Dynamic Nuclear Polarization of Deuterated Proteins

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Magic-angle spinning nuclear magnetic resonance (MAS NMR) has evolved as a robust and widely applicable technique for investigating the structure and dynamics of biological systems [1-3]. It is in fact rapidly becoming an indispensible tool in structural biology studies of amyloid, [4, 5] nanocrystalline,[6, 7] and membrane proteins [8]. Concurrently, it is clear that the low sensitivity of directly detected ^{13}C and ^{15}N signals in MAS spectra limits the utility of the approach, particularly when working with systems which are difficult to obtain in large quantities. This provides the impetus for the development of methods to enhance the sensitivity of MAS experiments, the availability of which will undoubtedly broaden the applicability of the technique. Remarkable progress towards this goal has been achieved by incorporating high frequency dynamic nuclear polarization (DNP) into the MAS protocol. [9-17]. This method exploits the microwave driven transfer of polarization from a paramagnetic center such as nitroxide free radical to the nuclear spins, and has been demonstrated to produce uniformly polarized macromolecular samples. In principle signal enhancements, $\varepsilon = (\gamma_e / \gamma_I) \sim 660$ can be obtained for ^1H and recently $\varepsilon=100\text{-}200$ was observed in model compounds. However, in applications of DNP to MAS spectra of biological systems which include studies of lysozyme[18], and bacteriorhodopsin [16, 19, 20] the enhancements have been smaller, $\varepsilon=40\text{-}50$. An exception to this statement is the amyloidogenic peptide GNNQQNY$_{7-13}$ which forms nanocrystals where the ^1H T_1 is long and $\varepsilon\sim100$ [21].

Almost a decade ago in studies of model systems, it was observed that deuteration of the solvent resulted in significant increases in ε [22] and subsequently many DNP experiments have employed ^2H labelled glasses such as d_6-DMSO or d_8-glycerol/D_2O/H_2O in a ~60:40:10 ratio [23-25]. The ~90% ^2H level slows the relaxation among ^1H's, while the ~10% ^1H level is sufficient to insure that ^1H-^1H spin diffusion distributes the enhanced polarization uniformly. The validity of this explanation explains the success of the DNP experiments on GNNQQNY even though the peptides are protonated.

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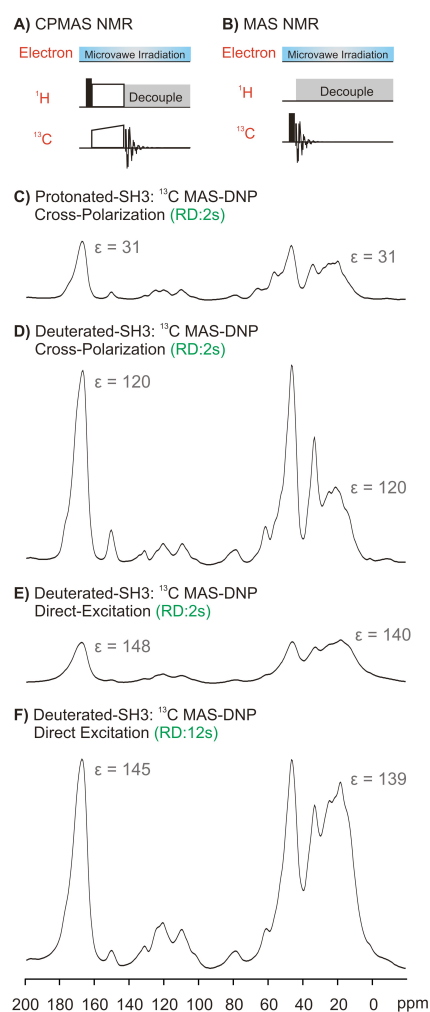Despite the success of deuteration in improving DNP

Figure 1. The pulse sequences used to record the dynamic nuclear polarization enhanced ^{13}C spectra with cross-polarization (CPMAS) (A) and direct-excitation (MAS) (B) at ~98 K and ~9 kHz MAS. C) ^{13}C CPMAS spectrum of protonated SH3 with DNP. DNP enhanced ^{13}C CPMAS (D) and MAS (E, F) spectra of the deuterated-SH3 with 50% exchangeable proton content. A relaxation delay (RD) of 2 s (C-E) and 12 s (F) were used. Continuous-wave microwave irradiation was used while acquiring the DNP enhanced spectra. For calculation of the DNP enhancement, the spectra were recorded with microwave irradiation and compared to the spectra recorded without microwave irradiation under exactly same experimental condition. The spectra are plotted with the same noise level, to allow direct comparison.

enhancements, it has not previously been employed in studies of proteins. In this communication, we demonstrate that deuteration of the protein itself results in factors of 3-5 larger DNP enhancements in its ^{13}C MAS spectra. This is a very significant

increase in the efficiency of DNP and may well evolve to the preferred means of performing DNP-MAS experiments in biological systems.

For the experiments reported here we used samples of the protein α−spectrin-SH3 that was fully deuterated at all sites and then recrystallized in appropriate H_2O/D_2O buffers to adjust the $^1H/^2H$ ratio at the exchangeable sites. Subsequently the protein
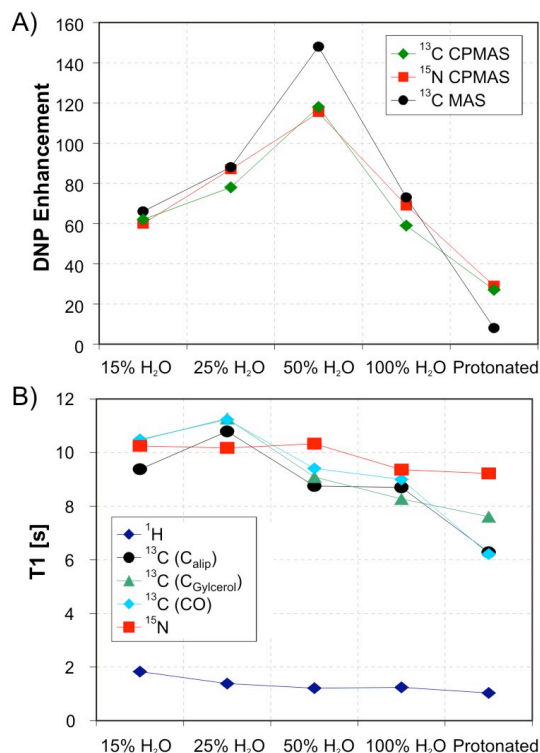

Figure 2. The dependence of the A) DNP enhancement and B) T_1 relaxation time (seconds) to the overall ^1H content. For comparison, a fully protonated (at exchangeable and non-exchangeable sites) and three different deuterated SH3 (at non-exchangeable sites) proteins were used. The proton content at the exchangeable sites of the protein was tuned by recrystallization of SH3 in buffers containing different H_2O/D_2O ratios. The H_2O contents were set around 15, 25, 50 and 100 % to be able to cover the full range of exchangeable proton content. The spectra of the data presented above were recorded at a temperature of ~98 K, an MAS frequency of ~9 kHz and with ~5W microwave irradiation at the rotor with zirconium rotors. The experimental data points are connected with lines as a guide to the eye, the data points are discontinuous.

was dispersed in a d_8-glycerol/D_2O/H_2O matrix. Figure 1 shows a comparison of one-dimensional ^{13}C MAS spectra recorded with microwave irradiation using the pulse sequences shown in Figure 1A-B, with CP for the protonated (1C) and deuterated (1D) SH3 samples. Figures 1E and 1F show MAS spectra recorded with direct ^{13}C excitation at different recycle delays. The DNP enhancement observed in the ^{13}C cross polarization (CP) MAS spectrum from a protonated and fully $^{13}C/^{15}N$ labeled sample is ε≈31 (1C), comparable to enhancements previously reported. [16, 19, 20]

In the deuterated protein, the efficiency of the DNP enhancement of the ^{13}C CPMAS experiments increases by a factor of ~3.9 (ε=120, 1D) compared to protonated samples. Furthermore, by using direct ^{13}C excitation on the deuterated sample, the enhancement is further increased by a factor of ~4.8 to ε=148 (1F) compared to the ^{13}C CPMAS experiment on the fully protonated SH3.

The sensitivity increase in a ^{13}C DNP-CPMAS experiment is determined by the enhancement of the proton spin reservoir which is subsequently transferred to ^{13}C and is limited by the ratio (γ_e/γ_{1H}). Similarly, in a ^{13}C direct excitation (MAS) experiment, the enhancement depends on (γ_e/γ_{13C}) which is a factor of four larger. Thus, the enhancements in direct excitation experiments are expected to be larger, although the required recycle delays could be
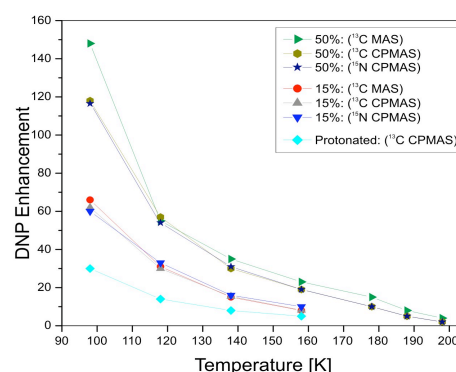

Figure 3. The dependence of the ^1H, ^{13}C and ^{15}N DNP enhancements on temperature. Results for the protonated and perdeuterated (15 and 50 % exchangeable proton content) SH3 are compared. Enhancement values are calculated at each temperature for each type of experiment. For the protonated SH3, only the ^{13}C CPMAS DNP enhancement values are shown.

longer because of slower spin diffusion in the ^{13}C reservoir. These ideas were recently confirmed experimentally [26] and it was also demonstrated that the maximum in the ^1H enhancement field profile is identical for ^{13}C. However, because of the lower value of γ_{13C}, the optimal field for the direct ^{13}C enhancements is on the opposite side of the profile. Nevertheless, in the case of SH3, the ^{13}C T_1's are short and in the MAS DNP experiment we observe ε≈148. This enhancement is significantly larger compared to the enhancement obtained from the CP experiment (1D). However, the ^{13}C DNP enhancement observed for the fully protonated SH3 sample recorded with direct ^{13}C excitation is ε≈8. These observations strongly suggest that protein deuteration, as well as direct ^{13}C excitation, is responsible for the further increase in ε. Supporting this hypothesis is the fact that the direct ^{15}N DNP enhancement is 207 for deuterated SH3 with 50 % protons at exchangeable sites. To quantify the amount of signal per unit time, we recorded a ^{13}C spectrum with direct excitation and short relaxation delay of 2 s for a deuterated protein with 50 % protons at the exchangeable sites (1E). The intensity at the CO and $C_{glycerol}$ signal is reduced compared to the CP spectrum of the fully protonated SH3 sample (1C), whereas, the aliphatic region is slightly stronger.

Figure 2A shows the dependence of the DNP enhancement on the exchangeable proton content in the protein and buffer. The enhancements obtained for various nuclei (^1H, ^{13}C, and ^{15}N) and by using different experimental approaches (MAS and CPMAS), depend strongly on the exchangeable proton content. A gradual increase of the ^{13}C and ^{15}N DNP enhancement is observed by increasing the exchangeable proton content from 15 % to 100 %. For all types of experiments, the fully protonated SH3 has lower DNP enhancements. The data suggests that a plethora of protons attenuates the enhancement, and a paucity interferes with the distribution of polarization via spin-diffusion. [23-25]

The fully protonated SH3 sample has a slightly shorter proton T_1 value compared to the deuterated SH3 samples. Using higher

concentrations of biradical in deuterated proteins can circumvent this problem, provided that it is sufficiently bulky and does not diffuse into the crystal lattice and broaden the ^{13}C lines. In addition, the possibility of using ^{2}H as an initial polarization transfer source could enhance the absolute sensitivity in deuterated proteins and help to exploit the increase in polarization enhancement further.

The resolution observed at the cryogenic temperature of MAS-DNP experiments at 400 MHz is currently not sufficient for assignment of a fully labelled protein. Accordingly, it is of interest to increase the temperature to achieve higher resolution, partially sacrificing enhanced DNP sensitivity. [27] Nevertheless, there might be a *compromise temperature* where there is sufficient resolution and a sufficiently high DNP enhancement. To study the temperature effect, we measured the ^{1}H, ^{13}C and ^{15}N enhancements at elevated temperatures, from 98 K up to 200 K, for fully protonated and perdeuterated SH3 samples (Figure 3). In this temperature range, an increase of 20 K results in a decrease of ~30-40 % in the enhancement. Above ~160 K, it becomes impractical to perform DNP MAS NMR for the full protonated or the deuterated protein with 15 % protonation level, since the DNP-enhancements decrease dramatically. For the SH3 sample with a 50 % protonation level, the enhancement decrease by 90 % in the temperature range of 98 to 178 K, nevertheless, the DNP enhancements are still $\varepsilon \approx 10$ and $\varepsilon \approx 15$ in ^{13}C cross-polarization and direct-excitation MAS-NMR spectra. Thus, this sample is suitable for *high-temperature* DNP.

In conclusion, we have shown that perdeuteration of a protein has remarkable effects on the observed DNP enhancements. Superior DNP enhancements are obtained for perdeuterated SH3 samples up to ~3.9, and ~18.5 times for ^{13}C CPMAS, and ^{13}C MAS experiments, respectively, compared to the same type of experiments in fully protonated SH3. The optimum exchangeable proton content is found to be ~50 % which results in the maximum enhancement of $\varepsilon \approx 148$ in a ^{13}C MAS spectrum by using a ZrO_2 rotor. By taking into account the ~20 % increase in enhancement by using sapphire rotors, higher ^{13}C DNP enhancement of $\varepsilon \approx 180$ can be expected. Moreover, by using the deuterated SH3 protein with 50 % proton content at the exchangeable sites, it is possible to increase the temperatures at which DNP experiments still yield considerable enhancements. We expect that, the use of perdeuterated proteins in MAS DNP NMR will open new possibilities in the application of these techniques to difficult biological problems.

Experimental Section

Details of the sample preparation by unfolding, exchanging and refolding of perdeuterated and protonated SH3 are described elsewhere. [28,29] The samples for the MAS-DNP measurements were prepared by dissolving the protein in 10-30-60 volume-% H_2O-D_2O-glycerol solution which forms a stable glassy matrix and cryoprotects the protein. [30] TOTAPOL biradical [31] is additionally dissolved in this solution at a concentration of 20 mM, corresponding to an electron concentration of 40 mM.

All solid-state MAS-DNP NMR experiments were performed on a commercial Bruker DNP spectrometer operating at a ^{1}H frequency of 400 MHz and microwave frequency of 263 GHz. Spectra were recorded using a triple resonance, low-temperature, HCN DNP probe employing 3.2 mm ZrO_2 rotors. Cryogenic temperatures were achieved and controlled with Bruker low-temperature MAS accessory. The signal enhancement is achieved in-situ, directly at the magnetic field inside the probe. The millimeter wave power, ~5 watts, is generated by Bruker gyrotron oscillator.

All of the DNP enhanced ^{13}C MAS spectra were recorded at $\omega_r/2\pi = 8888$ Hz and using $\pi/2$ pulses of 4 and 5 µs for ^{1}H and ^{13}C, respectively, and a CP contact time of 2 ms. A sapphire rotor was used to determine the enhancement difference between zirconia and sapphire rotors and found that use of sapphire rotor results in ~20 % increase in the observed DNP enhancement values. In addition, we note that the microwave irradiation reduces the apparent T_1 relaxation times by 30 %, most probably due to sample heating.

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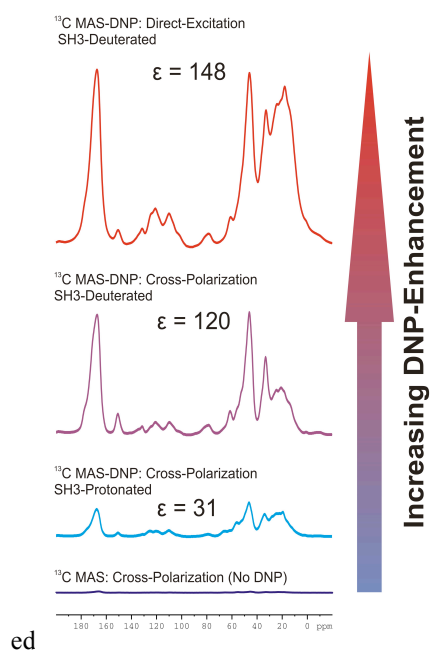[31]   C. Song, K.-N. Hu, C.-G. Joo, T. M. Swager, R. G. Griffin, *J. Am Chem. Soc* **2006**, *128*, 11385.

Entry for the Table of Contents

^{13}C MAS-DNP: Direct-Excitation SH3-Deuterated

$\varepsilon = 148$

^{13}C MAS-DNP: Cross-Polarization SH3-Deuterated

$\varepsilon = 120$

^{13}C MAS-DNP: Cross-Polarization SH3-Protonated

$\varepsilon = 31$

^{13}C MAS: Cross-Polarization (No DNP)

180 160 140 120 100 80 60 40 20 0 ppm

Increasing DNP-Enhancement

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Deuteration of proteins is shown to significantly increase the signal enhancements observed in DNP-MAS NMR experiments. In ^{13}C CPMAS spectra an enhancement of 120 is observed for perdeuterated SH3 with 50% exchangeable proton ratio, whereas an enhancement of only 31 is obtained for the fully protonated SH3. Thus, perdeuteration increases the DNP enhancement factor by ~4. Moreover, direct ^{13}C excitation on the perdeuterated sample, further increases the enhancement to 148. These results suggest that perdeuteration may be the optimal approach to performing DNP in biological macromolecules.