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High Frequency Dynamic Nuclear Polarization

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Conspectus

During the three decades 1980–2010, magic angle spinning (MAS) NMR developed into the method of choice to examine many chemical, physical and biological problems. In particular, a variety of dipolar recoupling methods to measure distances and torsion angles can now constrain molecular structures to high resolution. However, applications are often limited by the low sensitivity of the experiments, due in large part to the necessity of observing spectra of low-nuclei such as the $I = 1/2$ species ^{13}C or ^{15}N . The difficulty is still greater when quadrupolar nuclei, like ^{17}O or ^{27}Al , are involved. This problem has stimulated efforts to increase the sensitivity of MAS experiments. A particularly powerful approach is dynamic nuclear polarization (DNP) which takes advantage of the higher equilibrium polarization of electrons (which conventionally manifests in the great sensitivity advantage of EPR over NMR). In DNP, the sample is doped with a stable paramagnetic polarizing agent and irradiated with microwaves to transfer the high polarization in the electron spin reservoir to the nuclei of interest. The idea was first explored by Overhauser and Slichter in 1953. However, these experiments were carried out on static samples, at magnetic fields that are low by current standards. To be implemented in contemporary MAS NMR experiments, DNP requires microwave sources operating in the subterahertz regime — roughly 150–660 GHz — and cryogenic MAS probes. In addition, improvements were required in the polarizing agents, because the high concentrations of conventional radicals that are required to produce significant enhancements compromise spectral resolution.

In the last two decades scientific and technical advances have addressed these problems and brought DNP to the point where it is achieving wide applicability. These advances include the development of high frequency gyrotron microwave sources operating in the subterahertz frequency range. In addition, low temperature MAS probes were developed that permit in-situ microwave irradiation of the samples. And, finally, biradical polarizing agents were developed that increased the efficiency of DNP experiments by factors of ~4 at considerably lower paramagnet concentrations. Collectively these developments have made it possible to apply DNP on a routine basis to a number of different scientific endeavors, most prominently in the biological and material sciences. This Account reviews these developments, including the primary

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mechanisms used to transfer polarization in high frequency DNP, and the current choice of microwave sources and biradical polarizing agents. In addition, we illustrate the utility of the technique with a description of applications to membrane and amyloid proteins that emphasizes the unique structural information that is available in these two cases.

Keywords

DNP; solid state NMR; magic angle spinning

Introduction

Magic angle spinning (MAS) nuclear magnetic resonance (NMR) has emerged as a powerful, nondestructive method that can be used to characterize the structure and dynamics of systems that are not accessible by either solution NMR or crystallography. In particular, the last three decades have witnessed the development of MAS techniques to probe various anisotropic interactions at the molecular and atomic scale via dipole recoupling techniques¹. As a consequence, it is possible to measure internuclear distances in amorphous and powder samples as well as in crystals. In principle, these measurements provide copious high resolution information about the structure and dynamics of a variety of biological systems such as peptides², membrane proteins^{3–5}, nanocrystals⁶, amyloids^{7–10}, and materials science. Given this versatility, the recent rapid expansion of MAS NMR is expected to continue.

Despite the outstanding progress in this field, there remains an acute sensitivity problem since MAS NMR usually involves direct detection of ¹³C, ¹⁵N or another low- γ species. Cross polarization (CP) techniques and operation at higher magnetic fields have helped to address this issue. However, significantly higher sensitivity would help to bring MAS NMR into a regime where it is truly widely applicable. The subject of this Account is recent 10²–10³ fold improvements in MAS NMR sensitivity based on high frequency dynamic nuclear polarization (DNP). As we will see, high frequency DNP is significantly changing the landscape of what is possible with MAS NMR. This article illustrates this point with a discussion of polarization transfer mechanisms, polarizing agents, instrumentation, and recent applications of MAS DNP to complex heterogeneous systems.

DNP Mechanisms

DNP enhances NMR signals by transferring the large polarization of electrons to nearby nuclei via microwave (μ w) irradiation of electron-nuclear transitions¹¹. Contemporary MAS DNP experiments on *insulating* solids are usually based either on the solid effect (SE), coupling an electron-nuclear spin pair, or the cross effect (CE), utilizing a pair of electrons in the form of a biradical and a nuclear spin. A third mechanism, thermal mixing (TM), involves multiple electrons and a homogeneously broadened EPR spectrum. However, at the high fields and low temperatures (80–110 K) currently used in MAS experiments, TM has thus far not provided an important polarization pathway. In all of these mechanisms it is necessary to add a stable paramagnetic polarizing agent to the sample and the most commonly used radicals are shown in Figure 1. Trityl and BDPA (or water soluble BDPA¹²) support the SE, whereas the TEMPO based biradicals TOTAPOL¹³ and bTbk¹⁴ are used for the CE. The detailed polarization transfer schemes discussed below are closely linked to the shapes of the high field EPR spectra of these molecules.

The Solid Effect

The SE can be understood using a two-spin model involving one electron and one nucleus, interacting via an electron-nuclear dipole coupling, and irradiation at nominally forbidden electron transitions at $\mu_w = \omega_S \pm \omega_I$ illustrated in Figure 2. The Hamiltonian applicable to the two spin system is

$$\hat{H} = \omega_{oS} \hat{S}_z - \omega_{oI} \hat{I}_z + C \hat{S}_z \hat{I}_+ + C^* \hat{S}_z \hat{I}_-$$

where ω_S and ω_I are the electron and nuclear Larmor frequencies, respectively, $C = (-3/2)(\gamma_S \gamma_I / r_{IS}^3) \sin\theta \cos\theta e^{-i\theta}$ is the usual term in the electron-nuclear dipole Hamiltonian¹⁵ and $\hat{S}$ and $\hat{I}$ are spin operators for electrons and nuclei, respectively. First order perturbation theory yields the mixed eigenstates shown in the figure where the mixing coefficient $q = C / \omega_I \ll 1$. The other terms in the electron-nuclear dipolar Hamiltonian (A , B , E and F in Van Vleck notation) also mix states, but the contributions are relatively small. Irradiation of the partially allowed transitions by μ_w 's gives rise to either positive (double quantum) or negative (single quantum) enhancement of the nuclear polarization as illustrated at the bottom of Figure 2.

The SE is the dominant DNP mechanism in systems where the polarizing agent exhibits a homogeneous EPR linewidth ($\Delta\nu$) and an inhomogeneous spectral breadth ($\Delta\nu_{inh}$) smaller than the nuclear Larmor frequency (ω_I , $\Delta\nu, \Delta\nu_{inh} < \omega_I$). This condition is satisfied by radicals with high molecular symmetry such as BDPA¹⁶, SA-BDPA¹², and OX063¹⁷, where the g tensors are nearly isotropic and the hyperfine interaction is small. However, as the SE relies on the mixing of nuclear states by electron-nuclear coupling, the enhancement scales as ω_{oI}^{-2} . Therefore, the SE becomes less efficient at high magnetic fields (> 3 T). Nevertheless, recent research suggests that SE can be very efficient at high field provided that high microwave power and a large $\beta_{1S} = eB_{1S}$ is available. Enhancements as high as ~ 144 have been achieved at 5 T. Finally, the SE can also be the dominant mechanism when transition metals complexes, e.g. with Gd^{3+} , are used as polarizing agents. Since the broadening of the EPR line in these systems is mainly induced by the zero field splitting, the EPR line narrows at higher magnetic fields and metal-based polarizing agents may show improved performances at higher fields¹⁸.

The Cross Effect

When $\omega_I > \omega_S$, DNP is governed by the CE and scales with ω_{oI}^{-1} , leading to larger enhancements at higher magnetic fields. At highs, where the EPR spectrum is dominated by field g -anisotropy and inhomogeneously broadened, a three spin quantum mechanical treatment is possible^{19–21}. The Hamiltonian for the nuclear spin and two electrons is

$$\hat{H} = \omega_{oS_1} S_{1z} + \omega_{oS_2} S_{2z} - \omega_{oI} I_z + (A_1 S_{1z} + A_2 S_{2z}) I_z + (B_1 S_{1z} + B_2 S_{2z}) I_x + d \left(3 S_{1z} S_{2z} - \vec{S}_1 \cdot \vec{S}_2 \right) - 2 J \vec{S}_1 \cdot \vec{S}_2$$

where the first three terms represent electron and nuclear Zeeman interactions, the fourth and fifth describe the electron-nuclear coupling (with A and B denoting the secular and pseudosecular hyperfine couplings²²), the sixth represents the electron-electron dipolar coupling and the last describes exchange coupling. This leads to the energy level diagram for the CE is shown in Figure 3, and electron-electron-nuclear polarization transfer is maximized when the central energy levels are degenerate. This occurs when the matching

condition $|\omega_{S1} - \omega_{S2}| = \omega_I$ is fulfilled, where ω_{S1} and ω_{S2} are the Larmor frequencies of dipolar coupled electrons S_1 and S_2 . The degeneracy leads to saturation of the four connected levels and enhanced nuclear polarization. A field profile obtained from bTbk is shown at the bottom of Figure 3, and roughly represents the negative 1st derivative of the EPR spectrum. Also shown are the positions in the EPR powder pattern that are irradiated for optimal positive and negative enhancements.

Initially, high-field CE DNP experiments were performed with mono-radical species, such as TEMPO^{23,24}. In this situation, the frequency matching condition is fulfilled only for the fraction of the radicals that adopt the correct relative orientation of their g-tensors. In order to improve CE DNP, we introduced biradicals such as bis-TEMPO-n-ethylene glycol (BTnE)¹⁹ and TOTAPOL¹³, consisting of two tethered TEMPO moieties to obtain relatively short (~ 12 Å) electron-electron distances independent of concentration. With these polarizing agents, which have an e-e dipole coupling of 20–30 MHz, the enhancements were ~ 4 -fold higher at an ~ 4 -fold lower e concentration. Figure 4 shows recent results obtained using TOTAPOL from two standard samples, urea and proline. The observed $\rho = 181$ and $\rho = 134$ are ~ 2 -fold higher than we initially reported for TOTAPOL at this field²⁵ due to improvements in instrumentation – primarily gyrotron output power and lower temperatures (*vide infra*).

Optimizing DNP Signal Enhancements

DNP enhancements are governed by a number of factors, including microwave power, concentration and design of the polarizing agent, temperature, solvent, and the relaxation times of the solvent and solute. We now review recent results aimed at optimizing the efficiency of DNP experiments with a focus on the influence of these parameters on the enhancements.

Microwave power

Gyrotrons^{16,26,27} are capable of producing 10's of watts of μ w power with excellent frequency stability and low phase noise, making them the current microwave source of choice for DNP experiments. In particular, the low Q of the microwave circuit in the MAS NMR probe necessitates copious power to generate a sufficient B_1 to excite DNP transitions. Furthermore, since the gyrotron is a fast wave device, it can operate at high powers for extended periods of time, as is required for multidimensional NMR experiments that involve signal averaging. Figure 5 (left) shows the enhancement as a function of μ w power at 80 K obtained with a frequency tunable 250 GHz gyrotron²⁸. The enhancement increases with power, does not saturate at our maximum available power of 12.5 W, and extrapolates to a limiting value $\rho_{\max} \sim 240$. Similar dependences of ρ on μ w power have been published elsewhere.^{29,30} An alternative microwave source, that we explored sometime ago and currently in use in some labs³¹, is a low power (~ 10 – 100 mW) Gunn diode. However, the enhancements are lower: on one sample, $\rho \sim 25$ with 10 mW from the Gunn diode versus $\rho = 185$ with 1 W from the gyrotron³². Thus, the data in Figure 5 suggest that with current technology the gyrotron is the microwave source of choice for DNP experiments, especially at microwave/ ^1H NMR frequencies ~ 400 MHz/263 GHz for $^1\text{H}/e$. Currently gyrotron-based DNP spectrometers are operating at microwave/ ^1H NMR frequencies up to 460 GHz/700 MHz²⁹ and are expected to go still higher. Nevertheless, microwave technology does improve with time, and it is possible that alternatives to the gyrotron and Gunn diode will be available in the future.

Temperature and polarizing agents and

Both the sample temperature and the nature of the polarizing agent profoundly influence the DNP enhancements. Lower temperatures improve both the SE and CE enhancements, most

likely due to longer electron and proton relaxation times. Figure 5 (*right*) plots recent data showing the temperature dependence of the ^1H DNP signal enhancement in the range 80–140 K at 250 GHz/380 MHz with the TOTAPOL/urea sample described above. Note that the DNP enhancement increases as the temperature approaches 80 K, by a factor of 3.6 in the range 110 to 80 K.

It is well known that high concentrations of paramagnets dramatically broaden NMR linewidths and attenuate integrated NMR signal intensities. It is for this reason that we developed biradical polarizing agents with a ~20–30 MHz intramolecular e-e dipole coupling which yield ~4-fold larger enhancements at ~4-fold lower electron concentration than monoradicals such as TEMPO¹⁹. Our experiments performed on urea, proline, bacteriorhodopsin (bR), and PI3-SH3 fibrils^{33,34} suggest that optimal radical concentration of TOTAPOL is 10–20 mM.

Finally, we note that biradicals such as bTbk¹⁴ (Figure 1) and bTbk-py³⁵ have the TEMPO moieties locked at ~90° with respect to one another, and therefore yield a relative orientation of the two g-tensors that better satisfies the CE matching condition. These polarizing agents have produced enhancements as large as 250 (Figure 1)¹⁴.

Some time ago, some of us reported that ^2H labeled solvents improve ^1H DNP enhancements, and that even with 90–95% ^2H labeling we can still efficiently CP to low abundance nuclei in the target molecule^{19,36}. Thus, while ^1H dilution attenuates relaxation processes, even dilute protons mediate ^1H - ^1H spin diffusion, with the overall result of a higher enhancement. For example, Akbey et al.³⁷ reported that perdeuteration of the spectrin-SH3 domain led to three to five times higher DNP enhancement (~148) than obtained with protonated SH3. In a more recent example we prepared 98% perdeuterated U- $[\text{}^2\text{H}, \text{}^{13}\text{C}, \text{}^{15}\text{N}]$ bR and with 15 mM TOTAPOL and observed $\epsilon = 72$, whereas for U- $[\text{}^1\text{H}, \text{}^{13}\text{C}, \text{}^{15}\text{N}]$ -bR we obtained ~35–43^{33,38}. Thus, perdeuterated proteins will likely be important for biological applications of DNP.

Applications of DNP MAS NMR

To date one of the most interesting examples of the application of DNP is to the light-driven ion pump bR, which is a 26.6 kDa trans-membrane protein containing a retinal chromophore. bR has been studied intensively since its discovery in the 1970's, but the mechanism by which it enforces vectorial action is still not understood and MAS NMR studies can potentially elucidate the relevant structural details of the intermediates in its photocycle (Figure 6 (*left*)). However, many of the intermediates can only be cryo-trapped at low (~5%) concentrations, so that high signal-to-noise, and therefore DNP, is required to observe their MAS NMR spectra^{33,38}.

The retinal cofactor is covalently bonded to Lys216 via a protonated Schiff base linkage, and the sensitivity of the unique ^{15}N chemical shift of the Schiff base to its local environment provides an excellent marker and probe of each photocycle intermediate. In the dark-adapted (DA) state, bR exhibits two conformations: bR₅₅₅ and bR₅₆₈ in a ratio of 60:40 (Figure 6 (middle, A)). After irradiation at 532 nm, DA is converted to light adapted (LA) state in which only bR₅₆₈ remains (Figure 6 (middle, B)). Upon the absorption of a photon, the retinal isomerizes and cycles the protein through several intermediates that can be cryo-trapped for observation *in situ*. Figure 6 (middle, C) shows the 1D spectrum of M_0 . With low temperature DNP it is possible to perform 2D spectroscopy and a ^{13}C - ^{15}N spectrum of DA bR is shown in Figure 6 (*right*) showing that at 90 K there are actually four forms of bR present – two each of the bR₅₅₅ and bR₅₆₈.

We have also published the first DNP MAS NMR spectra of the K and L intermediates^{33,38}. While the K state showed just one Schiff base signal, it relaxed to several L states, of which all but one are dead ends (relaxing back to bR). The data for the functional L state (the one that relaxes to M) suggest that its Schiff base has a strong counterion. One of the possible explanations would support the hypothesis that bR is an inward OH⁻ pump, rather than an outward H⁺ pump.

With the sensitivity available from DNP, it is also possible to record 3D correlation spectra for the individual resonances in the DA state which has an effective molecular weight of ~ 85 kDa. Figure 7 shows cross-peaks between the Schiff base ¹⁵N and ¹³C-12,13,14,15,20 of the retinal and ¹³C- of Lys216 in the dark adapted state of bR. The experiment has been conducted by using a Gaussian pulse to select the signals arising from the ¹⁵N of Lys216 in both bR₅₆₈ and bR₅₅₅ followed by a ¹⁵N-¹³C and then ¹³C-¹³C diffusion via RFDR mixing. Again the spectrum of bR would not be accessible *sans* DNP.

Amyloid fibrils

MAS NMR is also essential for studies of the structure of amyloid fibrils. Intermolecular ¹³C-¹³C or ¹⁵N-¹³C distances derived from MAS DNP experiments provide otherwise unavailable structural constraints. The most straightforward approach is to measure long-range ¹³C-¹⁵N distances with a ZF-TEDOR experiment⁴⁰. However, for distances ~ 5 Å, the efficiency is low (< 5%), which vastly extends the acquisition time and severely limits the number of constraints that can be observed. The application of DNP to overcome this situation has been demonstrated on mixed samples of [¹⁵N, ¹²C] PI3-SH3 / [¹⁴N, ¹³C] PI3-SH3 (50:50 molar ratio). Figure 8 compares the ¹⁵N-¹³C intermolecular correlation spectra obtained with ZF-TEDOR recoupling ($t_{mix} = 16$ ms) at 750 MHz without DNP and at 400 MHz with DNP, collected in 16 days and 32 hours respectively. The number of intermolecular ¹⁵N-¹³C constraints detected was more than doubled due to the DNP with ~ 30 on ¹³C. The additional constraints obtained from DNP permitted us to establish that the PI3-SH3 protein strands are aligned in a parallel and in-register β -sheet arrangement³⁴.

In addition, it is clear that the approaches described here are widely applicable to other areas of science, in particular materials problems – polymers, zeolites, surfaces, semiconductors, etc. These experiments will likely include spectroscopy of quadrupolar species such as ¹⁷O⁴¹ and ²⁷Al⁴² as well as I=1/2 species. We refer the interested reader to other articles in this issue for a complete discussion of these very interesting applications.

Finally we note that, while most of the results described here were obtained at 250 GHz/380 MHz or 263 GHz/400 MHz, DNP experiments have recently been performed at 460 GHz/700 MHz²⁹ and at 395 GHz/600 MHz and 527 GHz/800 MHz [<http://www.bruker.com/products/mr/nmr/dnp-nmr/overview.html>]. Thus, DNP is rapidly moving to higher frequency where the chemical shift resolution will improve and additional systems will become accessible.

Conclusions

There are currently two important mechanisms that mediate high field DNP processes, namely the SE and the CE. In addition, there are a number of important experimental factors that influence the magnitudes of the enhancements, including microwave power, temperature, and the nature of the polarizing agent. With currently available technology — gyrotron microwave sources, MAS at 80 K, biradical polarizing agents, and partially deuterated proteins — it is possible to obtain enhancements of ~ 100 on many samples. This enhancement, together with the improved Boltzmann factor of 300 K/80 K=3.75 due to the

lower temperature, yields sensitivity gains of 375 and time savings of $>10^5$. Historically, increases in sensitivity of NMR experiments by factors of 10^2 – 10^3 have dramatically changed the landscape of what is possible with NMR, and we are beginning to witness the next step in this movement due to high frequency DNP. We have illustrated this point with applications of MAS DNP experiments to membrane proteins and fibrils which are typical of the biological materials that will be studied in the future. These results clearly illustrate that many experiments that are not possible *sans* DNP, become feasible *avec* DNP. Thus, it is clear that the increased availability of commercial instruments to perform DNP experiments will open many new avenues of scientific and technical endeavor.

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Figure 1. Polarizing agents commonly used for high field DNP experiments. (a) narrow line radicals trityl and BDPA used for the SE; (b) TEMPO based biradicals TOTAPOL and bis-TEMPO-bis-ketal (bTbk) used for the CE.

Figure 2.

(top) Energy level diagram illustrating DNP via the solid effect (SE). At thermal equilibrium (left), populations of the same electron spin subspaces are governed by the Boltzmann distribution. Mixing of states in the nuclear and electron spin subspaces (right), leads to partially allowed double quantum (DQ) and zero quantum (ZQ) transitions, and positive and negative enhancements, ϵ , respectively. The mixing of states is proportional to a constant q , which is inversely proportional to B_0 . Therefore, the enhancement in the Solid Effect DNP scales as B_0^{-2} . (bottom) A plot of the enhancement from SA-BDPA¹² as a function of magnetic field (^1H frequency) showing the positive and negative enhancements. ω_{NMR} and ω_{EPR} are the NMR and EPR frequencies and $\omega_e \pm \omega_n$ are the sum and difference of the EPR and NMR frequencies.

Figure 3.

(*top*) Energy diagram illustrating DNP via the CE. At equilibrium (left), under the matching condition, there is degeneracy and 1:1 population of the two shaded levels. The EPR spectrum of an ideal biradical for CE (middle) has two narrow lines separated by the nuclear Larmor frequency. Saturation of transitions near the first (second) EPR line gives rise to a positive (negative) DNP enhancement (right). (*bottom*) Field profile for bTbk with an enhancement $\varepsilon = 230$ ¹⁴

Figure 4.

^{13}C CP MAS NMR spectra of (A) 1M U- ^{13}C - ^{15}N urea and (B) 0.5 M U- ^{13}C - ^{15}N proline at 80 K with and without microwave irradiation. The DNP enhancements are ≈ 181 and ≈ 134 , respectively. Both samples contained 10 mM TOTAPOL in a 60/30/10 ratio of d_8 -glycerol/ D_2O / H_2O . Experimental parameters are: 4 scans, recycle delay 4 s, microwave power ~ 12.5 W, $B_1(^1\text{H})=83$ kHz, $B_1(^{13}\text{C})=71$ kHz, $\tau/2 = 5$ kHz.

Figure 5. ^{13}C CP DNP enhancements of U- ^{13}C , ^{15}N] urea with 10 mM TOTAPOL plotted as a function of μw power at 80 K (*left*) and as a function of temperature at 12.5 W μw (*right*). $\nu/2 = 7$ kHz.

Figure 6.

(*left*) The ion-motive photocycle of bR. The subscript for each intermediate represents the wavelength (in nm) of maximum visible absorption. (*middle*) ^{15}N CP DNP spectra [^{-15}N -Lys] bR prepared with 15 mM TOTAPOL in 60/30/10 ratio of d_8 -glycerol/ D_2O / H_2O in 0.3 M guanidinium hydrochloride at pH 10. (A) the dark adapted (DA) state comprises a thermal equilibrium mixture of bR₅₅₅ and bR₅₆₈ (B) LA (bR₅₆₈) accumulated by 532 nm irradiation of the rotating sample for 4 hours 273 K (C) the Mo intermediate created by 532 nm irradiation of rotating LA at 230 K. The spectra of all three intermediates were obtained in roughly 2 hours with a spinning frequency of 7 kHz. (*right*) 2D spectrum obtained from DA bR illustrating the splittings observed at low temperature due to inequivalent sites.

Figure 7. ^{15}N - ^{13}C spectrum obtained from dark adapted U- ^{13}C , ^{15}N -bR after selective excitation of the ^{15}N Schiff base, CP to the ^{13}C -15 of the retinal and ^{13}C of Lys216, followed by RFDR mixing. The spectrum shows cross-peaks between the Schiff base ^{15}N and ^{13}C -12,13,14,15,20 on the retinal chromophore and ^{13}C Lys216^{27,33}. The arrow indicates the *trans-cis* isomerization of the C13=C14 bond that occurs during the photocycle.

Figure 8.

Comparison between room temperature and DNP enhanced, low temperature correlation spectra of PI3-SH3. The spectra were obtained with ZF-TEDOR recoupling ($t_{\text{mix}} = 16$ ms) from sample prepared from partially labeled fibrils [^{15}N , ^{12}C] PI3-SH3 / [^{14}N , ^{13}C] PI3-SH3 (50:50 molar ratio). (a) ^{15}N - ^{13}C intermolecular correlations in PI3-SH3 fibrils at 300 K obtained at 750 MHz in 16 days of acquisition time. (b). Same sample and identical spectral regions were recorded at 100 K and 400 MHz with DNP enhancement in 32 h. (c). Illustration of the 23 interstrand contacts established from ^{13}C - ^{15}N peaks in the 750 MHz spectra acquired at 300 K in a. (d) the 52 interstrand contacts established from the 400 MHz DNP enhanced spectra recorded at 100 K shown in (d).³⁴