Introduction to Proton Detection in Biological Samples under Ultra-Fast Magic Angle Spinning

Venita Daebel
Bruker BioSpin GmbH, Rheinstetten, Germany

Protons are characterized by having a natural abundance of more than 99.9 %, as well as a high gyromagnetic ratio. These attributes provide much greater detection sensitivity than those of $^{13}$C or $^{15}$N, making $^1$H-detected NMR spectroscopy very attractive, not just for solution NMR.[1-6]

Soluble samples benefit from the fact that unwanted dipolar couplings are cancelled out by molecular tumbling, a feature that insoluble molecules lack. As a result, a strong network of homonuclear proton dipolar couplings dramatically broadens the linewidth of any $^1$H signal in solid-state NMR.

Two approaches especially have been shown to overcome this obstacle; sample preparation using perdeuteration-reprotonation to dilute the dense proton network[1-3], and the use of increased magic angle spinning (MAS) speeds in the order of 60 to 111 kHz (Figure 1).[3,5]

In collaboration with Guido Pintacuda and co-workers, Bruker BioSpin presents three basic $^1$H-detected pulse programs (‘pulprog’) that allow initial protein backbone assignment under fast MAS[3]:

1. hNH: 1D and 2D, Figures 1A and 3, pulprog: hNH2D.dcp
2. hCaNH: 1D-3D, Figures 1B and 4, pulprog: hCaNH3D.tcp
3. hCONH: 1D-3D, Figures 4 and 6B, pulprog: hCONH3D.tcp
Increasing 1H spin dilution will not only reduce unwanted dipolar couplings (Figure 2, purple), but the overall 1H signal-to-noise ratio. Furthermore, selective dipolar transfer pathways may not be possible, for example due to the low propensity of two protons being in close proximity. Moreover, in the regime of fast MAS, it depends on the experimental conditions whether dipolar or scalar coupling-based experiments are preferable (Figure 2, orange and green), and so when using fast MAS rates, experimental conditions should be carefully chosen. Alternatively, the use of strong dilution combined with moderate MAS rates or full reprotonation under (ultra-)fast MAS, where the spinning is sufficient to weaken the 1H dipolar couplings (Figure 2, red and black), is recommended.

Figure 2: Schematic illustration of different aspects of protonation (red) and MAS respectively. In proportion to the MAS rate and/or reprotonation level, spectral resolution increases and longer-lived 13C coherences allow the use of homonuclear scalar coupling-based polarization transfers (orange). Dipolar coupling-mediated transfer starts to become inefficient at MAS rates >20 kHz, due to averaging effects (green). Using strongly deuterated samples, 1H dipolar couplings are already effectively attenuated at moderate MAS rates (purple). Under fast to ultra-fast rates, MAS averages out 1H dipolar couplings efficiently, even in fully protonated samples (black).

Experiments and Pulse Programs

For 1H-detected experiments, the easiest way to begin with is the heteronuclear nNH experiment, which is comparable to a solution NMR HSQC experiment, but which is based on CP. This correlates the amide proton to its nitrogen (Figure 3B, purple). Using the hCaNH sequence (Figure 3B, orange), and Figure 4A), the Ca of a residue can be correlated to the amide
The off-resonance pulse causes phase evolution of the on-resonance spins, an effect often referred to as the ‘Bloch-Siegert shift’ in solution NMR\(^{10}\) (Figure 5A, purple). Furthermore, the initial evolution time, \(t_1(0)\), is greater than zero, resulting in unwanted initial chemical shift evolution for the first TD point (Figure 5A, red).

To compensate for both these effects, an on-resonance \(\pi\) pulse needs to precede \(t_1\) (Figure 5A, orange). For complete refocusing (Figure 5B), a symmetry construct (Figure 4, yellow) centered around this pulse has to be created, resulting in a second off-resonance \(\pi\) pulse and a symmetry delay (\(\Delta\)) which compensates for \(t_1(0)\). Both are applied consecutively to the initial \(^1\text{H}\) to \(^{13}\text{C}\) CP.

Any other TD point is acquired with a \(t_1\) larger than \(\Delta\), leading to desired additional chemical shift evolution, which is unaffected by refocusing (Figure 5C, cyan).

For heteronuclear decoupling (Figure 4, white pulses), a \(^{15}\text{N}\) \(\pi\) hard pulse and \(^1\text{H}\) low power decoupling are applied during \(t_1\), followed by a SPECIFIC \(^{13}\text{C}\alpha\) or \(^{13}\text{CO}\) to \(^{15}\text{N}\) CP\(^{10,11}\) transfer (Figure 4, purple pulses).

Afterwards, both sequences equal the ‘hNH2D.dcp’ (Figure 3A), where \(^{15}\text{N}\) evolution (\(t_1\) or \(t_2\)) is attended by heteronuclear decoupling using a \(^{13}\text{C}\) \(\pi\) hard pulse (white pulse) and \(^1\text{H}\) low power decoupling.

To suppress the water signal, continuous wave (cw, dark and light gray) pulses are applied alternately along X and Y, on-resonance with the water peak.

Figure 4: 3D pulse sequences of \(^1\text{H}\)-detected ‘hCaNH3D.tcp’ (A) and ‘hCONH3D.tcp’ (B) for obtaining intraresidue \(\text{\textalpha -N-H}\) and interresidue \(\text{CO-N-H}\) correlations respectively. Initial magnetization derives from \(^1\text{H}\), followed by \(^1\text{H}\) to \(^{13}\text{Ca}\) or \(^{13}\text{CO}\) CP (light blue). Centered during \(t_1\), an off-resonance band-selective \(\pi\) pulse is used for \(J_{\text{CO-Ca}}\) decoupling (light and dark green bell-shaped pulses). Phase evolution is compensated by a band-selective \(\pi\) pulse pair preceding \(t_1\) (see Figure 5 for details). Once magnetization is transferred back to \(^{15}\text{N}\) (purple CP step), the experiments follow the same scheme as that of the hNH experiment (Figure 3A). Pulses are applied along X unless otherwise stated (red letters).

With the exception of ‘hNH2D.dcp’, the pulse sequences are applied in triple channel mode with \(^1\text{H}\) on channel f1, \(^{15}\text{N}\) on f2 and \(^{13}\text{C}\) on f3. If the ‘-DTC’ (‘triple channel’) flag is set in the header of each pulprog, the carrier frequency \(f_2\) and \(f_3\) of Figure 3A, orange pulses), \(\nu_3\) or \(\nu_3\) pulses, which is followed by selective CP steps to \(^{13}\text{Ca}\) or \(^{13}\text{CO}\) (Figure 4, light blue pulses).

In ‘hCaNH3D.tcp’ and ‘hCONH3D.tcp’, the carrier frequency for RF irradiation (offset ‘o3’) is on-resonance with \(^{13}\text{Ca}\) or \(^{13}\text{CO}\) respectively. During the indirect evolution (\(t_1\)), homonuclear \(J_{\text{CO-Ca}}\) coupling must be decoupled using a \(t_1\)-centered, band-selective \(\pi\) pulse, which is applied off-resonance, i.e. a CO decoupling pulse during \(\text{Ca}\) evolution in the hCaNH experiment, and vice versa (Figure 4, light and dark green pulses).

Figure 5: Schematic representation of Bloch-Siegert shift and initial \(t_1\) (\(t_1(0)\)) phase evolution compensation. Numbers refer to ‘hCaNH3D.tcp’/‘hCONH3D.tcp’ pulprog of Figure 4. (A) After the initial CP, on-resonance \(^{13}\text{C}\) spins (blue) are oriented along X (1). The first off-resonance \(\pi\) pulse creates a Bloch-Siegert shift, \(\nu_{\text{vis}}\), (2, purple arrows). During the symmetry delay, \(\Delta\), which equals \(t_1(0)\), additional chemical shift, \(\nu_3\), evolves (3, red arrows). Overall, evolution is reversed by the on-resonance \(\pi\) pulse (4, orange arrow) to refocus both \(\nu_{\text{vis}}\) and \(\nu_3\) (5 and 7). Steps of \(\frac{1}{2}\nu_3\) are due to split \(t_1\). While during \(t_1(0)\) chemical shift evolution is refocused completely (B), it further evolves (‘\(\times\)’, cyan arrows) for all \(t_1\) times larger than \(\Delta\) (C).
During this so-called MISSISSIPPI H2O suppression, 15N magnetization is stored along Z by a π/2 hard pulse. With a second π/2 hard pulse, 15N magnetization is brought back into the XY plane to transfer polarization to 1H in a final 15N to 1H CP (dark blue pulses), followed by 1H-detection under low power heteronuclear decoupling on 15N and 13C.

Which Experimental Conditions to Choose?

The following recommendations have been tested on a u-[13C, 15N]-labeled, perdeuterated and 100% 1H back-exchanged ubiquitin sample at 60 kHz MAS using a Bruker 1.3 mm rotor and a Bruker Avance III HD 600 MHz Wide Bore system.

The sample temperature of approximately 294 K was regulated with a BCU II, using a N2 gas flow of ~1,100 l/h to reach a set temperature of 257 K. Because faster MAS produces more frictional heating, the ideal sample temperature and the N2 gas flow required should be adjusted beforehand using, for example, 79Br. Nevertheless, a gas flow of more than 1300 l/h should be avoided to not cause rotor imbalances at high spinning speeds. Additionally, when using temperature regulation, frame cooling should be used.

As known from ‘conventional’ solid-state NMR, the Hartmann-Hahn-condition \( n \times v_R = v_I \pm v_S \) must be fulfilled to achieve CP transfer, with \( n \) as an integer (usually ±1 or ±2), \( v_R \) the MAS rate and \( v_I \) and \( v_S \) the RF field strengths acting on the I and S spins respectively. Addition of \( v_I \) and \( v_S \) is known as a double-quantum (DQ) transition, and subtraction as a zero-quantum (ZQ) transition. Under (ultra-)fast MAS, selective CP is efficient already at low power irradiation, resulting in higher sensitivity for the DQ CP condition compared to ZQ (Figure 6A).

For all initial and final CP steps (\( v_R = 60 \text{ kHz} \)), RF field strengths of 50 kHz applied on 1H and only 10 kHz on 15N or 13C, respectively, have been proven well.

SPECIFIC 13C to 15N CP gave best results using 35 kHz applied on 13C and 25 kHz on 15N. Here, the 50/10 kHz condition results in poor transfer efficiency and is thus not suited (Figure 6B). Nevertheless, on unknown samples it is always recommended to test different Hartmann-Hahn conditions.

During CP, the use of a shaped pulse on one of the two nuclei involved increases transfer efficiency dramatically because several Hartmann-Hahn conditions can be passed and \( B_1 \) inhomogeneity compensated (Figure 6A). A shape can be applied on either of the two nuclei, while the other nucleus experiences a rectangular pulse in the form of, for example, a ‘square.1000’ shape.

Here, linearly ramped up and ramped down shapes were applied on 1H for all initial and final CP steps respectively. Theoretically, a flat ramp from 90 to 100 % (e.g. ‘ramp90100.1000’/’ramp10090.1000’) should perform well at high MAS rates, ensuring that magnetization is transferred during most of the contact time. However, sometimes, a steeper ramp from 70 to 100 % (e.g. ‘ramp70100.1000’/’ramp10070.1000’) can be more efficient, as in the presented data.

For SPECIFIC 13C-15N CP, a tangential amplitude modulated shape, for example ‘tacn80’, applied on 15N is most efficient. According to solution NMR, Gaussian pulse cascades (e.g. ‘Q3.200’) can be applied for 256 and 350 µs for selective 13Ca and 13CO π pulses. Note that a longer pulse is more selective. When setting the constants ‘cnst21’ and ‘cnst22’ as frequency offsets for 13CO and 13Cα in ppm, respectively, all necessary frequency changes are calculated and set automatically in the pulprog.
During MISSISSIPPI, the H₂O saturation time (\( t_{\text{sat}} \) in Figures 3 and 4, ‘d19’ in pulprogs), should be optimized between 100 and 300 ms. Cw decoupling programs (cpdprg) are called ‘cwX_13nofq’ and ‘cwY_13nofq’. As the names imply, parameter ‘plw13’ is the corresponding power level, which should be set to \( \frac{1}{4} \) of the MAS rate, for example 15 kHz at 60 kHz MAS.

Note: The suffix ‘nofq’ reveals that no frequency switch is taking place with the cpdprg itself.

Likewise, heteronuclear decoupling of only 15 kHz on \(^1\text{H}\) is sufficient during \(^{13}\text{C}/^{15}\text{N}\) evolution times using ‘sltppm_12nofq’ at power level ‘plw12’.\(^{[16]}\)

---

Table 1: Summary of recommended parameters for \(^1\text{H}\)-detected experiments recorded at 60 kHz MAS. Parameters in bold text should be optimized. For all other parameters, it is sufficient to calculate/set the recommended values.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nuclei</th>
<th>Parameter names in pulprog</th>
<th>Rule of thumb</th>
</tr>
</thead>
<tbody>
<tr>
<td>90° (180°) hard pulses</td>
<td>(^{1}\text{H})</td>
<td>p3 (p4) @ plw2</td>
<td>–100 kHz (2.5 µs)</td>
</tr>
<tr>
<td></td>
<td>(^{15}\text{N})</td>
<td>p21 (p22) @ plw21</td>
<td>–42 kHz (6 µs), optimize in hNH2D.dcp: flag ‘–DN90’ (zero crossing)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>p1 (p2) @ plw1</td>
<td>–72 kHz (3.5 µs), optimize in hCXNH3D.tcpx: flag ‘–DC90’ (zero crossing)</td>
</tr>
<tr>
<td>180° selective pulses</td>
<td>(^{13}\text{C}_\alpha)</td>
<td>p24* @ spw24</td>
<td>–350 us @ shape: ‘Q3.2000’; open shape in ‘shapetool&gt; stdisp&gt; Analysis&gt; Integrate Shape’ and enter: ‘length of p24, ’180.0’, length of p1’, result tells by how much pldb1 needs to be changed for spdb24</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{CO})</td>
<td>p23* @ spw23</td>
<td>–275 us @ shape: ‘Q3.2000’; open shape in ‘shapetool&gt; stdisp&gt; Analysis&gt; Integrate Shape’ and enter: ‘length of p23, ’180.0’, length of p1’, result tells by how much pldb1 needs to be changed for spdb23</td>
</tr>
<tr>
<td>CP transfers</td>
<td>H-N</td>
<td>p25 @ spw42 (H) &amp; spw43 (N)</td>
<td>1-3 ms @ 50 kHz &amp; 10 kHz, shapes: ‘ramp90100.1000’ &amp; ‘square.1000’</td>
</tr>
<tr>
<td></td>
<td>H-(\alpha)</td>
<td>p18 @ spw38 (H) &amp; spw52 (C)</td>
<td>2-6 ms @ 50 kHz &amp; 10 kHz, shapes: ‘ramp90100.1000’ &amp; ‘square.1000’</td>
</tr>
<tr>
<td></td>
<td>H-CO</td>
<td>p19 @ spw39 (H) &amp; spw53 (C)</td>
<td>2-6 ms @ 50 kHz &amp; 10 kHz, shapes: ‘ramp90100.1000’ &amp; ‘square.1000’</td>
</tr>
<tr>
<td></td>
<td>Ca-N</td>
<td>p16 @ spw5 (N) &amp; spw50 (C)</td>
<td>3-10 ms @ 25 kHz &amp; 35 kHz, shapes: ‘tacn80’ &amp; ‘square.1000’</td>
</tr>
<tr>
<td></td>
<td>CO-N</td>
<td>p17 @ spw6 (N) &amp; spw51 (C)</td>
<td>3-10 ms @ 25 kHz &amp; 35 kHz, shapes: ‘tacn80’ &amp; ‘square.1000’</td>
</tr>
<tr>
<td></td>
<td>N-H</td>
<td>p45 @ spw47 (N) &amp; spw46 (H)</td>
<td>400-800 µs @ 50 kHz &amp; 10 kHz, shapes: ‘square.1000’ &amp; ‘ramp10090.1000’</td>
</tr>
<tr>
<td>Decoupling</td>
<td>(^{1}\text{H})</td>
<td>cpdprg1 @ pcpd1 &amp; plw12</td>
<td>‘sltppm._12nofq’ @ 33.33 µs &amp; ( \frac{1}{4} ) ( \nu_t ) (15 kHz)</td>
</tr>
<tr>
<td></td>
<td>(^{15}\text{N})</td>
<td>cpdprg4/5 @ d19 &amp; plw13</td>
<td>‘cwX_13nofq’/’cwY_13nofq’ @ 100-300 ms &amp; 15 kHz (=MISSISSIPPI)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>cpdprg2 @ pcpd2 &amp; plw16</td>
<td>‘waltz16_18nofq’ @ 25 µs &amp; 10 kHz</td>
</tr>
<tr>
<td>Offsets</td>
<td>(^{1}\text{H})</td>
<td>o1</td>
<td>on resonance with the water peak (~5 ppm)</td>
</tr>
<tr>
<td></td>
<td>(^{15}\text{N})</td>
<td>o2</td>
<td>center of (^{15}\text{N}) signal (~119 ppm)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>o3 (hCaNH3D.tcpx)</td>
<td>on resonance with Ca region (~54 ppm)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>o3 (hCONH3D.tcpx)</td>
<td>on resonance with CO region (~174 ppm)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>cnst21</td>
<td>frequency offset for CO (~174 ppm)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>cnst22</td>
<td>frequency offset for Ca (~54 ppm)</td>
</tr>
<tr>
<td></td>
<td>(^{13}\text{C})</td>
<td>cnst26</td>
<td>frequency offset for CO-Ca (~114 ppm)</td>
</tr>
</tbody>
</table>

* The pulse length is linearly proportional to \( 1/B_0 \).
References