-state Nuclear Magnetic Resonance (ssNMR) spectroscopy has been successfully employed to acquire molecular insights on the structure and dynamics of many biomolecules. Most of these studies are focused on the structural determination of purified or reconstituted biomolecules such as amyloid fibrils, membrane proteins, large protein complex, ion channels and transporters, and nucleic acids [1–9]. Nevertheless, it is technically difficult to conduct high-resolution studies of biomacromolecules in their cellular environments. This limitation is the consequence of two technical issues: the inadequate

*Correspondence: Tuo Wang (tuowang@lsu.edu).

resolution due to the coexistence of many heterogeneous macromolecules and the unsatisfactory sensitivity due to the low concentration of the molecules of interest. In the past decade, the sensitivity-enhancing Dynamic Nuclear Polarization (DNP) technique has been integrated with specific isotope-labeling techniques and spectral editing approaches that efficiently attenuate spectral congestion. This has made it practicable to study protein folding, biopolymer interactions, and ligand binding using intact viruses and intact cells of bacteria, fungi, plants, and humans [10–15]. This review will summarize the technical feasibility of several emerging approaches and their applications to cellular samples as well as bio-composites. We will also elaborate on how structural restraints can be combined to conceptually comprehend the supramolecular architecture of biological constructs, which could facilitate the development of biopolymer-based materials, bio-sourced energy, and novel therapeutic agents.

MAS-DNP sensitivity enhancement enables new research avenues

NMR is a low-sensitivity technique whose signal-to-noise ratios greatly depend on the gyromagnetic ratio of spins. The cutting-edge technique MAS-DNP takes advantage of the several orders of magnitude higher gyromagnetic ratios of electrons over NMR-active nuclei, such as ^{13}C or ^1H , to boost NMR sensitivity [16–18]. An electron source, usually a stable nitroxide mono- or bi-radical, is physically or covalently incorporated into the sample, which allows the polarization of unpaired electrons to be transferred to protons under microwave irradiation (Figure 1A). The sensitivity enhancement factor ($\epsilon_{\text{on/off}}$) is measured by taking the ratio of intensity turning the microwave radiation on to the intensity keeping it off while accounting for the depolarization effects [19]. Nitroxide-based radicals can be chemically reduced in biological samples. As demonstrated by McDermott and colleagues, for cells and lysates at room temperature, less than a quarter of radicals can be retained after a short time of 10 minutes, which corresponds to a reduction rate of 0.18 mmol/(L*min) [20]. The reaction rate is substantially slowed down to 0.12 mmol/(L*min) at a moderately low temperature of 4°C. Because the short lifetime presents a barrier, MAS-DNP experiments are usually conducted at a very low temperature of 90–110 K. The use of cryogenic temperature also increases the signal-to-noise ratios of all NMR spectra following the Boltzmann distribution and improves DNP efficiency by elongating both electron and proton relaxation times [17, 21]. However, this also risks the loss of spectral resolution as a broad distribution of conformations will be trapped when the dynamic components are immobilized.

A careful choice regarding the composition of the glassy matrix (typically a mixture of water with glycerol or DMSO) and the concentration of radicals is essential because MAS-DNP efficiency is influenced by the way that radicals are dispersed in the biological medium [22–24]. A homogenous mixture of d_8 -glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ (60:30:10 vol%) has been widely used as the DNP juice for biomolecular samples. The formation of glassy matrix efficiently avoids the formation of the crystalline phase, thus evading radical aggregation. Multiple water-soluble bi-radicals (with two unpaired electrons), such as TOTAPOL and AMUPol [25, 26], have been widely used (Figure 1B). A recently developed, asymmetric biradical AsymPolPOK has shown meritorious performance: due to a substantial decline in the MAS-DNP buildup time, the absolute sensitivity is doubled when compared with the commercial

radicals (Figure 1 B, C) [27]. The DNP buildup time limits the recycle delays between two scans, which further determines the experimental time. The DNP buildup time depends on the concentration and property of radicals; it typically ranges in the scale of 2–6 seconds when the biomolecules are well mixed with the radicals but can be as long as tens of seconds in some challenging samples [28]. The buildup time is partially controlled by the strength of electron-electron interaction; the stronger as the shorter [29]. AsympolPOK has very strong electron dipolar and exchange interaction, which accounts for the very fast buildup even with a relatively low concentration of biradicals, without affecting other characteristic times [27]. In addition, there are significant efforts in covalently incorporating radicals to biomolecules at the location of interest, which provides efficient polarization of the embedded molecules and specific interaction sites [30, 31]. These efforts have made MAS-DNP a versatile technique for addressing key biochemical questions with structural relevance as discussed below.

Membrane protein and protein-ligand binding

DNP-assisted ssNMR is uniquely capable of analyzing heterogeneous mixtures as it circumvents crystallization or solubilization; however, this method has its own challenges in sample preparation. It often requires chemically modified radicals [32–34] or special procedures to ensure the homogeneous mixing of biomolecules and polarizing agents. For example, because of the limited water accessibility and the impermeability of membranes towards water-soluble biradicals, the enhancement factors achieved on membrane protein samples (those with a reasonably low peptide-to-lipid ratio and a sufficient membrane environment) are typically below 20-fold.

In 2016, an optimized protocol that mixes radicals and membranes by direct titration has resulted in a 40–100-fold of sensitivity enhancement ($\epsilon_{\text{on/off}}$) on a 400 MHz/263 GHz MAS-DNP instrument [22]. This approach has been successfully demonstrated on two ion channels: the influenza A M2 proton channel and an artificial designed protein channel that co-transport Zn^{2+} and H^+ ions [35]. The efficient gain of sensitivity is quantified to approach 100–160-fold by comparing MAS-DNP spectra with those collected at a higher temperature (243 K). This success has been attributed to the bimodal partitioning of radicals in the phospholipid membranes, with a surface-resided portion and a membrane-inserted fraction, which can be distinguished through the paramagnetic relaxation enhancement (PRE) effects of the unpaired electrons of biradicals on the signals of phospholipids [22].

At ambient temperature, quantification of the chemical shift perturbation allows us to locate the ligand-binding sites in proteins [36, 37], but this approach is no longer efficient under MAS-DNP conditions due to the broader linewidth. As a result, innovative strategies have been developed to probe the protein surface and topology for binding cholesterol and carbohydrate-based ligands. In 2013, we have developed a method that relies on differential isotope-labeling (^{13}C on carbohydrate components and ^{15}N , ^{13}C on recombinantly expressed proteins) to determine the binding of a nonenzymatic loosening protein expansin to *Arabidopsis* plant cell walls [12]. DNP helps to overcome the sensitivity barrier imposed by the low functional concentration of this protein, with spectral editing techniques used to detect the protein-bound carbohydrates. Expansins are recruited to the carbohydrate

junctions in which the hemicellulose xyloglucan is entrapped between multiple cellulose microfibrils or several glucan chains of a single microfibril, which turns out to be the polymer nexus being released during cell elongation [12].

Recently, Hong and colleagues have developed a strategy that integrates MAS-DNP with biosynthetic ^{13}C -labeling of cholesterol from the budding yeast *Saccharomyces cerevisiae* to investigate protein-cholesterol binding in lipid bilayers [38]. The yeast strain (RH6829) is genetically modified to produce cholesterol instead of ergosterol, and the cholesterol can be ^{13}C -labeled at alternate carbon sites using either 1- or 2- ^{13}C glucose (Figure 2A). With the sensitivity enhanced by MAS-DNP, two-dimensional (2D) ^{13}C - ^{13}C double-quantum filtered (DQF) spectra have explicitly resolved several cross peaks between the influenza A virus M2 protein and 1- ^{13}C cholesterol in lipid bilayers. These structural constraints were combined with previous findings on the helix orientation and binding stoichiometry [39] to reveal how the M2 protein utilizes its Ile, Leu and Phe sidechains on an annular binding site of the transmembrane helix to bind cholesterol asymmetrically through methyl-methyl and CH- π interactions (Figure 2B). These findings provide insights into the underlying mechanisms through which M2 proteins interact with membrane components, promote membrane curvatures [40–42], and facilitate the membrane scission process during viral budding and release [43].

There are tremendous efforts to covalently link mono- or bi-radicals to proteins or membranes, which, by expectation, should provide better DNP efficiency and site-specificity. Spin-labeled phosphocholine (PC) lipids have been used as the DNP polarizing agents and the constituent molecules of lipid bilayers [30, 44]. The mono-radicals are tethered to the lipids at multiple sites, including the phosphate headgroup (TEMPO-PC), the middle segments (5-Doxyl PC and 7-Doxyl PC), and the terminal part of acyl chains (16-Doxyl PC). Consequently, efficient and homogenous polarization has been observed across the lipid bilayers and to membrane-embedded peptides such as a lung surfactant mimetic peptide KL₄ inserted in these membranes. In addition, site-directed incorporation of polarizing agents has also been demonstrated on the potassium channel KcsA, the antibiotic gramicidin, and the sensory rhodopsin [45–47]. Targeted DNP has also been used to investigate the interaction between a radical-labeled ligand with a 20 kDa protein (Bcl-x_L) at a low concentration in crude cell lysates [48].

Because radicals are paramagnetic species, spins in their spatial vicinity experience faster NMR relaxation, which leads to line broadening and intensity suppression [49, 50]. The signal quenching, also called paramagnetic bleaching, can be quantified when the radical is directly bound to a protein (Figure 2C). This technique has been applied to determine the distance of a combined ligand-radical to a reductase in *E. coli* bacteria [51]. A comparison of the DNP spectra collected on two samples, one with a radical bound to the protein with high affinity and the other with the solvent-bearing AMUPol as a reference, has pinpointed the protein surface that is responsible for binding the target (Figure 2D).

Signal bleaching has also been employed to improve resolution when probing protein-ligand binding. This process has been successfully achieved by functionalizing the ligand (for example, carbohydrates) with a polarizing agent (TOTAPOL) covalently linked through a

phenylglycine linker (Figure 2E) [31]. The conceptual setup is consistent with the studies of the *E. coli* reductase as discussed above, with additional assistance from difference spectroscopy [52, 53] in which the spectrum measured using the paramagnetically tagged sample is subtracted from a reference spectrum measured on a sample only containing radicals homogeneously distributed in the solvent. A selective factor k is applied to the spectrum of the paramagnetically tagged sample before spectral subtraction, which renders selective DNP mimicking an atomic-resolution microscope with adjustable magnification: only the tightly bound residues ($<10 \text{ \AA}$) can be observed in the difference spectrum when $k=1$ is applied (Figure 2F), and the observable region gradually expands to 30 \AA with a decreasing k value. This method has been applied to the study of a galactose-specific lectin LecA [31]. The spectral subtraction provides unprecedented resolution for unambiguously locating the carbohydrate-binding spots on LecA (Figure 2G). This method requires no prior knowledge of the binding site and has no limitation on the protein size.

Protein structure in cellular fractions and intact cells

The magnificent sensitivity has made DNP a suitable tool for studying highly diluted biomolecules in cellular fractions or whole cells, which are otherwise “invisible”. In 2015, the type IV secretion system core complex (T4SScc), a megadalton protein complex, has been investigated in the cell envelope fractions of *E. coli* [54]. In the cellular system, this protein maintains its correct folding and assembles into a structure that is consistent with the X-ray crystallography results. Similarly, a protocol has been established to study protein folding in cellular lysates, which combines different labeling schemes to produce a sample containing NMR-active prion proteins and an NMR-silent cellular environment (Figure 3A) [13, 55]. The technique was employed to determine the folding of an intrinsically disordered region of the yeast prion protein Sup35. The Sup35 fibrils were found to be restructured and different from *in-vitro* templated assemblies [13].

Intact human cells have remained as a challenging system for DNP ssNMR, especially for the optimization of radicals and experimental conditions. In 2019, an original protocol has been designed to examine protein structures in mammalian cells. This approach comprises of three steps: isotope labeling the protein of interest, delivery of the protein into cells by electroporation techniques followed by a cell stimulus, and the introduction of biradicals for DNP measurement [56]. The cell integrity and biradical distribution have been simultaneously monitored with microscopy techniques. It is found that the model protein ubiquitin has remained correctly folded after delivery into the HeLa cell [56]. In the same year, another study has quantified the chemical reduction effect of nitroxide biradicals in *E. coli* pellets, suspensions, and lysates. Treatment of the cell using N-ethylmaleimide could neutralize pools of redox-active cysteines, suppress nitroxide reduction, and prolong the lifetime of radicals while adding potassium ferricyanide effectively re-oxidizes the reacted radicals back into their active state [20]. In 2018, a trimodal polarizing agent TotaFAM has been introduced, which contains a biradical, a targeting cell-penetrating peptide, and a fluorophore for tracking the localization of radicals in the cell (Figure 3B) [14]. The radical uptake is efficient in HEK293F cells (Figure 3C) and a high enhancement of 63-fold of the cellular signals was achieved using a low radical concentration (2.7 mM). In comparison, commercial radicals require a much higher concentration (20 mM) to reach a comparable

performance (Figure 3D). These ground-breaking advances have paved the way for understanding the molecular structure, functional mechanisms, and drug inhibition [57] of protein machinery and other biomolecules within their cellular environments.

Biopolymer packing in fungal and plant cell walls

There is a growing interest in characterizing cell walls of plants and microbes because these protective armors are the resources of new energy and the targets of antimicrobial therapeutic compounds [58–60]. During the past years, many organisms have been investigated using ssNMR, including the cell walls of many plants, pathogenic fungi, microalgae, and bacteria [61–75]. The highly rigid and semi-crystalline components, for example, cellulose microfibrils in plants and chitin in fungi, are capable of retaining decent resolution under cryogenic conditions [11, 12, 76], which has made the carbohydrate-rich cell walls a preferred system for DNP investigations. In addition, radicals mainly accumulate in the cell walls due to a high binding affinity to the carbohydrate components such as the peptidoglycan in bacteria and cellulose in plants [77–79]. We can either selectively detect the cell wall molecules using a low concentration of radicals (for example, 5 mM) or observe other cellular components by bleaching the cell wall signals using a saturated concentration of radicals (for example, 60 mM) [79]. Moreover, NMR fingerprints of the highly polymerized polysaccharides in the cell wall are uniquely different from those of intracellular and metabolic carbohydrates or other molecules (e.g. proteins and nucleic acids) [23]; therefore, cell walls are spectroscopically distinguishable from other cellular components.

Recently, we have been elucidating the structural organization of cell walls in several fungal pathogens, starting from a model fungus *Aspergillus fumigatus* [11] and progressively outspreading to other yeasts as well as molds. A 30-fold of sensitivity enhancement ($\epsilon_{\text{on/off}}$) allows us to highlight the highly polymorphic nature of biomolecules in intact fungal cell walls and efficiently probe their sub-nanometer packing. Despite its low abundance (~10% of the dry mass of *A. fumigatus* cell walls), chitin exists in three major forms as shown by the peak multiplicity of 2D ^{15}N - ^{13}C correlation spectra (Figure 4A). These signals deviate from the chemical shifts of model chitin crystallites from fungi or other sources [80, 81]. This unexpected level of structural polymorphism has been attributed to the complicated patterns of hydrogen-bonding (through the amide and carbonyl groups) that form parallel, anti-parallel, and mixed ways of packing in chitin microfibrils [82, 83]. The different forms are extensively mixed in individual microfibrils as evidenced by inter-form correlations using the 2D ^{15}N - ^{15}N Proton Assisted Recoupling (PAR) experiment [84–86]. When associated with difference spectroscopy, MAS-DNP has increased both spectral resolution and resolution so that many chitin-glucan interactions can be identified, unveiling a mechanical framework of tightly associated chitin and α -1,3-glucans. This is a novel feature that had never been discovered before [11]. Integrated with the conventional NMR data collected at room temperature, the chitin- α -1,3-glucan scaffold is further found to reside in a soft matrix of β -glucans and capped by a glycoprotein-rich shell [11].

When applied to the plant secondary cell walls, MAS-DNP is employed to probe the physical contacts between multiple polysaccharides and the aromatic polymer lignin, which

is a polymer interface with a low occurrence. Assisted by dipolar and frequency filters as well as a mechanical shutter that regulates microwave on the millisecond timescale [87], we have cleanly selected the aromatic signals from lignin and further determined the composition of polysaccharides in the vicinity. Contradictory to the prevailing knowledge, cellulose is found to lack interactions with lignin in maize stems because the signals from the internal and surface glucan chains in the microfibrils are either missing or weak (Figure 4B) [10]. The polysaccharide interactor of lignin is found to be the hemicellulose xylan, which relies on its twisted 3-fold conformers (3 residues per helical turn) to bind lignin and uses the 2-fold flat-ribbon domains to bind cellulose microfibrils [10]. The molecular information of the lignin-carbohydrate interface provides an understanding of the polymer interactions underlying the nanoscale architecture of this bio-complex, which has revised the structural concepts of lignocellulosic biomass. In addition, MAS-DNP has been employed to screen the carbohydrate and lignin constituents of poplar and its genetic variants following chemical treatments, which will aid the improvement of biomass conversion technology [88, 89].

Beyond these studies, there are many other DNP investigations focused on complex biosystems, for example, the DNA and coat proteins of the filamentous phage Pf1 in an intact virus, the supramolecular assembly of HIV capsid, the peptidoglycans of *Bacillus subtilis* bacterial cell walls, the nucleic acids in bones, the post-translational collagen modification in muscle cell extracellular matrix, and the interfaces of biominerals [15, 79, 90–96]. The scope of MAS-DNP should be substantially broadened by high-field DNP that provides better resolution, the natural-abundance technique that eliminates the need for isotope-enrichment, and the analytical software for spectral and structural comparisons [97–101]. These efforts have the potential for revolutionizing biomolecular and biomaterial research.

Perspectives

- **Importance of the field:** MAS-DNP has vastly broadened the horizon of solid-state NMR spectroscopy and enabled the atomic-level view of polymorphic biomolecules in their native cellular environments. The structural insights of protein machinery and structural carbohydrates also provide an in-depth understanding of many cellular processes to guide the development of functional biomaterials, bio-renewable energy, and novel inhibitors.
- **Summaries of the current thinking:** The molecular structure and binding interactions of many carbohydrate- or protein-based biopolymers have been successfully investigated using biomolecular mixtures, cellular fractions, and intact cells. Due to the complex nature of cellular polymers, MAS-DNP investigations should be coupled with selective labeling or site-directed polarization technology to efficiently alleviate spectral congestion.
- **Future directions:** The rapid development of high-field and fast-MAS DNP as well as natural-abundance approaches have established a new avenue of biochemical research, especially for the complex biosystems with a high demand for resolution or the biomedical samples that are difficult to replicate *in vitro*.

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Abbreviations used:

MAS	magic-angle spinning
ssNMR	solid-state Nuclear Magnetic Resonance
MAS-DNP	magic-angle spinning dynamic nuclear polarization
PRE	paramagnetic relaxation enhancement

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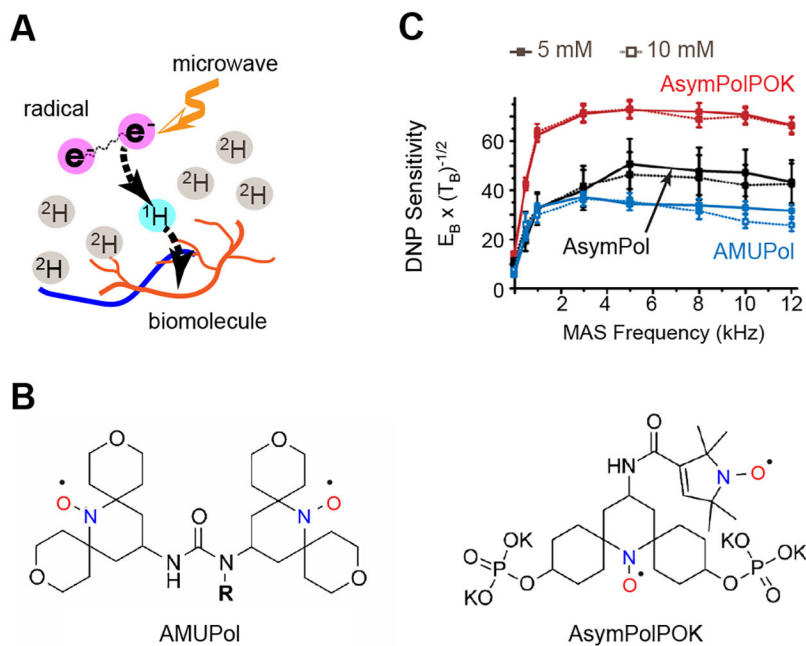


Figure 1. MAS-DNP technique boosts NMR sensitivity.

(A) Illustration of the MAS-DNP mechanism. (B) Representative structure of two bi-radicals, AMUPol and AsymPolPOK. (C) Enhancement of the sensitivity of spectra collected on ^{13}C -urea at a variety of magic-angle spinning frequencies at 9.4 T and 105 K using three different bi-radicals, including AMUPol, AsymPolPOK, and AsymPol at two different concentrations (5 and 10 mM). The MAS-DNP sensitivity is quantified as the signal intensity per unit square root of time. Figures 1B and 1C are adapted from reference [26] with copyright permission and reference [27] (an open-access article).

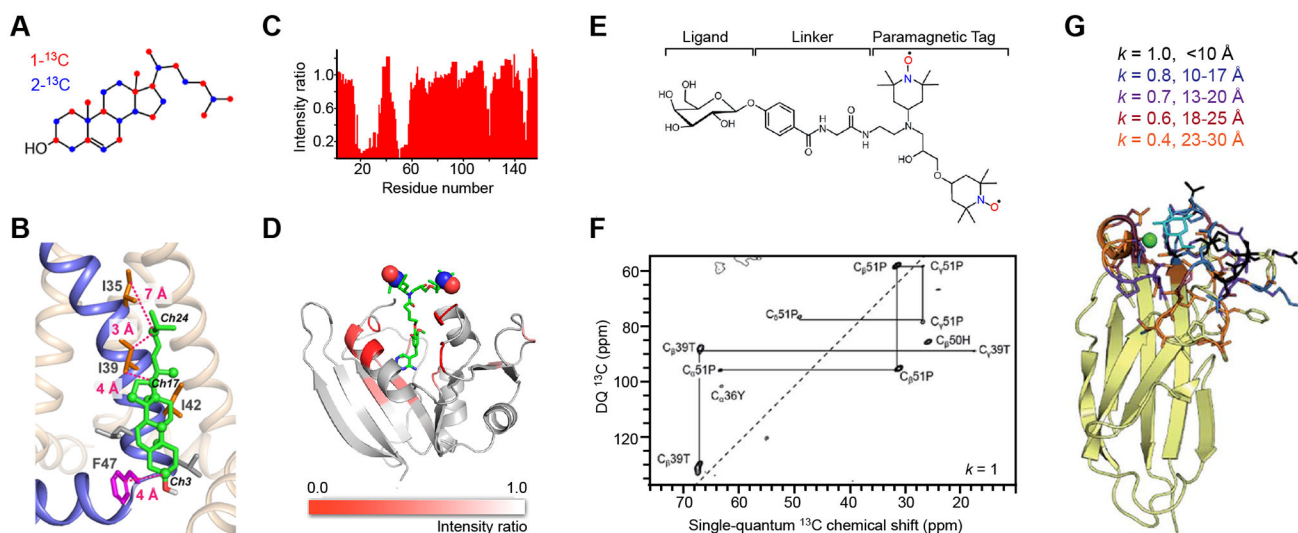


Figure 2. MAS-DNP methods for probing protein-ligand binding.

(A) Yeast-based ^{13}C labeling of cholesterol using site-specifically ^{13}C -labeled glucose. The labeled carbon sites on cholesterol are in red and blue for cholesterols produced from 1- ^{13}C and 2- ^{13}C glucose molecules, respectively. (B) A structural model of a cholesterol molecule bound to the influenza M2 proteins. The key Ile and Phe residues, as well as their distances to cholesterol carbons, are shown. (C) Signal bleaching quantified in solution ^1H - ^{15}N HSQC spectra due to the binding of radicals to dihydrofolate reductase. (D) A model of *E. coli* dihydrofolate reductase with DNP bleaching information represented by the intensity ratios of ^{13}C - ^{13}C DARR spectra collected on two samples containing either bound radicals or exogenous radicals. (E) Scheme for incorporating a carbohydrate ligand to a paramagnetic tag for selective DNP. (F) Selective DNP ^{13}C - ^{13}C INADEQUATE difference spectrum of LecA obtained using $k=1$: only the tightly bound residues are observed. (G) Sideview of LecA. Residues observed using selective DNP are highlighted, with the corresponding k values given. Figures 2A-2D are adapted from references [38, 51] with copyright permission. Figures 2E-2G are adapted from reference [31], an open-access article.

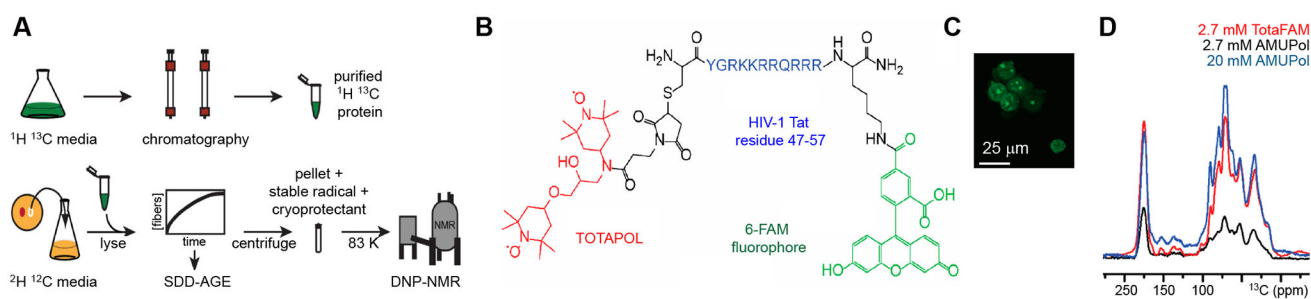


Figure 3. Cellular MAS-DNP of protein structure.

(A) Preparation of proteins at endogenous levels for MAS-DNP in biological environments.

(B) Chemical structure of the trimodal polarizing agent TotaFAM. **(C)** A fluorescent image

confirms the cellular uptake of TotaFAM. **(D)** $1\text{D } ^{13}\text{C}$ spectra of HEK293F cells at $<6\text{ K}$

using different radicals. Figures are adapted from references [13, 14] with copyright

permission.

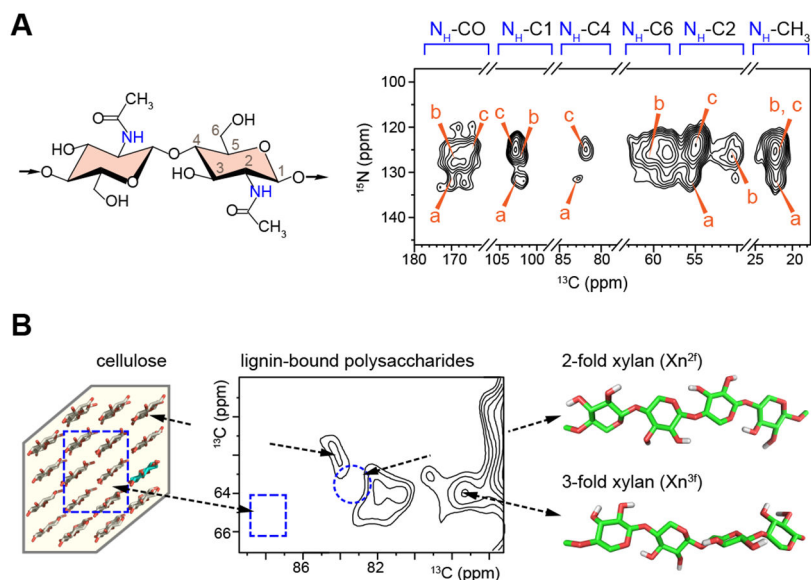


Figure 4. Polysaccharide structure and polymer binding in plant and fungal cell walls. (A) Representative structure and MAS-DNP spectra of chitin in cell walls of intact *A. fumigatus*. Three major types of chitin signals have been resolved (Types a-c). (B) The aromatic-edited spectrum of maize stems shows the signals of lignin-bound carbohydrates. Arrows and black dotted lines connect the spectral regions with polysaccharide structures. The dashline circle and rectangle on the spectrum highlight the missing signals of the carbohydrate components that are far from lignin. Figures are adapted from references [10, 11], which are open-access publications.