NMR Spectroscopy

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Rapid Natural-Abundance 2D ^{13}C–^{13}C Correlation Spectroscopy Using Dynamic Nuclear Polarization Enhanced Solid-State NMR and Matrix-Free Sample Preparation**

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Solid-state nuclear magnetic resonance (SSNMR) has been extensively used to characterize molecular structures at atomic scales.[1] Concerning biomolecular applications, structural studies are commonly performed on ^{13}C and/or ^{15}N enriched samples to compensate for their low natural abundance (1.11% for ^{13}C and 0.37% for ^{15}N). However, this strategy is mainly restricted to biomolecules that can be easily isotopically enriched and has proven difficult to expand to other types of systems.

To date, only a few examples of natural abundance (NA) 2D ^{13}C–^{13}C correlation experiments in solids have been reported using pulse sequences that rely on through-bond polarization transfer.[2] This type of experiment provides one-bond connections and is limited to small crystalline molecules, as it requires 2 to 10 days of experimental time. Owing to the low abundance of ^{13}C nuclei, cross-peak intensities are about four orders of magnitude smaller for experiments performed on NA systems compared to their labeled equivalents.

Recently, dynamic nuclear polarization (DNP) performed with a high-power high-frequency microwave source (gyrotron), a low-temperature (LT) magic-angle spinning (MAS) probe, and a suitable polarizing agent has emerged as an appropriate answer to the sensitivity limitation of SSNMR even at high magnetic fields.[3–5] This work by Griffin and coworkers has triggered a strong interest in the science community and high-field MAS-DNP has been used on many different types of systems ranging from biological systems[6–8] to materials.[9–11] Herein we will show that the sensitivity enhancement obtained with DNP can be significant enough to obtain 2D ^{13}C-^{13}C NMR correlation spectra on NA microcrystalline solids in 20 min,[12] that is, within an experimental time comparable to experiments routinely performed on isotopically labeled systems.

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The relevance of performing DNP experiments has so far mainly been judged by comparing the signal-to-noise ratio (S/N) with and without microwave (MW) irradiation. This "DNP enhancement" (ε_{DNP}) has shown factors of up to 200 at 9.4 T and 100 K,[5,13] but in most applications, a factor of 10 to 20 is obtained. However, we demonstrate here that the effective sensitivity gain in DNP experiments cannot be simply evaluated by measuring ε_{DNP}. Instead, we propose using the absolute sensitivity ratio (ASR) to evaluate the relevance of DNP by comparing the S/N per unit time obtained under optimized DNP conditions with the one obtained under standard NMR conditions (potentially using for example larger sample volumes and higher magnetic fields). Previously, Rossini et al. and Vitzthum et al. introduced an overall sensitivity factor[14] and a global DNP factor,[15] respectively, which take into account some of the important parameters. These factors are reduced forms of the ASR and are discussed in more detail in the Supporting Information, S4.

One of the main differences between conventional and DNP-enhanced SSNMR experiments is the effective sample volume. For DNP experiments, samples of interest are usually dissolved[3,5] or suspended[7,8] in glassy chemical matrices to achieve uniform radical distribution, which significantly reduces the effective amount of sample (for example, 45 μL of 0.1M glycine solution contains 0.34 mg of glycine). Impregnation of DNP matrices into porous materials reduces this sample volume reduction problem.[9] Another approach was reported using a solvent-free method that utilizes a spin-labeling technique where the biradical TOTAPOL[16] is covalently attached to a peptide. Enhancement factors of up to four were measured on a decapeptide.[15] The use of solvents also causes spectral line-broadening owing to the conformational distribution in frozen solutions.[17]

To optimize the sample volume and spectral resolution for MAS-DNP experiments, we developed a new sample preparation procedure that is based on a matrix-free (MF) approach where the polarizing agent is uniformly distributed around the microcrystals. This method maximizes the effective quantity of material observed and preserves its potential crystallinity, which then leads to narrower spectral lines at LT. This approach is demonstrated on a sample of microcrystalline cellulose using the TOTAPOL polarizing agent.

In Figure 1, we compare 1D ^{13}C CPMAS spectra of NA microcrystalline cellulose (a–c) and [2-^{13}C]glycine (d–f). For DNP experiments, the cellulose sample was prepared with the MF approach, while the standard frozen-solution preparation method was used for the glycine sample. Comparing the

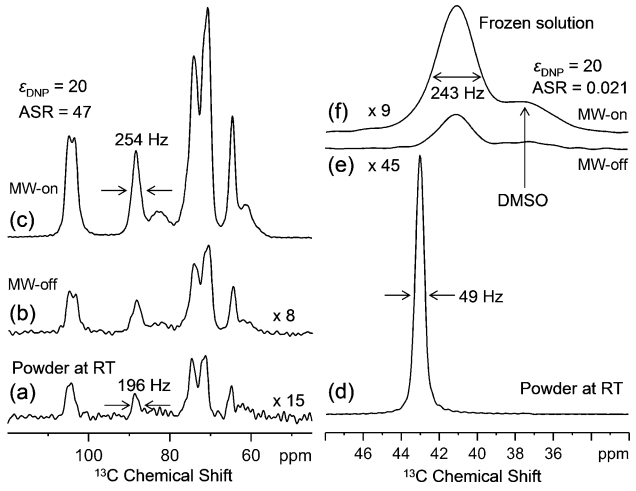

Figure 1. ^{13}C-CPMAS spectra of NA microcrystalline cellulose (a–c) and [2-^{13}C]glycine (d–f) recorded on a dried powder for conventional NMR experiments at RT (a, d), a MF sample (b, c), or a frozen solution (e, f) for DNP experiments at 105 K with MW off (b, e) and MW on (c, f). The MAS frequency was 8 kHz for all experiments. A DNP enhancement factor ε_{DNP} of 20 was observed with a zirconium rotor, although it is 30% less efficient compared to a sapphire rotor according to DNP experiments on ^{13}C urea (data not shown).

spectra with MW on and MW off, a similar DNP enhancement of 20 is obtained on both cellulose and glycine. However, when the DNP experiment is compared to the conventional NMR experiment at room temperature (RT) on powders, the ASR on glycine (0.021) is more than three orders of magnitude smaller than on cellulose (47). Notably, the S/N of the cellulose C2 site (the biggest peak) using DNP reaches 747 for a recycle delay of 13 s (5 times T_1) and four scans, whereas it was 15 for the same number of scans with conventional NMR at RT (recycle delay of 11.5 s).

The linewidths of the cellulose sample remain almost unchanged because it keeps its crystalline structure with the MF approach. On the other hand, the glycine peak is strongly broadened at low temperature, as the dissolved molecule is frozen in many different conformations. Furthermore, the strong solvent peaks present in the frozen-solution spectrum of glycine (Figure 1 f) do not appear in the case of MF cellulose (Figure 1 c). This is because the MF approach does not require the use of a cryoprotectant (such as glycerol, DMSO) to form glassy matrices at LT and uniformly distribute the polarizing agent. This is very important for NA experiments because signals of interest are often buried under large solvent peaks.

The substantial effective enhancement obtained on the MF NA cellulose allowed us to acquire a DNP-enhanced 2D double-quantum–single-quantum (DQ–SQ) ^{13}C homonuclear dipolar correlation experiment. The 2D spectrum, which was obtained in only 20 min, is shown in Figure 2 a. All one-bond correlations are observed with good resolution and sensitivity using the POST-C7[18] recoupling sequence with a total mixing time of 1 ms. In this case, the 20 min experimental time is not limited by sensitivity but rather by the number of repetitions required to complete the phase cycling for DQ selection, the number of points necessary in the indirect dimension for sufficient resolution, and the probe duty cycle. An experiment on a fully ^{13}C-labeled system would require the same experimental duration.

By increasing the recoupling mixing time to 3.5 ms, we were able to obtain a spectrum in only 2 h (see Figure 2 b) containing all two-bond correlations in NA cellulose (C1–C3, inter-unit C1–C4, C1–C5, C2–C4, C3–C5, and C4–C6). As dipolar truncation is negligible for ^{13}C–^{13}C polarization transfer in NA systems, long-range correlations are easier to measure than on fully ^{13}C-labeled systems. Furthermore, in samples at 1% natural abundance, no relayed mechanism is involved in two-bond polarization transfers. This will enable the performance of long-range distance measurements with high accuracy. A DNP-enhanced 2D SQ-SQ ^{13}C homonuclear dipolar correlation experiment using DARR[19] recoupling was also performed (Supporting Information, S3).

Finally, we would like to discuss the essential concept of sensitivity enhancement in the context of DNP-enhanced NMR experiments. To evaluate the true sensitivity gain of performing DNP experiments, we have introduced above the experimental quality factor, dubbed ASR. All the different contributions to the effective enhancement have to be considered to rationalize the measured ASR. We separate them into eight factors (Supporting Information, S4):

1) ε_{DNP}: the standard DNP enhancement factor (MW on/off);
2) ε_T: the gain obtained at LT, whose origin is twofold: the increase of magnetization owing to the Boltzmann distribution and the reduction of thermal noise at LT;
3) η_{T1}: takes into account the different repetition times that can be used in the two experiments. It depends on the longitudinal relaxation time constant T_1 for the RT NMR

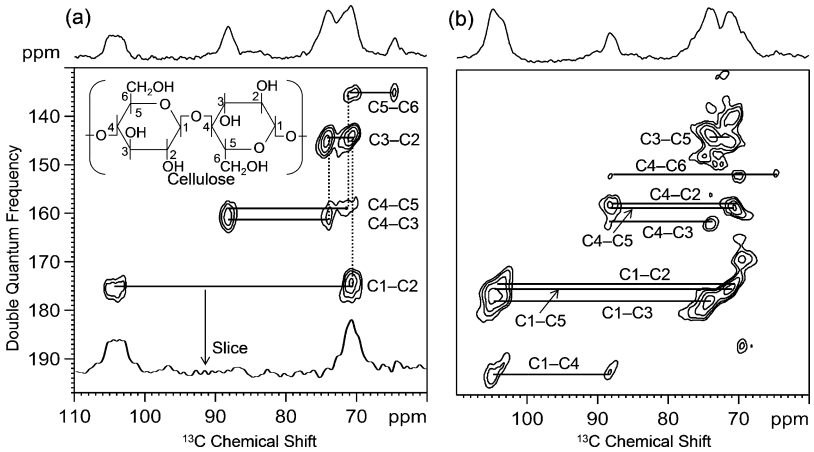

Figure 2. DNP-enhanced 2D DQ–SQ ^{13}C–^{13}C POST-C7 dipolar correlation spectra of MF NA microcrystalline cellulose recorded at 105 K. MAS frequency and the number of scans are 8 kHz and 16, respectively. a) The spectrum is obtained in 20 min with a recycle delay of 0.9 s, 1.4 ms evolution time, and 1 ms POST-C7 mixing. b) The experimental time is 2 h with a recycle delay of 3.4 s, 2.3 ms evolution time, and 3.5 ms of POST-C7 mixing. Projections are shown on the top.

experiment ($T_{1,RT}$) and on the DNP build-up time constant (τ_{DNP}) for the DNP experiment. The square root of their ratio accounts for the S/N per unit time: $\eta_{T1} = (T_{1,RT}/\tau_{DNP})^{1/2}$;

4) χ_{bleach}: the factor accounting for signal "bleaching"[14,15] caused by paramagnetic effects resulting from the presence of the radical in the DNP sample;
5) χ_{LW}: the ratio of the line widths;
6) χ_{weight}: the ratio of the effective sample weights taking into account the amount of material of interest in both experiments;
7) χ_{seq}: the ratio of the effective magnetization after decays during the pulse sequence, such as mixing times and cross-polarization (CP) contact times;
8) χ_{ex}: the enhancement/reduction factor caused by extra effects if applicable (for example when using a different probe or magnetic field).

The effective DNP enhancement factor, or ASR, can be expressed as the product of the different contributions:

$$ASR = \varepsilon_{DNP}\,\varepsilon_T\,\eta_{T1}\,\chi_{bleach}\,\chi_{LW}\,\chi_{weight}\,\chi_{seq}\,\chi_{ex} \quad (1)$$

In most cases, both ε_{DNP} and ε_T lead to sensitivity enhancement (ε_{DNP}, $\varepsilon_T > 1$) and χ_{bleach}, χ_{LW}, χ_{weight}, χ_{seq},and χ_{ex} to sensitivity reduction (factors < 1). Rigid samples without mobile/rotating groups, such as a methyl group, exhibit long T_1 times at RT that can be shortened by the addition of the polarizing agents. This then contributes positively to the ASR, as $\eta_{T1} \gg 1$.

The eight different factors contributing to the ASR were measured for glycine and cellulose, and are summarized in Table 1. Large sensitivity reductions are observed on glycine in a frozen solution owing to limited sample amount and line broadening. These factors are minimized in the MF cellulose. The microcrystalline structure is kept almost unchanged, leading to $\chi_{LW} \approx 0.77$. As the sample is not dissolved in a matrix, the same sample amount can be used for DNP experiments as for conventional NMR experiments. In the case of the MF cellulose, a factor $\chi_{weight} > 1$ could even be obtained by moisturizing the sample for the DNP experiment (Supporting Information, S1). Using Equation (1), ASRs of 0.020 and 47 are estimated for the glycine and cellulose samples, respectively. These values are in excellent agreement with the experimental values (0.021 for glycine and 47 for cellulose). This clearly demonstrates that the ASR can be fully rationalized by the different contributions presented herein.

Table 1: Summary of enhancement/reduction factors.[a]

	ASR[m][b]	ε_{DNP}	η_{T1}	χ_{bleach}	χ_{LW}	χ_{weight}	χ_{seq}[c]	ASR[e][d]
Glycine	0.021	20	0.38	0.60	0.20	0.0064	0.97	0.020
Cellulose	47	20	0.94	0.60	0.77	1.5	1.0	47

[a] Taking into account the inefficient and incomplete probe circuit cooling, ε_T was estimated to 3.6 ± 0.9, as the gain is at a maximum $(285\ K/105\ K)^{3/2} = 4.5$ (the entire circuit is cooled down to 105 K) and at a minimum $(285\ K/105\ K) = 2.7$ (the circuit is not cooled at all and considered to be at 285 K). [b] Experimentally measured ASR. [c] Comparison of spin-lock decays during the CP period of 1 ms. [d] Estimated ASR using Equation (1). Further details are given in the Supporting Information, S4.

In summary, we have introduced a new approach to record efficient multidimensional SSNMR correlation experiments on NA microcrystalline samples. Notably, we introduced a matrix-free sample preparation method that allows reaching excellent absolute sensitivity and the acquisition of 2D correlation spectra in a time equivalent to that needed for isotopically labeled systems. Thus, a 2D dipolar correlation experiment recorded in only 20 min has been presented on a MF NA cellulose sample. This method applied to NA correlation experiments also allows the detection of long distance transfer thanks to the lack of dipolar truncation. This sample preparation method can be further applied to labeled systems, opening up a new world of 4D (or higher dimensional) SSNMR experiments that can be designed to improve resolution in complex systems.

Experimental Section

Sample preparation: Biradical TOTAPOL was synthesized according to the literature.[16] NA microcrystalline cellulose (crystal size of 20 μm) and [2-^{13}C]glycine powder were purchased from Sigma–Aldrich. For conventional NMR experiments, cellulose was used without further treatment and fully packed in a rotor. For DNP experiments, 105 mg of cellulose was suspended in 1.5 mL of 3 mM TOTAPOL solution (D_2O) and dried in a desiccator under vacuum. During this process, a fraction of TOTAPOL molecules are bound to the cellulose surface. The obtained sample was fully packed in a rotor with 2 μL of D_2O to moisturize the sample (Supporting Information, S1). Glycine powder was dissolved in [D_6]DMSO/D_2O/H_2O (6:3:1 v/v/v) and TOTAPOL was added to obtain a 0.1M glycine/20 mM TOTAPOL solution for DNP experiments. Glycine powder (53 mg) and the above solution (45 μL) were fully packed into a rotor.

NMR experiments: All experiments were performed on a Bruker AVANCE™ III 400 MHz wide-bore NMR system equipped with a 263 GHz gyrotron, a transmission line and a LT (ca. 100 K) triple-resonance 3.2 mm MAS probe.[5] Details are provided in the Supporting Information, S2.

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