NMR Spectroscopy

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Dynamic Nuclear Polarization of Deuterated Proteins**

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Magic-angle spinning nuclear magnetic resonance (MAS NMR) spectroscopy 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 indispensable tool in structural biology studies of amyloid,[4,5] nanocrystalline,[6,7] and membrane proteins.[8] However, it is clear that the low sensitivity of MAS experiments to directly detected ^{13}C and ^{15}N signals limits the utility of the approach, particularly when working with systems which are difficult to obtain in large quantities. This limit provides the impetus to develop 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 NMR technique.[9–17] The DNP 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, $\varepsilon = (\gamma_e/\gamma_I) \approx 660$ can be obtained for ^{1}H and recently signal enhancements of $\varepsilon =$ 100–200 were observed in model compounds. However, in applications of DNP to MAS spectra of biological systems, including studies of lysozyme,[18] and bacteriorhodopsin,[16,19,20] the enhancements have been smaller, $\varepsilon = 40$–50. An exception is the amyloidogenic peptide $GNNQQNY_{7-13}$ which forms nanocrystals for which the proton T_1 time is long and $\varepsilon \approx 100$.[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 ^{2}H-labelled glasses, such as $[D_6]$DMSO or $[D_8]$glycerol/D_2O/H_2O in an approximately 6:3:1 ratio.[23–25] The approximately 90% ^{2}H concentration level slows the relaxation among protons, while the approximately 10% ^{1}H concentration level is sufficient to ensure that ^{1}H–^{1}H spin diffusion distributes the enhanced polarization uniformly through the sample. The validity of this explanation explains the success of the DNP experiments on GNNQQNY even though the peptide is protonated.

Despite the success of deuteration in improving DNP enhancements, to date it has not been employed in studies of proteins. Herein, we demonstrate that deuteration of the protein itself results in three to five times larger DNP enhancements in its ^{13}C MAS spectra. This is a very significant increase in the efficiency of DNP and may well become the preferred means of performing DNP-MAS experiments in biological systems.

For the experiments reported herein we used samples of the SH3 domain of the protein α-spectrin in which at all the amino acids were deuterated, the samples were then recrystallized in appropriate H_2O/D_2O buffers to adjust the ^{1}H/^{2}H ratio at the exchangeable sites. Subsequently the protein was dispersed in a $[D_8]$glycerol/D_2O/H_2O matrix. Figure 1 shows a comparison of one-dimensional (1D) ^{13}C MAS NMR spectra recorded with microwave irradiation using the pulse sequences shown in Figure 1A and B, with cross-polarization (CP) for the protonated SH3 samples (Figure 1C) and deuterated (Figure 1D) SH3 samples. Figures 1E and F show MAS spectra recorded with direct ^{13}C excitation at different recycle delays (RDs). The DNP enhancement observed in the ^{13}C CP-MAS spectrum from a protonated and fully ^{13}C/^{15}N labeled sample is $\varepsilon \approx 31$ (Figure 1C), comparable to enhancements previously reported.[16,19,20]

In the deuterated protein, the efficiency of the DNP enhancement of the ^{13}C CP-MAS experiments increases by a factor of approximately 3.9 ($\varepsilon = 120$, Figure 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 around 4.8 to $\varepsilon = 145$ (Figure 1F) compared to the ^{13}C CP-MAS experiment on the fully protonated SH3.

The sensitivity increase in a ^{13}C DNP-CP-MAS experiment is determined by the enhancement of the proton spin reservoir which is subsequently transferred to ^{13}C and is limited by the ratio ($\gamma_e/\gamma_{^1H}$). Similarly, in a ^{13}C direct excitation MAS experiment, the enhancement depends on $\gamma_e/\gamma_{^{13}C}$ 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 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 ^{1}H enhancement field profile is identical for ^{13}C. However, because of the lower value of $\gamma_{^{13}C}$, the optimal field for the direct ^{13}C enhancements is on the

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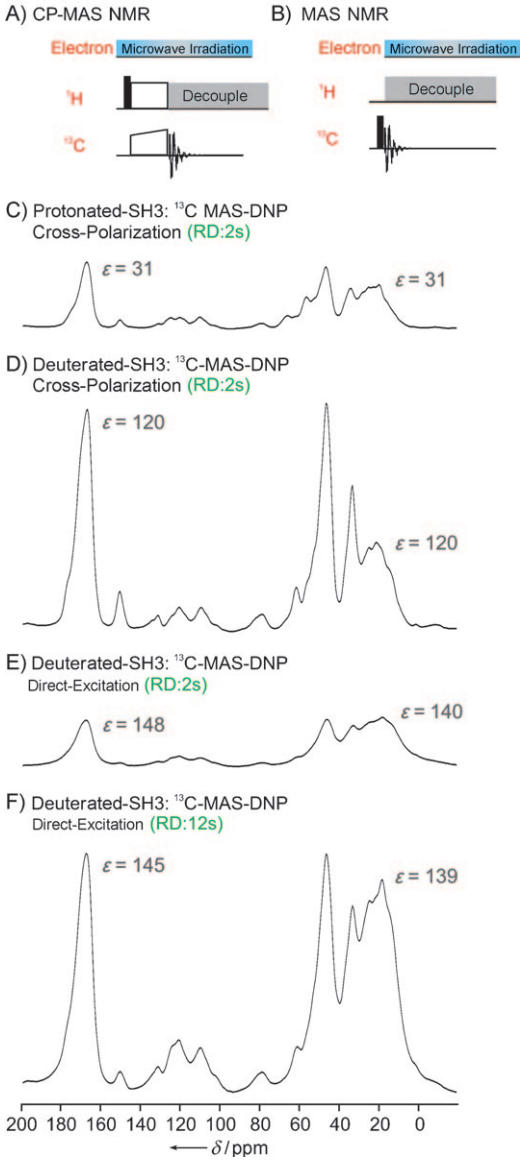

Figure 1. The pulse sequences used to record the DNP enhanced ^{13}C spectra with A) CP-MAS and B) direct ^{13}C excitation, at approximately 98 K and approximately 9 kHz MAS. C) ^{13}C CP-MAS spectrum of protonated SH3 with DNP. DNP enhanced D) ^{13}C CP-MAS and E),F) MAS spectra of the deuterated-SH3 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 conditions. The spectra are plotted with the same noise level, to allow direct comparison.

opposite side of the profile. Nevertheless, in the case of SH3, the ^{13}C T_1 times are short and in the MAS DNP experiment we observe $\varepsilon \approx 148$ (Figure 1E). This enhancement is significantly larger than the enhancement obtained from the CP experiment (Figure 1D). However, the ^{13}C DNP enhancement observed for the fully protonated SH3 sample recorded with direct ^{13}C excitation is $\varepsilon \approx 8$. 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 $\varepsilon = 207$ for deuterated SH3 with protons at 50% of the exchangeable sites. To quantify the signal per unit time, we recorded a ^{13}C spectrum with direct excitation and a short relaxation delay of 2 s for a deuterated protein with 50% protons at the exchangeable sites (Figure 1E). The intensity at the CO and $C_{glycerol}$ signal is reduced compared to the CP spectrum of the fully protonated SH3 sample (Figure 1C), whereas, the intensity in the aliphatic region is slightly increased.

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 (^{1}H, ^{13}C, and ^{15}N) and by using different experimental approaches (MAS and CP-MAS), 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 approximately 50%. For all types of experiments, the fully protonated SH3 has lower

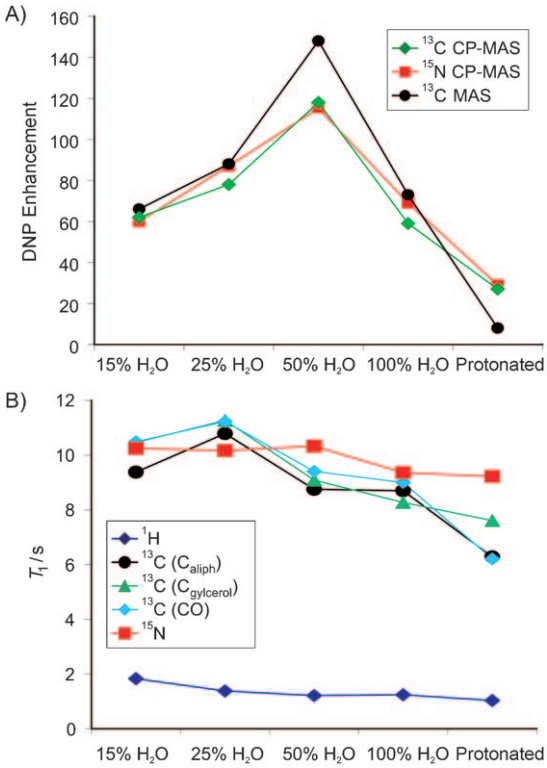

Figure 2. The dependence of the A) DNP enhancement and B) T_1 relaxation time (seconds) on the overall ^{1}H content. For comparison, a fully protonated (at exchangeable and non-exchangeable sites) and three different deuterated SH3 (at non-exchangeable sites) proteins were used. The proton content at 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 were recorded at a temperature of approximately 98 K, an MAS frequency of approximately 9 kHz, and with approximately 5 W microwave irradiation in zirconium rotors. The experimental data points are connected with lines as a guide to the eye, the data points are discontinuous.

DNP enhancements. The data suggests that a plethora of protons attenuates the enhancement, and a paucity interferes with the distribution of polarization through spin diffusion causing a reduced signal enhancement.[23–25]

The fully protonated SH3 sample shows slightly shorter T_1 values (except for ^{15}N) than the perdeuterated SH3 samples, which all show similar values. Using higher concentrations of biradical in deuterated proteins can circumvent this problem, provided that the radical 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 complete assignment of the signals of a fully labeled 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 around 30–40 % in the enhancement. Above 160 K, it becomes impractical to perform DNP-MAS NMR for the fully protonated or the perdeuterated protein with 15 % protonation level, since the DNP enhancements decrease dramatically. For the SH3 sample with a 50 % protonation level, the enhancement decreases by 90 % between 98 and 178 K, nevertheless, the DNP enhancements are still $\varepsilon \approx 10$ and $\varepsilon \approx 15$ in ^{13}C CP and direct-excitation MAS-NMR spectra. Thus, this sample is suitable for high-temperature DNP.

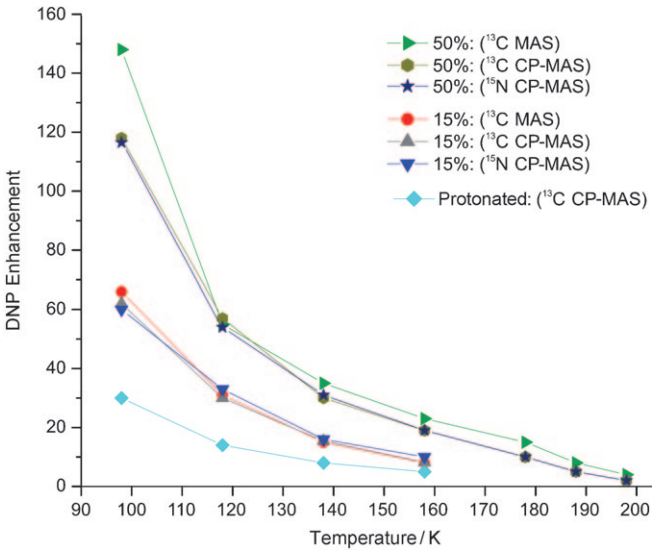

Figure 3. The dependence of the ^{1}H, ^{13}C, and ^{15}N DNP enhancements on temperature. Results for the protonated and perdeuterated (15 and 50% exchangeable proton content) SH3 are shown. Enhancement values are calculated at each temperature for each type of experiment. For the protonated SH3, only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

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 of up to 3.9 and 18.5 times for ^{13}C CP-MAS, 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 approximately 50 % which results in the maximum enhancement of $\varepsilon \approx 148$ in a ^{13}C MAS NMR spectrum obtained 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 DNP-MAS 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 DNP-MAS measurements were prepared by dissolving the protein in 1:3:6 vol % 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 DNP-MAS 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, approximately 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 an approximately 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 because of sample heating.

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[1] R. G. Griffin, *Nat. Struct. Biol.* **1998**, *5*, 508.
[2] A. E. McDermott, *Curr. Opin. Struct. Biol.* **2004**, *14*, 554.
[3] R. Tycko, *Quart. Rev. Biophys.* **2006**, *39*, 1.

[4] C. P. Jaroniec, C. E. MacPhee, V. S. Bajaj, M. T. McMahon, C. M. Dobson, R. G. Griffin, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 711.
[5] C. Wasmer, A. Lange, H. Van Melckebeke, A. B. Siemer, R. Riek, B. H. Meier, *Science* **2008**, *319*, 1523.
[6] F. Castellani, B. van Rossum, A. Diehl, M. Schubert, K. Rehbein, H. Oschkinat, *Nature* **2002**, *420*, 98.
[7] A. J. Nieuwkoop, B. J. Wylie, W. T. Franks, G. J. Shah, C. M. Rienstra, *J. Chem. Phys.* **2009**, *131*.
[8] J. M. Griffiths, K. V. Lakshmi, A. E. Bennett, J. Raap, C. M. Vanderwielen, J. Lugtenburg, J. Herzfeld, R. G. Griffin, *J. Am. Chem. Soc.* **1994**, *116*, 10178.
[9] A. W. Overhauser, *Phys. Rev.* **1953**, *92*, 411.
[10] T. R. Carver, C. P. Slichter, *Phys. Rev.* **1953**, *92*, 212.
[11] T. R. Carver, C. P. Slichter, *Phys. Rev.* **1956**, *102*, 975.
[12] R. A. Wind, M. J. Duijvestijn, C. Vanderlugt, A. Manenschijn, J. Vriend, *Prog. Nucl. Magn. Reson. Spectrosc.* **1985**, *17*, 33.
[13] M. Afeworki, R. A. Mckay, J. Schaefer, *Macromolecules* **1992**, *25*, 4084.
[14] L. R. Becerra, G. J. Gerfen, B. F. Bellew, J. A. Bryant, D. A. Hall, S. J. Inati, R. T. Weber, S. Un, T. F. Prisner, A. E. McDermott, K. W. Fishbein, K. E. Kreischer, R. J. Temkin, D. J. Singel, R. G. Griffin, *J. Magn. Reson. Ser. A* **1995**, *117*, 28.
[15] G. J. Gerfen, L. R. Becerra, D. A. Hall, R. G. Griffin, R. J. Temkin, D. J. Singel, *J. Chem. Phys.* **1995**, *102*, 9494.
[16] M. Rosay, J. C. Lansing, K. C. Haddad, W. W. Bachovchin, J. Herzfeld, R. J. Temkin, R. G. Griffin, *J. Am. Chem. Soc.* **2003**, *125*, 13626.
[17] A. B. Barnes, G. D. Paëpe, P. C. A. van den Wel, K.-N. Hu, C.-G. Joo, V. S. Bajaj, M. L. Mak-Jurkauskas, J. R. Sirigiri, J. Herzfeld, R. J. Temkin, R. G. Griffin, *Appl. Magn. Reson.* **2008**, *34*, 237.
[18] D. A. Hall, D. C. Maus, G. J. Gerfen, S. J. Inati, L. R. Becerra, F. W. Dahlquist, R. G. Griffin, *Science* **1997**, *276*, 930.
[19] M. L. Mak-Jurkauskas, V. S. Bajaj, M. K. Hornstein, M. Belenky, R. G. Griffin, J. Herzfeld, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 883.
[20] V. S. Bajaj, M. L. Mak-Jurkauskas, M. Belenky, J. Herzfeld, R. G. Griffin, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9244.
[21] P. C. A. van der Wel, K. N. Hu, J. Lewandowski, R. G. Griffin, *J. Am. Chem. Soc.* **2006**, *128*, 10840.
[22] M. M. Rosay, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, MA, **2001**.
[23] K. N. Hu, H. H. Yu, T. M. Swager, R. G. Griffin, *J. Am. Chem. Soc.* **2004**, *126*, 10844.
[24] K.-N. Hu, V. S. Bajaj, M. M. Rosay, R. G. Griffin, *J. Chem. Phys.* **2007**, *126*, 044512.
[25] K.-N. Hu, C. Song, H.-h. Yu, T. M. Swager, R. G. Griffin, *J. Chem. Phys.* **2008**, *128*, 052321.
[26] T. Maly, A.-F. Miller, R. G. Griffin, *ChemPhysChem* **2010**, *11*, 999.
[27] A. Linden, W. T. Franks, U. Akbey, B. J. van Rossum, H. Oschkinat, unpublished results.
[28] U. Akbey, S. Lange, W. T. Franks, R. Linser, K. Rehbein, A. Diehl, B. J. van Rossum, B. Reif, H. Oschkinat, *J. Biomol. NMR* **2010**, *46*, 67.
[29] U. Akbey, H. Oschkinat, B. J. van Rossum, *J. Am. Chem. Soc.* **2009**, *131*, 17054.
[30] A. Baudot, L. Alger, P. Boutron, *Cryobiology* **2000**, *40*, 151.
[31] C. Song, K.-N. Hu, C.-G. Joo, T. M. Swager, R. G. Griffin, *J. Am. Chem. Soc.* **2006**, *128*, 11385.

