**Accurate Determination of 1H-15N Dipolar Couplings using inaccurate settings of the Magic Angle in Solid State NMR**

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**Abstract**

Magic Angle Spinning is an essential ingredient in every solid-state NMR experiment. The standard procedures to adjust the rotor angle are not highly accurate resulting in a slight mis-adjustment of the rotor from the magic angle () on the order of a few millidegrees. This small missetting has no significant impact on the overall spectral resolution, but is sufficient to reintroduce anisotropic interactions. We show here that site specific 1H-15N dipolar couplings can be accurately measured in a heavily deuterated protein. This method can be applied at arbitrarily high MAS frequencies, since neither rotor synchronization nor particularly high radiofrequency field strengths are required. The off-magic angle spinning (off-MAS) method allows to quantify order parameters for very dynamic residues, which often escape an analysis using existing methods.

**Keywords:** Solid State NMR, Off Magic Angle, Spin Echo, Microcrystalline proteins

**Introduction**

Solid-state NMR has undergone impressive development during the last decade. With the aid of new hardware and the advent of robust NMR techniques, it has been possible to determine the structure of fully protonated proteins using state-of-the art commercially available probes that can spin up to 111 kHz 1-2. The angle that the rotor axis adapts with respect to the main magnetic field is ideally adjusted to the magic angle ()3-4. Under this condition, anisotropic components of second rank dipole-dipole, chemical shift and quadrupolar interaction tensors vanish to the first order, which is essential when high resolution NMR spectra shall be acquired. Therefore, adjusting the rotor angle to the magic angle is a prerequisite for solid state NMR.

In routine biomolecular applications, the spinning axis of the rotor is adjusted to the magic angle () by mechanically changing the orientation of the stator with respect to the B0 field. The correct angle is achieved when the number of sidebands (or the intensities of the sidebands) are maximal in a 79Br spectrum of a KBr powder sample5. Hall effect magnetic flux sensors or Satellite-transition-MAS (ST-MAS) on 23NaNO3 can alternatively be used6 to set the proper spinning angle. However, Hall devices are not available for most of the commercially available probes. Such devices also suffer from the temperature sensitivity of its electrical components which are difficult to control.

The KBr method yields an accuracy of c.a. 0.1° in the determination of the rotor angle5. For solid-state NMR probes that require a flip of the stator upon sample insertion, the error can increase up to 0.2°. Experiments performed under large amplitude off magic-angle yield poor spectra with poor resolution and sensitivity, as anisotropic dipole-dipole and chemical shift interactions are not completely averaged out by sample spinning. Spin-State transfer and selection methods have to be applied to restore some resolution. It was shown that spin state selective experiments carried out at high B0 fields can be used to restore resolution under slightly misadjusted rotor angle5.

Levitt and co-workers7 have demonstrated that the residual anisotropic interactions from rotor spinning angles away from the magic angle can be used to measure the dipolar coupling in molecules containing a pair of 13C atoms.

Measurement of heteronuclear dipolar coupling for fully protonated biosolids, e.g., among 13C-1H, 15N-1H, 13C-15N, by solid-state NMR can be performed either by monitoring oscillations in Lee-Goldburg (LG-CP)8 type polarization transfer experiments or by measuring the dephasing of NMR signals from recoupled dipolar couplings. From the later, several schemes such as transverse MREV (T-MREV)9, symmetry based recoupling schemes10 such as R1871 or R1852, TEDOR11 have been implemented to measure heteronuclear dipolar couplings. However, in fully protonated samples, homonuclear dipolar coupling among protons is hard to average out and leads to error in the quantification of dipolar couplings.

Perdeuteration schemes yield excellent resolution in proton detected solid-state NMR experiments for biosolids12-16. Partially back substituted protons in an otherwise deuterated protein provides a matrix of almost completely isolated 15N-1H spin pairs. Such systems are well suited to measure 15N-1H dipolar couplings accurately in a protein using phase inverted CP (CPPI) 17 or REDOR 16 experiments.

In CPPI, Hartmann-Hahn matching condition alternates between the +1 and -1 rotary resonance conditions on the 15N channel. REDOR type experiments 18-19 on the other hand rely on recoupling of dipolar interactions by rotor synchronized π pulses. The effect of radio frequency (RF) inhomogeneity and the presence of remote protons on both these methods have been analysed in details20. Although robust in the presence of RF inhomogeneity and remote protons, the CPPI method cannot be applied for MAS frequencies above 60 kHz due to RF power limitations in solid-state NMR probes. In addition, CPPI is unable to detect signals from very flexible residues. Cross polarization with Variable Contact time (CP-VC)21 has been introduced to probe 15N-1H and 13C-1H distances employing 100 kHz MAS for fully protonated samples. Numerical simulations have shown that REDOR methods can be applied to MAS frequencies beyond 100 kHz. However, in REDOR based methods, four π pulses (three on 1H and one on 15N) are applied in each rotor period. The total duration of the recoupling period can extend up to 2 ms or more. When the MAS frequency exceeds 100 kHz, *i.e.*, the rotor period is less than 10 μs, 800 or more strong π pulses are needed to record a standard REDOR curve. The applied RF power during the recoupling period result in significant sample heating.

In high-resolution MAS solid-state NMR experiments, the rotor angle is slightly misadjusted. We make use of this mis-adjustment and show how 1H-15N spin echo curves can be employed to quantify order parameters. The method is not significantly affected by RF inhomogeneity and can be applied at high MAS rotation frequencies.

**Results and Discussion**

When the rotor angle is exactly set to 54.74° () with respect to B0, the anisotropic dipolar and Chemical Shift Anisotropy (CSA) interactions vanish. Under off-MAS, the anisotropies are not refocused any longer after each rotor period, and sensitivity is compromised. Obviously, resonances of atoms that experience a large anisotropy are affected more than those that feel only weak interactions.

../../Figure.1.Spectrum.pdf

**Figure 1.** (A)1H-15N correlation spectra recorded using a uniformly 2H, 13C, 15N labelled αSH3 sample, where amide protons are back substituted with 20% H2O / 80 % D2O. Experiments are carried out at spinning off-angle and and respectively, at a B0 field of 9.4T (400 MHz for 1H) and a MAS frequency of 20 kHz. (B) Traces through the cross peaks of residues G51 and G5 residues along the 1H dimension at different spinning angle. (C) Signal intensities of G5 (black, asterisks), T4 (red, square), D62 (purple, triangle) and G51 (blue, circles) as a function of the rotor off-angle. The intensity for a particular was estimated using an exponential fit. Intensities for all residues at has been normalized to 1 to allow for a better comparison of the sensitivity decay under off-MAS.

The imperfect setting of the magic angle translates into a poor signal to noise ratio in the decoupled HSQC experiment. HSQC experiments recorded (Figure 1A) at show lower intensities compared to an experiment recorded at . When , only a few resonances can be observed. We acquired decoupled HSQC experiments for a series of off-angles . Cross peak intensities for a few representative residues are shown in Figure 1C. Notably, the intensities of G51 decreases more rapidly as a function of the off-angle compared to D62 It has been shown previously that G51 has a 15N-1H dipolar coupling (10255.16 Hz) that is close to the rigid limit (11477.6 Hz, corresponding to a 15N-1H bond length of 1.02 A) 20, whereas D62 shows a reduced dipolar coupling (6780 Hz) as it undergoes motion on the ns-μs timescale. As the anisotropy for G51 is larger than for D62, the intensity of G51 decreases more rapidly as increases. Intensities of very flexible residues such as T4 and G5, which are only visible in INEPT based HSQC experiments, are least affected if the spinning angle is off from the magic angle. Notably, it was not possible to quantify the 15N-1H dipolar coupling for T4 and G5 using the CPPI method. Also REDOR yields a large uncertainty for these residues20.

In commercial probes, the magic angle adjustment has an accuracy of ca. 0.04°5. A more accurate setting of the spinning angle would allow to increase sensitivity of rigid amides by approximately a factor of 1.6 (Figure 1C).

The observation that the residue-specific signal intensities decay differently as a function of motivated us to investigate whether the residual 15N-1H dipolar couplings can be related to the order parameter of particular residues.

To obtain a more quantitative picture how the spin-echo curve depends on the dipolar coupling, we carried out numerical simulations (Figure 2) following the description introduced by the Levitt group. If the sample rotates at the magic angle, the spin echo modulation frequency is determined solely by the scalar coupling. Under off-MAS condition, the oscillation frequency of the spin echo curve is not only governed by the scalar coupling, but also by the dipolar coupling between 1H and 15N scaled by. Following the work of Pileio et. al.7, the powder averaged echo modulation of the signal under off magic angle spinning for a pair of scalar coupled spins can be written as:

Equation 1

Where and denote the decay time constants. accounts for the decay of the orientation dependent component, where does not depend on powder averaging. In the phenomenological expression of the spin echo signal, p is the fraction of signal component independent of crystallite orientations, whereas J denotes the scalar coupling between the involved spins and b is the dipolar coupling.

The modulated component is given by:

Equation 2

The analytical solution is derived as follows:

Equation 3

Where

Notably, positive results in a faster oscillation whereas negative yields slower oscillation when compared to . Frequency modulation from dipolar coupling to the ideal J oscillation curve is introduced through off angle and it is what we used for order parameter determination.



**Figure 2.** Numerical simulations of spin echo curves for a 15N-1H spin system assuming a dipolar and scalar coupling of 11477.6 and 90 Hz, respectively. The echo modulation frequency is increased for positive (A) and decreased for negative (B), respectively. As a result, the position of the echo maxima (3rd, as denoted by a black dashed line) moves to the left when increases (A) and to the right (B) when compared with simulations carried out for .

1H-15N spin echoes were recorded (Figure S1A and S1B) site specifically in a pseudo 3D experiment. 2D cross peak intensities at a given spin echo delay were fitted using six parameters with the analytical function (Equation 1). The experimental spin echo signals together with the best fit are shown in Figure 4 for G51 and D62.

Out of the six fit parameters {p, J, , b, T20and T2J}, {p, T20 and T2J} are empirical parameters, which govern the decay of the spin echo curves. On the other hand, {J, , b} determine the frequency of the spin echo oscillations. We show that for a wide range of {p, T20 and T2J} values, the relative error on the determination of {J, , b} is negligible (Figure S4).

We note that b and appear as one single parameter (b\*) in the analytical Equation 1. In order to determine the dipolar coupling b for a given residue, the knowledge of is necessary. In the experiments presented here, we use very small spinning off angles. These small deviations from the magic angle produce a significant effect on the 15N-1H spin echo modulation while the spectral resolution is not compromised. Initially, we determined in a reference experiment using u-13C-glycine7. However, C-C dipolar couplings (~2.1kHz) are far less sensitive for an accurate determination of small off-angles. N-H dipolar couplings (~11.5 kHz), on the other hand, gives a 5 times larger modulation to the scaler coupling oscillation at a defined value. We therefore decided to fit together with the other parameters that determine the spin echo modulations. The residues M25, L31 and V44 yield the largest 15N-1H dipolar couplings. We therefore assumed the rigid limit values for these residues to yield . At the same time T2’ for the deuterated sample is increased with respect to the protonated sample, allowing a more sensitive acquisition of the echo modulation curves.



**Figure 3.** Experimental spin echo evolution curves represented for G51 (black) and D62 (red) respectively. The experiment was carried out at a spinning off-angle at a B0 field of 18.8T (800 MHz for 1H) and rotor spinning frequency of 55 kHz. 1D traces through the cross peak of residue G51 are shown for several echo delays.

1H-15N dipolar coupling values measured by the off-MAS method as a function of residues are shown in Figure 4A. We obtain an excellent correlation (Figure 4B) if the off-MAS dipolar coupling values are correlated to dipolar coupling derived using CPPI by Chevelkov et. al 17.



**Figure 4.** (A)Site specific 1H-15N dipolar couplings measured using a uniformly 2H, 13C, 15N labelled αSH3 sample, where amide protons are back substituted with 80 % D2O. Experiments were carried out at employing 55 kHz MAS and B0 field of 18.8 T (800 MHz for 1H). (B) Comparison of dipolar coupling values using two different methods, CPPI and Off-MAS, respectively. It was not possible to determine order parameters for T4 and G5 using CPPI as these residues are very dynamic. However, it is possible to measure the 1H-15N dipolar couplings for these residues using the off-MAS approach.

**Conclusions**

We have shown that order parameters can be extracted from 1H-15N correlation spectra that are recorded with a subtle mis-setting of the spinning angle. The magic angle mis-adjustment (on the order of 0.03°) is so small that the quality of the correlation spectrum is not compromised.  The presented method is applicable at any rotation frequency, and will allow to circumvent limitations of pulsed recoupling techniques at very fast spinning. Using off-magic angle spinning, very small order parameters can be measured very reliably, which is not possible with conventional methods.

**Materials and Methods**

**Sample Preparation:** SH3 domain of chicken α-spectrin was produced following procedure described earlier22. Protein was expressed using 15NH4Cl, u- [2H, 13C] glucose as the nitrogen and carbon sources, respectively. All buffers used for the M9 medium contained 100 % D2O. The sample was lyophilized twice in 20 % H2O / 80 % D2O at pH 3.5 before crystallization. To increase crystallization, pH was shifted to 7.5 using NH4OH. Subsequently, the crystals were soaked in 75 mM Cu(II)-EDTA buffer to reduce the 1H T1 times.

**NMR Spectroscopy:** NMR experimentswere carried out using Bruker Avance III spectrometers operating at a 1H Larmor frequency of 400 (9.4 T) and 800 MHz (18.8 T) employing 20 kHz and 55 kHz MAS, respectively. In order to measure the scalar coupling in solution, we employed a heteronuclear spin echo method.

**800 MHz:** 1H and 15N hard pulses were applied using a RF field strength of ω1H/2π = 166.7 kHz, ω15N/2π = 62.5 kHz RF fields, respectively. The sample temperature was maintained at 10 °C with a Bruker BCU-X unit, setting the cooling gas flow rate to 1100 litres per hour. 16 spin echo delays were measured with 32 scans each employing recycling delay of 1s (d1). The maximum echo delay was set to 81.9 ms. Each of 16 spin echo delays increase delay time of 5.45 ms (rotor synchronize to 55 kHz MAS); acquisition times in the 1H, 15N dimensions were set to 40 and 35 ms, respectively. The increments in the indirect dimension were rotor synchronized for every 2D plane.

**400 MHz:** 1H and 15N hard pulses were applied using a RF field strength of ω1H/2π = 100 kHz, ω15N/2π = 33 kHz RF fields, respectively. The sample temperature was maintained to 10 °C by Bruker BCU-X, setting the cooling gas flow rate to 935 litres per hour. 16 spin echo delays were measured with 32 scans with recycling delay of 1s (d1), where the maximum echo delay was set to 90 ms. Each of 16 spin echo delays increase delay time of 6 ms (rotor synchronize to 20 kHz MAS); acquisition times in the 1H, 15N dimensions were set to 50 and 35 ms, respectively. The increments in the indirect dimension were rotor synchronized for every 2D plane.

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