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Setting the magic angle for fast magic-angle spinning probes

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ABSTRACT

Fast magic-angle spinning, coupled with ¹H detection is a powerful method to improve spectral resolution and signal to noise in solid-state NMR spectra. Commercial probes now provide spinning frequencies in excess of 100 kHz. Then, one has sufficient resolution in the ¹H dimension to directly detect protons, which have a gyromagnetic ratio approximately four times larger than ¹³C spins. However, the gains in sensitivity can quickly be lost if the rotation angle is not set precisely. The most common method of magic-angle calibration is to optimize the number of rotary echoes, or sideband intensity, observed on a sample of KBr. However, this typically uses relatively low spinning frequencies, where the spinning of fast-MAS probes is often unstable, and detection on the ¹³C channel, for which fast-MAS probes are typically not optimized. Therefore, we compare the KBr-based optimization of the magic angle with two alternative approaches: optimization of the splitting observed in ¹³C-labeled glycine-ethylester on the carbonyl due to the Ca-C *J*-coupling, or optimization of the H–N *J*-coupling spin echo in the protein sample itself. The latter method has the particular advantage that no separate sample is necessary for the magic-angle optimization.

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1. Introduction

In recent years, solid-state NMR has made considerable advancements towards being able to obtain "solution"-like spectra, i.e., proton-detected spectra with high resolution in the proton dimension [1–6]. This improvement has been made possible by the development of probes capable of magic-angle spinning [7,8] (MAS) at high frequencies, upwards to 110 kHz and beyond [9–11], and both accelerates assignment [12–18] and structure determination of proteins [19,20], as well as enabling detailed studies of protein dynamics [21–24].

To realize the full resolution enhancement by MAS the magic angle must be set with high precision. The residual dipolar coupling under sample rotation about an axis θ_r scales as

$$\omega_0^{lS} \propto \frac{3\cos^2(\theta_{\rm r}) - 1}{2},\tag{1}$$

leading to $\omega_0^{lS} = 0$ when $\theta_r = \arccos \sqrt{(1/3)} \approx 54.74^\circ$, the magic angle. Without having this condition met, one may spin arbitrarily fast without being able to eliminate coherent contributions to linewidth due to ω_0^{lS} .

Often the magic angle is set by detecting ⁷⁹Br in KBr on the ¹³C channel as described by Frye and Maciel [25]. The broadening in

* Corresponding authors. E-mail addresses: maer@ethz.ch (M. Ernst), beme@ethz.ch (B.H. Meier). K⁷⁹Br is primarily the result of a small first-order quadrupole coupling due to lattice defects in the cubic lattice of crystalline K⁷⁹Br. Since the broadening is almost fully heterogeneous, MAS fully refocuses the contribution due to the quadrupole coupling, leading to rotational echoes appearing in the free-induction decay (FID) once a rotor period. In the Fourier-transformed spectrum, this appears as a large central peak, with spinning side bands spaced at the MAS frequency. However, when spinning off the magic angle, then the residual quadrupole coupling, ω_0^Q , is not averaged to zero, so that it results in an incomplete refocusing of the rotational echo (equivalently, weaker and broadened lines in the spectrum). Thus, one may optimize the magic angle by adjusting it such that $\omega_0^Q \approx 0$. Practically, one optimizes the number of rotational echoes in the FID (while sitting on resonance with the ⁷⁹Br) or by comparing the amplitude of the second spinning sideband intensity to the height of the central peak in the spectrum. Rather low spinning frequencies are typically used, since spinning significantly faster than the size of the quadrupole coupling will almost eliminate the spinning sidebands [26]. Note that, in the case of faster MAS, one could maximize the length of the FID, or minimize the linewidth of the central peak, but typically such linewidth characterization is difficult to quantify reliably. One implements magic-angle optimization either by adding KBr into the same rotor as the actual sample, or more often, and especially for smaller rotors, uses a separate rotor filled completely with KBr to gain better signal to noise for the angle optimization. The estimated accuracy of the latter





method was estimated to be within 0.1° of the magic angle [25]. However, other methods exist, for example, using quadrupolar nuclei with larger quadrupole couplings in combination with satellite transition magic-angle spinning (STMAS) [27,28].

For fast-MAS, ¹H-detection optimized probes, the KBr approach has three problems:

- (1) Often, stable spinning frequencies can only be obtained above 10–30 kHz MAS, so that rotational echoes are not obtained (and direct linewidth measurement is often inaccurate).
- (2) Small rotor size reduces the signal available from KBr. Furthermore, circuit design of fast-MAS probes usually sacrifices the sensitivity of the ¹³C channel in favor of optimizing the ¹H channel sensitivity for better ¹Hdetection. Since ⁷⁹Br is detected by re-tuning the ¹³C channel to the ⁷⁹Br resonance, this leads to lower sensitivity when using ⁷⁹Br for magic-angle optimization.
- (3) One cannot typically include KBr in the same rotor as the sample of interest due to the limited rotor volume. Therefore, one requires an additional rotor, with the risk of changing the magic-angle setting when changing samples.

We compare two alternative approaches for setting the magic angle with the more common KBr method. In the first approach, one optimizes the splitting observed on the carbonyl of ¹³Clabeled glycine-ethylester, which is due to the $C\alpha$ -C' J-coupling. This addresses the first problem and second problem in part, by not requiring a particular spinning frequency, and increasing signal to noise via the use of a fully labeled ${}^{13}C$ sample, use of ${}^{1}H{-}^{13}C$ cross-polarization, narrower linewidths, and elimination of sidebands. In the second approach, one optimizes the intensity of the J_{NH}-coupling mediated spin echo in a deuterated protein by eliminating the orientation-dependent ω_0^{IS} term of the N–H dipole coupling. The latter approach can be used under fast-MAS conditions $(\sim 100 \text{ kHz})$, and uses ¹H detection to increase signal to noise. It has the advantage that it can be performed on the sample of interest (therefore we refer to it as the on-sample method), with the best performance on partially deuterated samples. This solves the third problem by eliminating the need for an additional sample. Here, this method is demonstrated with deuterated and backexchanged ubiquitin, but also with a fully protonated N-formyl-MLF tri-peptide (although with better results with the former sample). The two discussed approaches are related to methods used for the characterization of the rotation angle in off-MAS experiments to measure homonuclear dipolar couplings [29,30].

2. Background

In all three approaches to magic-angle optimization (KBr, glycine-ethylester, and on-sample), our goal is to minimize the relevant anisotropic interaction. Minimization of ω_0^Q in KBr is achieved simply by using a one-pulse experiment (Fig. 1A) and optimizing the ratio of the second sideband of KBr and the main peak. Using glycine-ethylester, one minimizes primarily the residual chemical-shift anisotropy (CSA) of the carbonyl, ω_0^l , where spectra are then acquired with a ¹H-¹³C cross-polarization (CP) experiment with ¹³C detection and ¹H decoupling (Fig. 1B). One then optimizes the magic angle to obtain maximum resolution between the two C' peaks (and consequently the peak heights) that results from the $C\alpha$ –C' J-coupling of 55 Hz (the glycine-ethylester molecule is shown in Fig. 1B, with the C' highlighted). Maximum separation of these two peaks indicates that the residual CSA has been minimized (additional contributions also arise from the $C\alpha$ –C' dipole coupling). Note that alternatively, one could apply a



Fig. 1. Experimental pulse sequences for setting the magic angle. (A) shows a simple, one-pulse sequence for optimization using KBr. (B) shows a cross-polarization sequence used to polarize ¹³C' magnetization before acquiring a 1D spectrum to measure the C' peak separations. Also shown is the glycine-ethylester molecule, with the C' highlighted. (C) shows the on-sample method, using *J*-coupling evolution under a spin-echo sequence. This experiment can be either run as a 1D with a fixed value of τ , or as a pseudo-2D with variable τ . Small and wide black rectangles represent 90 and 180° pulses, respectively and open rectangles stand for CP transfer spin-lock fields. MISSISSIPPI water suppression (grey rectangle) and WALTZ64 decoupling are used. Orange rectangles denote the spin-echo periods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spin-echo with total delay τ , after ${}^{1}H{-}{}^{13}C$ CP, and find the maximum of the negative signal for $\tau = 1/J$ (similar to the on-sample method proposed below, and as described in [29]), although this approach was not used in this study.

Minimization of ω_0^{IS} using the on-sample method is based on the spin-echo method as originally proposed by Pileio et al. [29] (Fig. 1C). After CP transfer to ${}^{15}N$, a spin-echo period (red overlay) with π -pulses both on ${}^{1}H$ and ${}^{15}N$ is implemented. This allows magnetization to evolve under the *I*-coupling from in-phase into anti-phase and back (assuming the magic angle is correctly set). With the transverse magnetization on ¹⁵N one benefits from the longer ¹⁵N transverse relaxation times and facilitates the implementation of water suppression afterwards, e.g. by using MISSIS-SIPPI where necessary [31], before the magnetization is transferred back to ¹H for detection. When the echo time is incremented, the result is a ¹H-detected oscillation curve with the typical frequency of the ¹H-¹⁵N *J*-coupling of 92 Hz. However, if the magic angle is not correctly set, then evolution occurs both under the *J*-coupling and under the residual dipole coupling, ω_0^{lS} , resulting in an attenuation of the oscillating curve (vide infra) due to the anisotropic contribution, which indicates a maladjustment of the magic angle.

 $\omega_0^{I_5}$ depends on the orientation of the H–N bond relative to the rotor axis, so that different crystallites will oscillate with different frequencies unless $\omega_0^{I_5}$ is zero. This is shown in Fig. 2, where the spin-echo period in the pulse sequence of Fig. 1C is simulated assuming a ¹H–¹⁵N spin pair with dipole coupling anisotropy of $\delta_{IS} = -22,944$ Hz, and *J*-coupling of 92 Hz, using SIMPSON [32]. In Fig. 2C, a simple oscillation with frequency of *J*/2 is observed when the rotor angle is at the magic angle. However, in Fig. 2A and E, one observes attenuations to the oscillations when the angle is offset by ±0.1°. Smaller attenuations are observed when the angle offset is set to ±0.03°. The attenuation of the signal due to anisotropic contributions can be detected with sufficient signal to noise on top of the attenuation due to relaxation that appears independent of the angle setting. Thus, in simulation, we see improvement com-



Fig. 2. Simulations of magic-angle calibration as a function of τ and rotor angle (pulse sequence in Fig. 1(C). (A) and (E) show the amount of S_x magnetization as a function of τ with the rotor angle set off of the magic angle by ±0.1°, respectively. (B and D) show an offset of ±0.03°, and (C) shows the rotor angle set to the magic angle. Dotted lines show expected zero-crossings for a *J*-coupling of 92 Hz (without dipole couplings). Simulations were performed for an H–N pair, with a dipole coupling anisotropy of $\delta_{1S} = -22,944$ Hz, and *J*-coupling of 92 Hz, MAS frequency of 100 kHz, and a π -pulse with 50 kHz field strength, using SIMSPON [32].

pared to the accuracy of KBr, which was originally suggested to be 0.1° [25].

3. Materials and methods

Hardware: All experiments were performed on a Bruker Biospin Avance III 850 MHz (¹H Larmor frequency) spectrometer. The two-dimensional (2D) ubiquitin spectra, and one-dimensional (1D) MLF spectra were acquired at 93 and 90 kHz MAS, respectively using a home-built fast-MAS, proton-detection optimized, triple-resonance probe (Ago Samoson, Tallinn, Estonia) with 0.8 mm rotors. Other 1D data were acquired using a commercially available Bruker 0.7 mm probe, where KBr, glycine-ethylester, and ubiquitin spectra used spinning frequencies of 3.2, 60, and 100 kHz MAS respectively. In order to acquire spectra at 3.2 kHz spinning frequency, manual adjustment of the bearing and drive was used.

The angle increment per one complete turn of the magic-angle setting rod of the 0.7 mm probe was calculated to be 0.08° , so that each of 100 markings yields an increment of 0.0008° . Steps between rotor angles used for optimization were either 10 or 20 markings (0.008° or 0.016°). The step for a complete turn was calculated based on a 0.05 mm stroke of magic-angle setting rod, which has a distance to the stator rotation axis of 24 mm.

Samples and rotor filling: 100%-HN-[²H,¹³C,¹⁵N] ubiquitin was prepared by over-expression of uniformly ²H,¹³C,¹⁵N-labeled ubiquitin in *E. coli* and crystallization in protonated 2-Methyl-2,4-pentanediol (MPD) with H₂O to re-protonate the exchangeable sites as previously described [33]. The rotors were filled by ultracentrifugation with filling tools [34] adapted to the 0.8 and 0.7 mm rotors. The finely ground powdered samples of glycine-ethylester (1,2-¹³C,¹⁵N labeled), KBr, and MLF (U-¹³C,¹⁵N-labeled N-formyl-methionyl-leucyl-phenylalanine) were packed into the rotors directly, using the provided filling tools.

NMR Experiments: KBr 1D spectra were acquired with 2048 scans using a 90° pulse of 2.5 μ s length with an acquisition time of 0.02 s and a recycle delay of 0.094 s (determined to be optimum, based on a T_1 relaxation time of 0.075 s).

For the glycine-ethylester ¹³C-detected 1D spectra at 60 kHz MAS a DQ cross-polarization [35] transfer was optimized to 100 kHz RF-field on ¹H and 20 kHz RF-field on ¹³C using a contact time of 6.5 ms with a tangential ramp on the ¹H channel. Pulses were set to 100 kHz RF-field strength on the ¹H and ¹³C channel. During the acquisition time of 0.16 s, frequency-swept low-power TPPM [36] of 3.4 kHz was used on the ¹H channel. Typically, 32 scans were acquired with a recycle delay of 1.26 s.

J-coupling oscillating pseudo-2D spectra and magic-angle optimizing 1D spectra of ubiquitin were performed on the 0.7 mm probe at 100 kHz MAS. CP transfers were optimized to 1 ms at the ZQ condition of 125 and 15 kHz RF-field strength on the ¹H and ¹⁵N channel, respectively, and pulses set to 125 and 62.5 kHz RF-field strength on the ¹H and ¹⁵N channel, respectively. MISSIS-SIPPI [31] water suppression was used for 100 ms at 5 kHz on the ¹H channel and during the acquisition of 70 ms WALTZ64 [37] decoupling of 5 kHz was employed on the ¹⁵N channel. The recycle delay was 1.0 s.

The HNH 2D spectra of ubiquitin were acquired at 93 kHz MAS using the 0.8 mm probe at 16.7 °C sample temperature as determined by the relative chemical shift of the bulk water line to the MPD line referenced to 4.1 ppm. Polarization transfer was achieved by a DQ CP of 1.2 ms with 16 and ~ 80 kHz RF-field strength on the ¹⁵N and ¹H channel, respectively and an increasing and decreasing tangential ramp on the ¹H channel for the transfer from and to ¹H. 50 ms MISSISSIPPI at 5 kHz was used for water suppression. ¹⁵N decoupling during 70 ms of direct acquisition (5550 data points, 46.7 ppm spectral width) was achieved with WALTZ64 at 2.5 kHz, and xix-CW [38] ¹H decoupling was used during 58 ms of indirect acquisition (300 data points, 30 ppm spectral width). The KBr-optimized spectrum was acquired with 32 scans, and the on-sample optimized spectra was acquired with 16 scans, both with a recycle delay of 1.0 s.

Data processing and analysis: Initial processing (apodization with QSINE 2.5 and Fourier transformation of the 2D spectra) were done in TopSpin (Version 3.5pl5, Bruker) while plotting and further analysis were performed in MATLAB (Release 2016b, The Math-Works Inc. Natwick, MA, USA, 2016) using INFOS spectrum fitting software [39]. Median linewidths for spectra shown in Fig. 5 were obtained by fitting using the FitSpec function, using 71 peaks to fit each of the two spectra. Gaussian broadening was used for fitting in the ¹⁵N dimension, and Lorentzian broadening was used in the ¹H dimension.

Simulations: Simulations were performed with SIMPSON [32]. In Fig. 2, a ¹H-¹⁵N spin-pair was simulated, with a J-coupling of 92 Hz, and a dipole coupling of $\delta_{1S}/2\pi = 22,944$ Hz. Initial ¹⁵N magnetization along the *x*-axis was allowed to evolve for a delay $\tau/2$, followed by a 50 kHz π -pulse on both the ¹H and ¹⁵N channels, a second delay of $\tau/2$, and subsequent detection of ¹⁵N *x*-magnetization. The MAS spinning frequency was set to 100 kHz, and τ was incremented by 300 µs. In Fig. 5E, transverse magnetization evolution of a single ¹H or ¹⁵N spin was calculated with the rotor angle set to 54.65°, with a 100 kHz spinning frequency, 2000 time points, and a time step of 5 ms. CSA parameters for ¹⁵N were δ = 120 ppm, η = 0.4 [40], and CSA parameters for ¹H were δ = 9 ppm, η = 0.9 [41,42] ($\gamma_{1H}B_0$ = 850 MHz). 1 Hz of Lorentzian broadening was applied to the resulting signal before Fourier transform.

4. Results

Fig. 3 shows spectra for each of the three magic-angle optimization methods (KBr, glycine-ethylester, and on-sample), where the rotor angle has been gradually incremented. We begin comparing KBr and the glycine-ethylester methods. KBr spectra were acquired at a spinning frequency of 3.2 kHz, using a Bruker 0.7 mm probe. Note that to obtain stable spinning at 3.2 kHz, we use manual adjustment of drive and bearing pressure, since the minimum spinning frequency with the automatic unit is 30 kHz. Both KBr and glycine-ethylester require detection on the ¹³C channel on the ¹H detection-optimized probe. However, the sensitivity of the glycine-ethylester compared to natural abundance KBr is much higher. This is mainly because only $\sim 4\%$ of the KBr signal is found in the second sideband, whereas the full signal of glycineethylester is split between only two resonances resulting from the *I*-coupling (50% of signal in each resonance).

Then, whereas for KBr about 2048 scans were necessary to obtain a 1D spectrum sufficient for analysis (233 s), 32 scans suffice for the ¹³C-detected spectrum of glycine-ethylester (45 s), in which time on obtains signal to noise that is 3.5 times larger than that obtained with KBr (28 times higher signal to noise per scan). As a further advantage, the spectrum can be acquired at any spinning frequency, therefore, allowing a spinning frequency high enough to be stable and eliminate spinning sidebands, but low enough to avoid possible problems with frictional sample heating (so that one does not require waiting for stabilization of cooling via variable temperature (VT) gas). As a result, this method is less time-consuming compared to setting the magic angle with KBr. Fig. 3A and B show series of spectra for with the first sideband of KBr and the CO region of glycine-ethylester at different angle settings, respectively, each differing from each other by 0.016°. Then,



Fig. 3. Experimental comparison of optimization methods. (A) shows the second sideband of KBr spectra (normalized to the central peak) for 16 rotor angles, incremented by 0.016°. (B) shows the intensity and splitting of the C' resonance for glycine ethyl-ester for 14 rotor angles, incremented by 0.016°. (C) shows the intensity of the on-sample method for 15 rotor angles, incremented by 0.008°. Note that spikes in the noise appear due to incomplete water suppression. All x-axes are given as an offset from the spectrum with the highest intensity (so that 0 corresponds approximately to the magic angle, 54.74°). Y-axes are arbitrary, scaled so that the maximum (minimum) is ±1.0.

in addition to reducing experimental time, the glycine-ethylester method has another benefit: the sensitivity to magic-angle deviations is significantly higher than for KBr.

On the other hand, several challenges remain when using the glycine-ethylester method: (1) The splitting also depends on the ¹H decoupling during acquisition which needs to be carefully optimized and introduces further parameters, not allowing for a straightforward comparison between different setups and experiments. (2) If performed at lower spinning frequencies without VT cooling gas, the experimental conditions are different than the ones used for measuring proteins under fast MAS. This difference in temperature can lead to changes in the probe material and, therefore, to changes of the magic angle. In principal, the same experimental conditions could be used, but this adds extra time requirements to the setup experiments.

The on-sample method saves time because the final experimental conditions are the same as those required for magic-angle optimization, and one does not risk potential disturbance of the magic angle due to sample changes. The experimental time is longer, however, 128 scans were required for the on-sample method in Fig. 3, so that each spectrum required 137 s, and had 2.3 times lower signal-to-noise than glycine-ethylester. As we have shown in Fig. 2, the frequency and shape of the oscillation is affected, when the magic angle is not properly set. One may either optimize the first maximum of negative intensity ($\tau = 10.87 \text{ ms} = 1/92 \text{ Hz}$) or the zero-crossing ($\tau = 5.43 \text{ ms} = 1/(2 * 92 \text{ Hz})$). The zero-crossing can be easier to optimize, however, it is more sensitive to variations in the J-coupling, and furthermore, complete mis-set of the magic angle may also lead to zero signal. In this case, it may be more practical to optimize the negative maximum of the signal, especially during initial optimization, where the rotor angle may be far away from the magic angle. Also, compared to the first positive maximum, the absolute value of the signal is higher due to lower relaxation losses during the spin-echo evolution. The procedure used here was to first find the maximum negative intensity in 1D experiments with τ = 10.87 ms, and subsequently optimize on the zero-crossing with τ = 5.43 ms. Then, one may verify the angle quality by checking the frequency of the complete oscillation by performing a pseudo-2D experiment for which τ is incremented. One should be aware that relaxation will damp the curve; although we saw in simulations in Fig. 2B/D, that angle offsets look like damping of the curve, this is accompanied by shifting of the zero-crossings, which will not occur if pure relaxation leads to curve damping.

In Fig. 3C, the negative maximum signal using the on-sample method is shown ($\tau = 10.87$ ms), as a function of rotor angle. We see that the precision of the magic-angle setting is similar to that obtained with glycine-ethylester (Fig. 3B). We may further verify the accuracy of the magic-angle setting by varying τ as a pseudo-2D experiment, after optimizing the zero-crossing as well ($\tau = 5.43$ ms). The integrated intensity as a function of τ is shown in Fig. 4A, where we see that although the signal decays, zero-crossings appear in the expected positions, indicating that angle mis-set is not a large source of the signal decay (also verifying that the average *J*-coupling in the sample is ~92 Hz).

In principle, the on-sample method can also be used on fully protonated samples. For example, we have optimized the negative maximum using the on-sample method with a sample of fully protonated MLF. The resulting signal as a function of τ is shown in Fig. 4B (red curve). One observes a distorted oscillation, and zero crossings no longer occur exactly at the expected positions. However, when the sample is exchanged with deuterated ubiquitin, without readjustment of the magic angle, one obtains an undistorted oscillation, with the zero crossings occurring at the correct positions. This suggests that optimization of the negative maximum using the on-sample method with fully protonated samples



Fig. 4. Integrated intensity of signal as a function of τ using the on-sample method. (A) shows the signal as a function of τ obtained by optimizing the angle and measuring on deuterated ubiquitin (pulse sequence in Fig. 1(C). (B) shows the signal as a function of τ obtained by optimizing the angle using fully protonated MLF. The solid red curve shows the signal as a function of τ obtained on MLF, and the blue dotted line shows the signal obtained on deuterated ubiquitin, using the angle setting obtained with MLF. Gray dotted lines in (A) and (B) indicate the expected zero-crossings. Note that proximity of the zero-crossings to the expected values indicate that signal decay is not due to mis-setting of the magic angle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can be used for magic-angle optimization. However, the distortion shows that fast-MAS (in this case, 90 kHz) is not sufficient to eliminate residual coherent effects resulting from the dense proton network. As a result, although one can optimize the magic-angle, it is not straightforward to verify the quality of the optimization via measurement of signal as a function of τ . Additionally, the faster damping of the fully protonated sample reduces the sensitivity of this method (a problem that could be exacerbated by the more complex dynamics of a protein sample).

5. Discussion and conclusion

Our comparison of three methods of optimization, when using probes optimized for fast-MAS and ¹H detection, suggests that considerable improvement may be made on the traditional KBr optimization method. Although with patience, one may still achieve 0.1° accuracy using KBr, as previously indicated, both the glycine-ethylester methods and on-sample methods achieve better quality angle optimization (an alternative approach would be to achieve improved angle optimization would be to use quadrupole nuclei with a larger quadrupole coupling at utilized STMAS [27,28], but this requires a more complex optimization and yet another sample). On the other hand, glycine-ethylester and on-sample methods yield similar quality optimization of the magic angle. With this in mind, it is hard to ignore the convenience of the on-sample method. One does not require an additional sample, and all experimental conditions can be the same for the magic-angle optimization and the experiments of interest.

Note that, so far we have shown the variation in several spectra as the rotor angle is adjusted, but after performing the sweep of the rotor angle as in Fig. 3, one must still go back to re-locate the magic angle. Then, to find the correct magic-angle setting, we suggest the usual procedure: acquiring 1D spectra after each adjustment of the angle as in Fig. 3, until one is beyond the optimum spectrum (as determined by highest second-order sideband to central band ratio in KBr, largest splitting in glycine-ethylester, and largest negative maximum or zero-crossing in ubiquitin). To get the correct angle then, turn backwards the same increments and again acquire 1D spectra at each turn, until the optimum is reached again.

In a final comparison using a model sample of deuterated, and back-exchanged ubiquitin, two-dimensional spectra are shown in Fig. 5, where the magic angle was taken at the optimum angle setting from a series of spectra (such as is shown in Fig. 3A/C for the KBr and on-sample methods, respectively. Fig. 5A/B show the resulting 2D spectra for the KBr and on-sample methods. Peaks appear narrower in Fig. 5B compared to A, and this is verified by overlaying slices taken through the largest six peaks, shown in Fig. 5C. We may also compare the linewidths in the ¹H and ¹⁵N dimensions for the two spectra, obtained via fitting [39]. The distributions are shown as histograms in Fig. 5D, where ¹H and ¹⁵N median linewidths are found to be 75 and 52 Hz, respectively for the KBr-optimized magic angle, and 57 and 39 Hz when the magic angle is optimized via the on-sample method. To verify that these changes in linewidth are due to magic-angle mis-set, we simulate the broadening of ¹H and ¹⁵N resonances due to the CSA for a small mis-set (0.09°) in Fig. 5E. This mis-set yields an increase in ¹⁵N full width at half max of 13 Hz, consistent with the observed broadening, although one sees that the base of the lineshape is broader, so the actual mis-set may be less than that used in simulation.



Fig. 5. Two-dimensional spectra acquired after magic-angle optimization with KBr and on-sample methods. (A) shows a 2D ubiquitin spectrum, acquired after setting the magic angle to the best setting using KBr. (B) shows a 2D ubiquitin spectrum acquired after setting the magic angle using the on-sample method. (C) selects the six largest peaks in the spectrum, and shows an overlay of the two spectra as slices through the ¹H and ¹⁵N dimensions (KBr: blue, on-sample: red). Peaks have been centered on top of each other and scaled to the same maximum for better linewidth comparison. (D) shows histograms of the ¹⁵N (top) and ¹H (bottom) linewidths using KBr (blue) and on-sample (red) methods. A black line in each plot indicates the median linewidth. (E) shows simulated lineshapes of ¹⁵N and ¹H, with broadening due to an angle mis-set of 0.09° ($\theta_r = 54.65^\circ$). Simulations are 1-spin simulations, with broadening due to the CSA of the respective spin (signal was processed with 1 Hz Lorentzian broadening). CSA parameters: ¹⁵N: $\delta = 120$ ppm, $\eta = 0.4$ [40], ¹H: $\delta = 9$ ppm, $\eta = 0.9$ [41,42]. The full width at half the peak maximum, and the width are at the base are indicated in each plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Simulated ¹H broadening is less than observed for the same rotor angle, but this can result from ¹H–¹H couplings not included in the simulation. Overall, the expected linebroadening due to angle misset is consistent with the observed differences and the increased precision supported by the on-sample method is indeed relevant in actual experiments. Thus, we obtain a significant improvement in linewidth due to improvement of the magic-angle setting.

Clearly, the on-sample and glycine-ethylester methods of magic-angle setting offer improvements over KBr. In the case of glycine-ethylester, this is primarily attributed to improvement in signal to noise (or optimization time) and does not require special setups to achieve slow spinning frequencies (3–4 kHz for KBr). The on-sample method has the added benefit that one can skip the additional step of changing samples for magic-angle optimization, although its quality is only easily verified for partially deuterated samples and for samples with reasonably high signal-to-noise ratio.

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