Rapid access to RNA resonances by proton-detected solid-state NMR at >100 kHz MAS†

Alexander Marchanka,a,†§ Jan Stanek,a,† Guido Pintacuda,b and Teresa Carlomagno,ab,†c

Fast (>100 kHz) magic angle spinning solid-state NMR allows combining high-sensitive proton detection with the absence of an intrinsic molecular weight limit. Using this technique we observe for the first time narrow 1H RNA resonances and assign nucleotide spin systems with only 200 μg of uniformly 13C,15N-labelled RNA.

The discovery of the three-dimensional structure of ribonucleic acids (RNA), either in isolation or in complex with proteins, is essential to explain their numerous functions in cells. The structure of RNA is best studied by Nuclear Magnetic Resonance (NMR) spectroscopy, which can cope with the inherent flexibility of the polymer. However, unlike proteins, RNA consists of building blocks of low chemical diversity, resulting in poor chemical shifts dispersion. Consequently, NMR analysis of RNAs >40–50 nucleotides requires laborious selective isotopic labeling. Furthermore, the sensitivity and the resolution of solution NMR decrease with the molecular mass, posing a practical limit of about 60–80 nucleotides.

Magic-angle spinning (MAS) solid-state NMR spectroscopy does not have an intrinsic molecular weight limit and holds the potential of being applicable to RNAs of any size. Recently, MAS NMR was employed for the first time to determine de novo the structure of a RNA in a non-diffraeting solid preparation, the 26mer Box C/D RNA in complex with the protein L7Ae from Pyrococcus furiosus (Pf).2,3 The strategy comprised of 13C- and 15N-detected experiments for both resonance assignment and measurement of structural restraints. The low sensitivity of 13C,15N nuclei rendered high-dimensional NMR methods impractical, even when using >6–8 mg of RNA.2,3 The severe overlap of the resonances in two-dimensional spectra was lifted using single (A, U, C, G) nucleotide-type selective labeling for the assignment of nucleotide spin systems, and double (AU, CG, etc.) nucleotide-type selective labeling for sequential assignment and collection of structural restraints. Despite successful, this strategy necessitated the measurement of several samples, resulting in a substantial commitment of laboratory and instrument times. 1H-detection provides the highest sensitivity; nonetheless, it has long been a unique mark of solution NMR spectroscopy, because of the extreme line broadening caused in solids by the dense network of strong 1H–1H dipolar couplings. 1H-detection at moderate (24 kHz) MAS rates was shown to be practical with perdeuteration and partial reintroduction of exchangeable protons.4–6 However, in RNA this labeling scheme limits the NMR active probes to labile imino and amino 1Hs, and prevents efficient resonance assignment strategies.5,7,8 Faster MAS rates are necessary to lift the requirement of proton dilution and at the same time ensure sensitivity and resolution. Recently, 1H detection was used on a uniformly labeled 23mer DIS HIV-1 RNA to observe base pairs through 15N–15N proton assisted recoupling (PAR); however, at the MAS rate of 40 kHz, the proton resolution (500 Hz) was insufficient to enable site-specific assignments.9

The availability of hardware that is capable to reach fast (60–100 kHz) spinning rates has been critical for the development of 1H-detected MAS NMR experiments for proteins, allowing faster backbone and side-chain assignments, as well as structural and dynamics studies.10–12 Despite its lower proton density when compared to proteins, RNA is a challenge for the spatial clustering of protons and the intrinsic flexibility. Here we show that MAS rates exceeding 100 kHz open an avenue for the study of fully protonated RNA at high resolution and sensitivity. 2D 3H-detected fingerprint spectra can be acquired in minutes to hours using as little as 200 μg of RNA. This enables the exploitation of high-dimensional NMR techniques, which allow

---

‡ Electronic supplementary information (ESI) available: Details on sample preparation, NMR spectroscopy; RF pulse sequence schemes; list of chemical shifts. See DOI: 10.1039/c8cc04437f

A. M. and J. S. contributed equally.
the identification of $^1$H, $^{15}$N and $^{13}$C resonances of nucleotide spin-systems in the 26mer Box C/D RNA from a single, uniformly $^{13}$C, $^{15}$N-labelled sample.

Fig. 1 shows the remarkable effect on $^1$H resolution and sensitivity observed for the Box C/D RNA upon increase of the magic-angle spinning rates. 2D dipolar-based $^{13}$C–$^1$H cross-polarization (CP)-HSQC experiments, 13 tailored for ribose shifts and line widths of 150 Hz ($^1$H) and 120 Hz ($^{13}$C). In the 2D $^{13}$C–$^1$H correlations of base resonances are more overlapped due to the magic-angle spinning rates. 2D dipolar-based $^{13}$C–$^1$H cross-polarization (CP)-HSQC experiments, 13 tailored for ribose resonances on a 800 MHz spectrometer, improve from a featureless spectrum at 20 kHz to a resolved fingerprint at 110 kHz MAS. $^1$H line widths decrease substantially due to effective averaging of dipolar $^1$H–$^1$H interactions, which is reflected by a nearly linear increase of the $^1$H coherence lifetime ($T_2^*$) with MAS rates up to 110 kHz (Fig. 1c). In this forefront regime, anomic ribose $^1$H$^1$ and base H6/H8/H2 resonances reach above 4 ms of bulk $T_2^*$; this corresponds to an 80 Hz homogeneous contribution to the $^1$H line width of 130–170 Hz. At comparable MAS conditions, such long coherence lifetimes have been reported only for proteins in microcrystalline preparations featuring low dynamics. 14 The resolution of ribose H2’–H5’/H5” resonances is lower, as these protons are involved in a stronger network of dipolar couplings; however, they feature better chemical shift dispersion than their carbon counterparts. 15, 16 Seven C5–H5 correlations are visible out of the nine pyrimidines (Fig. S1a, ESI†).

Fig. 2 shows a magnification of the two anomeric and base $^{13}$C–$^1$H fingerprints recorded on a 1 GHz spectrometer. Analogously to solution NMR, non-helical regions ($\delta$($^1$C$'$) < 90 ppm, Fig. 2b) display the best dispersion of C1’–H1’ resonances. 2D $^{13}$C–$^1$H correlations of base resonances are more overlapped (Fig. 2c), with only two bases showing distinct $^{13}$C chemical shifts and line widths of 150 Hz ($^1$H) and 120 Hz ($^{13}$C). In the C2–H2 region (Fig. S1b, ESI†), five resonances can be resolved out of the nine As. The crowded appearance of the spectrum indicates a broader line width of most H8/H6 (purine/pyrimidine) with respect to the ribose $^1$Hs. We hypothesized that the broadening of the base resonances could stem from incomplete suppression of the dipolar interactions with either (i) the hydrogens of water molecules entering the major groove 17 or (ii) the close-by H2’s in helical regions. To test these two hypotheses we acquired CP-HSQC spectra on two additional samples: (i) L7Ae-Box C/D RNA complex dissolved in 90%/10% D2O/H2O rather than 100% H2O. For this sample the H6/H8 line widths should not suffer from the vicinity to $^1$Hs of the bound water; (ii) the L7Ae-Box C/D RNA complex with $^{2}$H$_2$, $^{14}$N-C,U,A and $^{1}$H$_2$, $^{15}$N-G, dissolved in 90%/10% D2O/H2O. For this sample the H8 line width of the $^{13}$C, $^{15}$N-Gs should not suffer from the vicinity with the H2’s of the preceding nucleotides. The fingerprint in the base region is
almost identical for all samples (Fig. S1, ESI†), with a slight improvement of the line width in deuterated buffer. Thus, we conclude that the largest contribution to line broadening must be due to local structural heterogeneity that mostly affects the aromatic $^1$H shifts.

In the following, we demonstrate that, despite the limited chemical shift dispersion of RNA, 14 spin systems can be established out of 19 nucleotides in the structured regions of the 26mer Box C/D RNA (excluding the loop and the ends) without resorting to nucleotide-specific labeling. This is possible due to efficient magnetization transfers at MAS $>$ 100 kHz and high-dimensional experiments.

Leveraging the high-sensitivity of the 2D (H)CH spectrum, we implemented an (H)NCH experiment. This is comparable to the (H)NCAHA experiment for proteins,13,18 which we adapted to the topology of $^1$H, $^{13}$C and $^{15}$N spins in RNA and to the distribution of $^{13}$C chemical shifts. The experiment starts with a long-range $^1$H–$^{15}$N transfer, followed by a band specific CP step tuned to either ribose or aromatic $^{13}$C offsets, and yields either ribose-specific N1/N9–C1′–H1′ or base-specific N1–C6–H6/N9–C8–H8 correlations (Fig. 3a and b). The $^{15}$N chemical shifts allow distinguishing pyrimidine from purine correlations (Fig. 3c and d) as well as associating C1′–H1′ with C6–H6/C8–H8 resonances. These correlations are similar to those established in solution NMR, with the advantage that the dipolar-based long-range $^1$H-$^{15}$N and the one-bond $^{15}$N–$^{13}$C transfers used here maintain high efficiencies even for large RNAs, due to the sufficiently long $T_{1p}$ relaxation times at fast MAS ($>$ 12 and 50 ms for ribose $^1$H and $^{13}$C, respectively; Fig. S3, ESI†). The ribose-specific (H)NCH spectrum shows excellent sensitivity (Fig. 3e–g); in less than 12 hours, resonances could be correlated for 17 out of the 19 previously assigned11 spin systems (10 N9–C1′–H1′ and 7 N1–C1′–H1′ spin sets belonging to the region 3–10 and 15–23, with only nucleotides G14 and G24 missing, Table S4, ESI†). In the base-specific (H)NCH spectrum, the sensitivity of the transfer is affected by the multiple competitive coherence transfer pathways from the N1/N9 spins towards C2/C4 and C6/C8, as previously observed in CNC transfer schemes.7 Nonetheless, we obtained correlations for 19 nucleotides in 26 hours.

At fast MAS rates, the long $^{13}$C $T_{1p}$ relaxation times enable efficient scalar transfers between bonded $^{13}$C atoms. These transfers allow establishing entire ribose spin systems in an efficient manner, as scalar $^{13}$C transfers are easier to interpret than those based on proton-driven spin diffusion (PDSD) at slower MAS rates.19 Scalar transfers can be triggered by inserting into the (H)CH module a rotor-synchronized, low-power TOBSY mixing,20,21 or even less power-demanding schemes, such as WALTZ-16.22 In the resulting HCCH-TOCSY18,23 experiment, long mixing times (25 ms) are used to spread magnetization to all ribose $^{13}$C spins. The information content can be maximized if both $^1$H and $^{13}$C shifts of starting and ending CH groups on
Notes and references

1. Y. C. Su, L. Andreas and R. G. Griffin, *Annu. Rev. Biochem.*, 2015, 84, 465–497.
2. A. Marchanka, B. Simon and T. Carlonagom, *Angew. Chem., Int. Ed.*, 2013, 52, 9996–10001.
3. A. Marchanka, B. Simon, G. Althoff-Ospelt and T. Carlonagom, *Nat. Commun.*, 2015, 6, 7024.
4. V. Chevelkov, K. Rehbein, A. Diehl and B. Reif, *Angew. Chem., Int. Ed.*, 2006, 45, 3878–3881.
5. Ü. Akbay, S. Lange, W. T. Franks, R. Linser, K. Rehbein, A. Diehl, B. J. van Rossum, B. Reif and H. Oschkinat, *J. Biomol. NMR.*, 2010, 46, 67–73.
6. S. Asami, M. Rakwalska-Bange, T. Carlonagom and B. Reif, *Angew. Chem., Int. Ed.*, 2013, 52, 2345–2349.
7. J. R. Lewandowski, J. N. Dumez, Ü. Akbay, S. Lange, L. Emsley and H. Oschkinat, *J. Phys. Chem. Lett.*, 2011, 2, 2205–2211.
8. A. J. Nieuwkoop, W. T. Franks, K. Rehbein, A. Diehl, Ü. Akbay, F. Engkelke, L. Emsley, G. Pintacuda and H. Oschkinat, *J. Biomol. NMR.*, 2015, 61, 161–171.
9. Y. Yang, S. Xiang, X. Liu, X. Pei, P. Wu, Q. Gong, N. Li, M. Baldus and S. Wang, *Chem. Commun.*, 2017, 53, 12886–12889.
10. L. B. Andreas, T. Le Marchand, K. Jaudzems and G. Pintacuda, *J. Magn. Reson.*, 2015, 253, 36–49.
11. A. Böckmann, M. Ernst and B. H. Meier, *J. Magn. Reson.*, 2015, 253, 71–79.
12. T. Schubeis, T. Le Marchand, L. B. Andreas and G. Pintacuda, *J. Magn. Reson.*, 2018, 287, 140–152.
13. D. H. Zhou, G. Shah, M. Cormos, C. Mullen, D. Sandoz and C. G. Rienstra, *J. Am. Chem. Soc.*, 2007, 129, 11791–11801.
14. L. B. Andreas, K. Jaudzems, J. Stanek, D. Lalli, A. Bertarello, T. Le Marchand, D. C. D. Paêpe, S. Kotolevica, I. Akopjana, B. Knott, S. Wegner, F. Engkelke, A. Lesage, L. Emsley, K. Tars, T. Herrmann and G. Pintacuda, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, 113, 9187–9192.
15. B. Fürtig, C. Richter, J. Wöhntert and H. Schwalbe, *ChemBioChem*, 2014, 15, 936–962.
16. A. Marchanka and T. Carlonagam, *eMagRes*, 2014, 3, 119–128.
17. M. Egli, S. Portmann and N. Usman, *Biochemistry*, 1996, 35, 8489–8494.
18. J. Stanek, L. B. Andreas, K. Jaudzems, D. Cala, D. Lalli, A. Bertarello, T. Schubeis, I. Akopjana, S. Kotolevica, K. Tars, A. Pica, S. Leone, D. Picone, Z. Q. Xu, N. E. Dixon, D. Martinez, N. El Merriani, A. Nobbhan, S. Saupe, B. Habenstein, A. Loquet and G. Pintacuda, *Angew. Chem., Int. Ed.*, 2016, 55, 15503–15509.
19. V. Agarwal, S. Penzel, K. Szekely, R. Cadalbert, E. Testori, A. Oss, J. Past, A. Samoson, M. Ernst, A. Böckmann and B. H. Meier, *Angew. Chem., Int. Ed.*, 2014, 53, 12253–12256.
20. E. H. Hardy, R. Verel and B. H. Meier, *J. Magn. Reson.*, 2001, 148, 459–464.
21. K. O. Tan, V. Agarwal, N. A. Lakomek, S. Penzel, B. H. Meier and M. Ernst, *Solid State Nucl. Magn. Reson.*, 2018, 89, 27–34.
22. J. A. Shaka, J. Keeler, T. Frenkel and R. Freeman, *J. Magn. Reson.*, 1983, 52, 335–338.
23. A. Bax, G. M. Clore and A. M. Gronenborn, *J. Magn. Reson.*, 1990, 88, 425–431.
24. M. Huber, S. Hiller, P. Schanda, M. Ernst, A. Böckmann, R. Verel and B. H. Meier, *ChemPhysChem*, 2011, 12, 915–918.
25. S. Xiang, V. Chevelkov, S. Becker and A. Lange, *J. Biomol. NMR*, 2014, 50, 85–90.
26. R. Linser, B. Bardiaux, L. B. Andreas, S. G. Hyberts, V. K. Morris, G. Pintacuda, M. Sunde, A. H. Kwan and G. Wagner, *J. Am. Chem. Soc.*, 2014, 136, 11002–11010.
27. S. Paramasivam, C. L. Sutter, G. Hou, S. Sun, M. Palmer, J. C. Hoch, D. Rovnyak and T. Polonen, *J. Phys. Chem. B*, 2012, 116, 7416–7427.
28. I. V. Sergeev, B. Itin, R. Rogowski, L. A. Day and A. E. McDermott, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, 114, 5171–5176.
29. J. C. J. Barna, E. D. Laue, M. R. Mayer, J. Skilling and S. J. P. Worrall, *J. Magn. Reson.*, 1987, 73, 69–77.
30. D. Rovnyak, J. C. Hoch, A. S. Stern and G. Wagner, *J. Biomol. NMR*, 2004, 30, 1–10.
31. J. M. Lamley, D. Iuga, C. Oster, H. J. Sass, M. Rogowski, A. Oss, J. Past, A. Reinhold, S. Grzesiek, A. Samoson and J. R. Lewandowski, *J. Am. Chem. Soc.*, 2014, 136, 16800–16806.
32. P. Schanda, S. Triboulet, C. Laguri, C. M. Bougault, I. Ayala, M. Callon, M. Arthur and J. P. Simorre, *J. Am. Chem. Soc.*, 2014, 136, 17852–17860.
33. H. R. W. Dannatt, M. Felleti, S. Jehle, Y. Wang, L. Emsley, N. E. Dixon, A. Lesage and G. Pintacuda, *Angew. Chem., Int. Ed.*, 2016, 55, 6638–6641.

Conflicts of interest

There are no conflicts to declare.