DOI: 10.1002/anie.201108132

Dual Acquisition Magic-Angle Spinning Solid-State NMR-Spectroscopy: Simultaneous Acquisition of Multidimensional Spectra of Biomacromolecules**

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Magic-angle spinning solid-state NMR (MAS ssNMR) spectroscopy is a powerful method for structure determination of biomacromolecules that are recalcitrant to crystallization (membrane proteins and fibrils).[1,2–4] Developments in pulse sequence design,[5–10] probes,[11] isotopic labeling schemes,[12] sensitivity enhancement using paramagnetic effects,[13] dynamic nuclear polarization (DNP),[14–16] and sample preparations have made it possible to advance resonance assignments and structure determination by ssNMR spectroscopy. However, as the biological systems under investigation increase in complexity, new methods are needed to improve spectral resolution and sensitivity as well as to speed up NMR data acquisition.

Here we present a novel approach (which we refer to it as "dual" acquisition MAS ssNMR or DUMAS ssNMR spectroscopy) for parallel acquisition of multidimensional ssNMR experiments without the need of additional hardware. DUMAS ssNMR spectroscopy is able to combine several of the above advancements to almost double the capability of the NMR spectrometers. Central to this technique are the following phenomena: 1) the long-living ^{15}N polarization in biological solids, 2) simultaneous Hartmann–Hahn (HH) cross-polarization (CP) from ^{1}H to ^{13}C and ^{15}N, and 3) ^{15}N–^{13}C dipolar decoupling under MAS conditions. DUMAS is not limited to specific pulse sequences, rather it represents a general approach to concatenate various multidimensional ssNMR experiments.

The classical ssNMR experiments start with HH-CP,[17,18] which enhances the sensitivity of rare nuclei coupled to an abundant ^{1}H spin bath. However, during CP only part of the polarization from the ^{1}H spin bath is transferred to dilute spins (^{13}C or ^{15}N). Recently, Oschkinat and co-workers proposed to enhance ^{13}C sensitivity with triple resonance (^{1}H, ^{2}H, ^{13}C) CP using perdeuterated proteins.[19] Analogously, for DUMAS we utilize simultaneous CP (SIM-CP) to polarize ^{13}C and ^{15}N nuclei. We then exploit the polarization to acquire two ^{13}C- and ^{15}N-edited multidimensional experiments in parallel. Since the ^{15}N nuclei have relatively long longitudinal spin relaxation times (T_1),[20] the ^{15}N z magnetization can be stored for several milliseconds (while acquiring ^{13}C coherences) and recovered for an additional multidimensional experiment.

A schematic of DUMAS is reported in Figure 1 A. A 90° pulse on ^{1}H is followed by SIM-CP and creates the initial polarization of the ^{13}C and ^{15}N nuclei. After SIM-CP, the ^{15}N magnetization is tilted along the z axis by a 90° pulse, whereas the ^{13}C magnetization is used to record a ^{13}C-edited spectrum with t_1' indirect evolution and t_2' acquisition under two-pulse phase-modulated (TPPM) heteronuclear decoupling.[21]

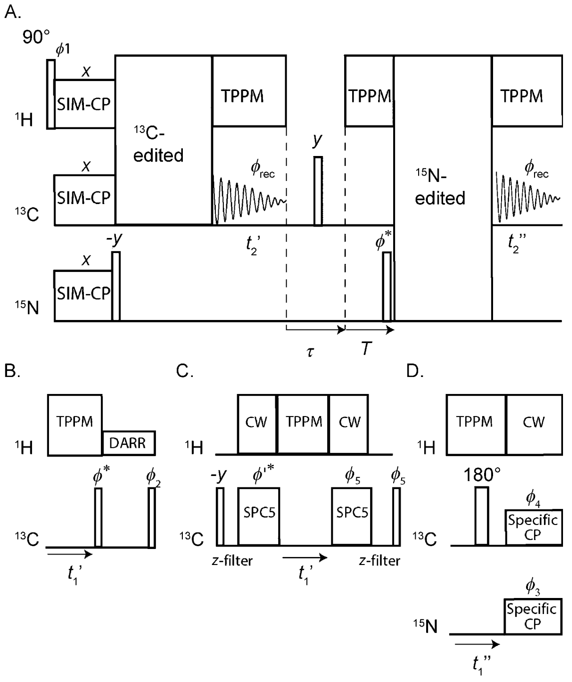

Figure 1. A) General scheme for the DUMAS ssNMR spectroscopy. The ^{13}C- and ^{15}N-edited blocks are combined to give DUMAS-DARR-NCA (B and D blocks) or DUMAS-DQSQ-NCO (C and D blocks).

After the acquisition of the first experiment, a τ delay of 2 ms is used to dephase any residual magnetization. Namely, in the first $\tau/2$ period, the transverse magnetization is dephased, whereas the longitudinal magnetization is dephased by a 90° pulse, followed by another $\tau/2$ period. A delay $T = [t_{1\max}'\text{-}dw(t_1')]$ is applied under heteronuclear decou-

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[**] This work was supported by the National Institutes of Health (grant numbers GM64742 and GM072701). We acknowledge M. Gustavsson, K. Mote, and F. Chao for preparing the samples and Dave Rice (Agilent) for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201108132.

pling, where $t_{1\max}'$ is the maximum t_1', and $dw(t_1')$ is the dwell time of the t_1' evolution period. This delay makes the total time of the ^{13}C-edited acquisition identical for all of the t_1' increments, whereas the ^{15}N magnetization is stored along the z axis. A 90° pulse recovers the ^{15}N magnetization stored along the z axis and places it in the transverse plane. At this point, a ^{15}N-edited sequence block is implemented followed by a second acquisition on the ^{13}C channel during t_2''. Due to the different relaxation behaviors, the evolution times (t_1) are usually longer for ^{15}N than for ^{13}C nuclei. Our scheme uses separate evolution time periods (t_1' and t_1''), which can be optimized for both sensitivity and resolution according to Table S1 in the Supporting Information. Although it is possible to combine various multidimensional experiments, we show the DUMAS method concatenating DARR,[22] NCA,[23] DQSQ,[24] and NCO,[23] four of the most widely used pulse sequences for structure determination of proteins. Specifically, we incorporated the ^{13}C- and ^{15}N-edited blocks to create the DUMAS-DARR-NCA and DUMAS-DQSQ-NCO pulse sequences (Figure 1).

To test the performance of these experiments, we used uniformly (U) ^{13}C,^{15}N-labeled ubiquitin in microcrystalline form, which is the benchmark for MAS ssNMR experiments on proteins.[25] U-^{13}C,^{15}N-labeled ubiquitin powder sample (10 mg) was dissolved in citrate buffer (20 mM, pH 4.1) to obtain a solution of 35 mg mL^{-1}. The protein was then crystallized by adding 2-methyl-2,4-pentanediol (60% final volume).[25,26] Approximately, 5 mg of microcrystalline ubiquitin was loaded into the MAS rotor. We also show that DUMAS pulse sequences can be applied to more challenging systems such as the integral membrane protein phospholamban (PLN) reconstituted in the lipid membrane. Phospholamban (52 residues)[27–29] was expressed recombinantly in E. coli[30] and reconstituted in lipid membranes as previously reported.[31]

For the DARR, DQSQ, NCA, and NCO pulse sequences (see Figure 1S, panels A–C in the Supporting Information), the initial ^{1}H–^{13}C or ^{1}H–^{15}N CP was optimized using the conventional CP experiment shown in Figure 1S in the Supporting Information. During SIM-CP, ^{1}H–^{13}C, or ^{1}H–^{15}N CP, the radio-frequency (RF) field strengths of the ^{1}H, ^{13}C, and ^{15}N nuclei were 45, 36.7, and 36.7 kHz, respectively, which corresponds to a $n=1$ side band matching condition. The ^{1}H RF amplitude was ramped by 10% with the center of the slope equal to 45 kHz. For comparison, the spectra obtained with ^{1}H/^{13}C CP and ^{13}C-detected ^{1}H/^{13}C/^{15}N SIM-CP at various contact times are reported in Figure 2S in the Supporting Information. Notably, the sensitivity in the ^{13}C spectral region between 0 and 80 ppm is nearly identical for both CP and SIM-CP at all contact times. The ^{13}C sensitivity in the carbonyl region of the ^{13}C spectrum (see Figure 3S in the Supporting Information) is 5–15% higher for the spectrum acquired with CP. However, the carbonyl region of the ^{13}C–^{13}C correlation spectra is often neglected due to the inherent low sensitivity and resolution. The comparison of ^{1}H/^{15}N CP and ^{15}N-detected ^{1}H/^{13}C/^{15}N SIM-CP spectra at various contact times (Figure 4S in the Supporting Information) shows a sensitivity loss of around 12–23% for SIM-CP. The integrated peak intensities from the spectra (see Figures 2S and 4S in the Supporting Information) as a function of the contact time are plotted in Figure 5S in the Supporting Information. Note that we have also tested CP and SIM-CP experiments on a U-^{13}C,^{15}N-labeled *N*-acetyl valine–leucine dipeptide powder. Indeed, we obtained relative sensitivities similar to those obtained for microcrystalline ubiquitin and phospholamban in lipids. Although it is difficult to derive a theoretical (quantitative) expression for SIM-CP, it should be noted that the rate of ^{13}C and ^{15}N polarization enhancement versus the contact time is similar for both CP and SIM-CP experiments (see Figure 5S in the Supporting Information). The latter indicates that the spin dynamics for CP and SIM-CP are qualitatively similar. Since only a moderate loss of sensitivity is observed for ^{15}N spectra (10–23%), we deduce that more polarization from the ^{1}H bath to the heteronuclei is transferred during SIM-CP compared to double-resonance CP (^{1}H–^{13}C or ^{1}H–^{15}N). In fact Pines et al.[17] showed that more ^{1}H polarization can be transferred using multiple contact periods of ^{1}H–^{13}C CP. For SIM-CP, the ^{13}C and ^{15}N spin lock periods do not have to be identical. For example, a longer ^{1}H–^{13}C or ^{1}H–^{15}N spin lock period can be used in SIM-CP to improve the sensitivity of dynamic regions of the biopolymers or for side chain detection. For the ubiquitin sample, we found that contact times of 400 and 600 μs give optimum sensitivity for ^{1}H–^{13}C and ^{1}H–^{15}N CP, respectively (see Figures 2S and 4S in the Supporting Information), whereas 400 μs was optimal for SIM-CP. For PLN, we used CP contact times of 300 and 500 μs for ^{1}H–^{13}C and ^{1}H–^{15}N CP, respectively, and 300 μs for SIM-CP. With the exception of the contact time, all of the other experimental parameters used for DUMAS were identical to the conventional pulse sequences. Moreover, we tested the decay of ^{15}N magnetization stored along the z axis after SIM-CP. Indeed, we found that the magnetization decay is negligible during the first ^{13}C-edited acquisition (see Figure 6S in the Supporting Information).

Figure 2 A shows the NCA and DARR spectra recorded using conventional 2D pulse sequences (see Figure 1S in the Supporting information). The total experimental time for both spectra was 16.03 h. Figure 2 B shows the corresponding spectra recorded simultaneously using the DUMAS-DARR-NCA experiment. The total experimental time was 8.12 h. As expected, the sensitivity of DUMAS-DARR is identical to the conventional DARR, whereas the sensitivity of DUMAS-NCA is about 80% of the NCA spectrum, which is due to the implementation of the SIM-CP. A similar situation is presented for the NCO and DQSQ spectra. Figure 3 A shows the two spectra acquired with the conventional methods for a total experimental time of 15.57 h. Figure 3 B shows the corresponding spectra acquired simultaneously with DUMAS-DQSQ-NCO with a total experimental time of 8.06 h, which is roughly half the time spent with conventional methods. As for the NCA, the sensitivity loss for the DUMAS-NCO is approximately 20%, whereas the DUMAS-DQSQ spectrum is similar to the conventional DQSQ. Figure 4 shows the application of DUMAS on the U-^{13}C,^{15}N-labeled membrane protein phospholamban. Each of the conventional DARR and NCA spectra was recorded in about 16 h, whereas the DUMAS spectra were acquired

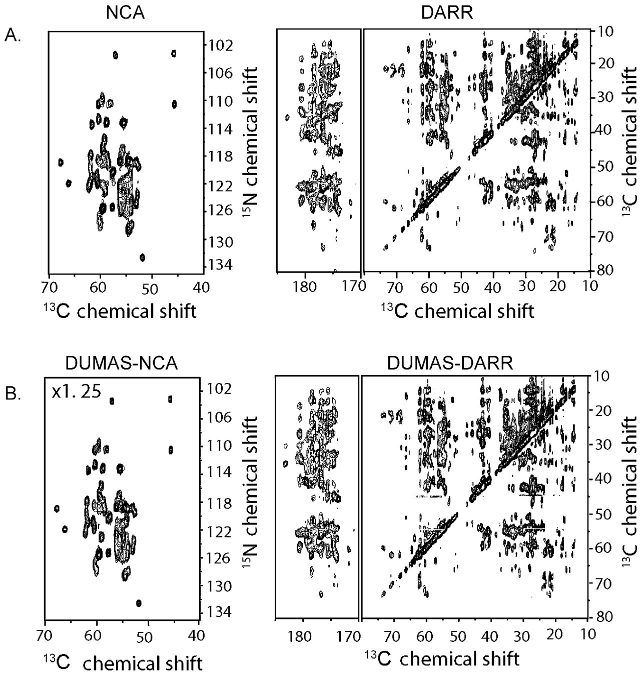

Figure 2. A) 2D NCA and DARR spectra of U-^{13}C,^{15}N-labeled ubiquitin acquired using conventional methods (see Figure 1S in the Supporting Information). B) NCA and DARR spectra acquired simultaneously using the DUMAS-DARR-NCA pulse scheme. DARR and DUMAS-DARR are plotted with the identical noise level. DUMAS-NCA is plotted at 1.25 times the signal intensity of NCA to account for 20% signal loss of ^{15}N during SIM-CP.

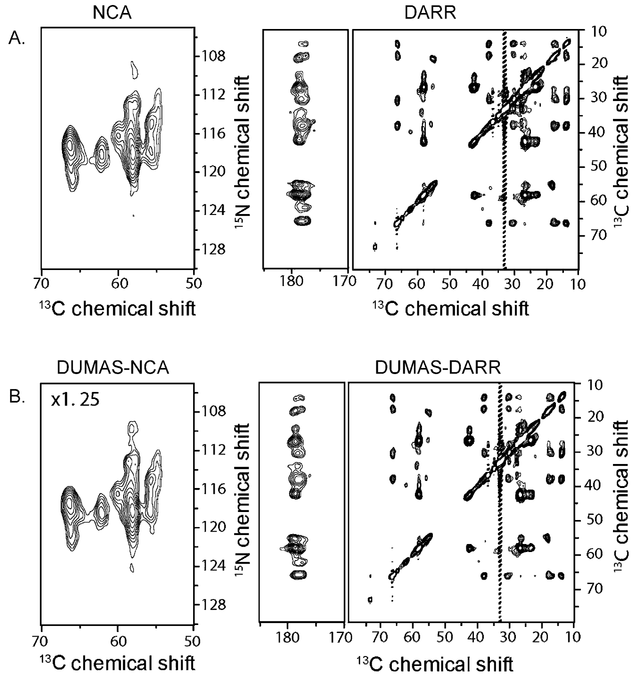

Figure 4. A) 2D NCA and DARR spectra of U-^{13}C,^{15}N-labeled phospholamban reconstituted in lipid membranes acquired using conventional methods (Figure 1S in the Supporting Information). B) NCA and DARR spectra acquired simultaneously using the DUMAS-DARR-NCA pulse scheme. As for the previous spectra, DARR and DUMAS-DARR are plotted with the identical noise level. DUMAS-NCA is plotted at 1.25 times the signal intensity of NCA to account for 20% signal loss of ^{15}N during SIM-CP.

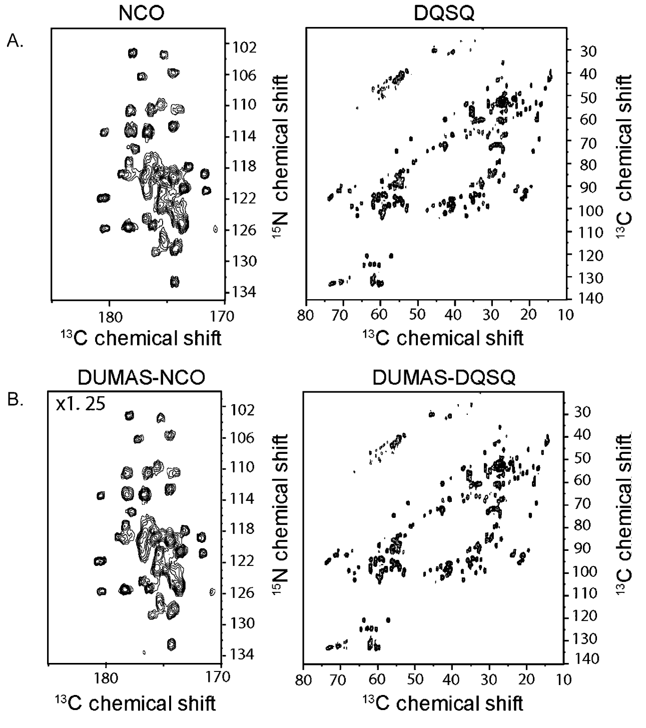

Figure 3. A) 2D NCO and DQSQ spectra of U-^{13}C-^{15}N ubiquitin using conventional methods (see Figure 1S in the Supporting Information). B) NCO and DQSQ spectra acquired simultaneously using DUMAS-DQSQ-NCO. DQSQ and DUMAS-DQSQ are drawn at the same noise level. To account for 20% signal loss of ^{15}N nuclei during SIM-CP, DUMAS-NCO is plotted with 1.25 times higher scale compared to NCO.

simultaneously in about 16 h. These results show that the DUMAS approach is extendable to integral membrane proteins as well.

Note that for the DUMAS experiments, the parameters for the indirect acquisition of ^{13}C and ^{15}N magnetization are such that $\mathrm{ni}(t_1')\cdot\mathrm{nt}(t_1') = \mathrm{ni}(t_1'')\cdot\mathrm{nt}(t_1'')$ according to Table S1 in the Supporting Information (ni = number of increments, nt = number of transients). In general, the optimum number of scans and increments (total number of 1D experiments) depends on the type of ^{13}C- and ^{15}N-edited experiments. Since the ^{13}C- and ^{15}N-edited blocks are in different time periods, DUMAS gives the flexibility to acquire an insensitive ^{13}C-edited experiment using a greater number of scans, and multiple (two or more) ^{15}N-edited 2D experiments, or vice versa. In other words, a longer ^{13}C-edited experiment can be combined with multiple ^{15}N-edited experiments, or vice versa.

In the past years, there has been a consistent effort to make the best out of polarization for both solution- and solid-state NMR experiments.[32–35] An approach involves the recovery of discarded coherences to acquire an additional experiment[36–38] or obtain sensitivity enhancement of the NMR signals.[39–43] Another example is the simultaneous acquisition of ^{13}C- and ^{15}N-edited experiments in liquid-state NMR using single acquisition on the ^{1}H channel.[44] In the latter experiment, the evolution of ^{13}C and ^{15}N nuclear spins is coupled to ^{1}H spins through two-spin operators, limiting its performance. In ssNMR spectroscopy, ^{15}N nuclei have long T_1 relaxation values in the order of a few hundred milliseconds.

For the first time, we used this unique property in combination with a SIM-CP scheme to exploit an untapped source of polarization and generate multiple experiments simultaneously. More importantly, DUMAS can be applied in combination with several sensitivity and resolution enhancement methods. For instance, it can benefit from protein perdeuteration.[45] To this extent, it has been shown that the values of ^{15}N T_1 for perdeuterated protein samples are not significantly different from the protonated ones.[46] A recent paper by Oschkinat and co-workers[47] shows that triple CP of $^{1}H/^{2}H/^{13}C$ is substantially more sensitive than conventional $^{1}H/^{13}C$ CP. Based on this work, we can anticipate that quadruple $^{1}H/^{2}H/^{13}C/^{15}N$ CP using a four channel probe with DUMAS acquisition will further enhance the sensitivity of these experiments.

DUMAS can also be used in concert with paramagnetic relaxation enhancement (PRE) methods,[13] obtaining faster data acquisition. In fact, it has been shown that for PRE samples the loss of ^{15}N z magnetization in the absence of ^{1}H decoupling is less than 10% for 100 ms.[48] Therefore, DUMAS can also be successfully applied in combination with PRE enhancement.[49,50]

Another recent improvement for sensitivity enhancement of ssNMR spectroscopy is the implementation of DNP.[14–16,51] This technique boosts the signal-to-noise ratio (S/N) of NMR spectra taking advantage of the polarization transfer from electronic spins to nuclear spins, doping the samples with nitroxide spin labels. If DNP is applicable to the sample under analysis, DUMAS can be coupled with methods to further speed up data acquisition and push the sensitivity limits of MAS ssNMR spectroscopy.

In conclusion, we introduce a novel approach for the simultaneous acquisition of ^{13}C- and ^{15}N-edited multidimensional MAS ssNMR experiments without additional hardware. Multidimensional DUMAS experiments are shown on microcrystalline ubiquitin as well as PLN in lipid vesicles. The DUMAS scheme opens up new avenues for reducing the acquisition time for multidimensional experiments for biomolecular ssNMR spectroscopy by combining the sensitivity and resolution advancements obtained in the past few years. We anticipate that the combination of DUMAS with dynamic nuclear polarization,[15,16,51] proton detection,[8,52] PRE, and multiple receivers[53] will further increase the sensitivity, resolution as well as the number of experiments that can be acquired simultaneously.

Experimental Section

All of the NMR experiments were performed using a 700 MHz VNMRS spectrometer (Agilent Technologies). The spectrometer was equipped with a 3.2 mm bioMAS scroll coil probe with reduced RF heating.[11] A spinning rate (ω_r) of 8.333 kHz was used for all of the experiments. The temperature was held constant at 5 °C. Further details on data acquisition are provided in the Supporting Information.

Received: November 18, 2011

Keywords: magic-angle spinning · NMR spectroscopy · proteins · solid-state structures · structure elucidation

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