Protein–RNA Complexes Hot Paper

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A Suite of Solid-State NMR Experiments for RNA Intranucleotide Resonance Assignment in a 21 kDa Protein–RNA Complex**

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Solid-state NMR (ssNMR) spectroscopy is becoming a very important instrument for the elucidation of structure–function relationships in large biomolecular complexes. To date, substantial progresses have been made in the structure determination of membrane proteins[1–3] and amyloid fibrils,[4–7] whereas significantly fewer studies have addressed the structure of RNA[8–17] or protein–RNA complexes (RNP)[13–16,18] by ssNMR spectroscopy. Nevertheless, the application of ssNMR spectroscopy to study large RNP complexes holds excellent promises, because of the independence of the ssNMR line widths from the molecular size.

Here, we present a comprehensive ssNMR-based intranucleotide resonance assignment of the 26mer box C/D RNA in complex with L7Ae (21 kDa in molecular weight, Figure 1 a). This small RNP is part of the 390 kDa large box C/D sRNP,[19–21] which performs site-specific methylation of 2′-O-ribose.[20] The sRNA contains two conserved sequence elements—the box C (RUGAUG) and box D (CUGA),[22,23] and additional copies of these elements, box C′ and box D′. A specific methylation guide sRNA recognises each ribosomal RNA (rRNA) methylation site. The sRNP particle[19,24] is assembled around a binary complex, which, in archaea, is constituted by the protein L7Ae and the kink-turn (K-turn) motif in the box C/D RNA. In this study we use the L7Ae—box C/D RNA sub-complex (Figure 1 a) to develop a suite of ssNMR experiments and assign the intranucleotide resonances of the RNA.

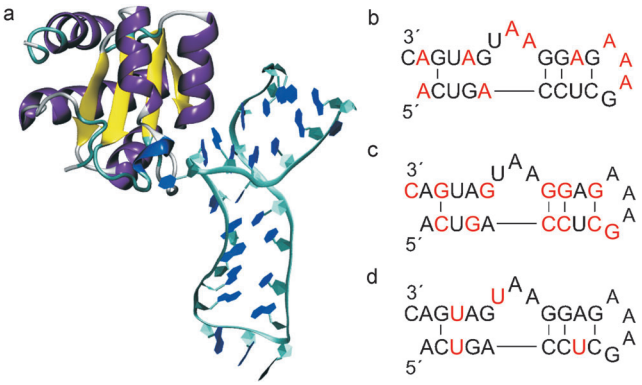

Figure 1. a) Molecular structure of the L7Ae—box C/D RNA complex, obtained after MD minimization of a model based on the crystallographic structure of the homologous complex from *Archaeoglobus fulgidus*.[19] Labeling schemes for the 26mer box C/D RNA with b) $^{13}C,^{15}N$-labeled adenosines, A^{lab}-RNA c) $^{13}C,^{15}N$-labeled guanosines and cytidines, $(G,C)^{lab}$-RNA and d) $^{13}C,^{15}N$-labeled uridines, U^{lab}-RNA. Labeled nucleotides are indicated in red.

To our knowledge, this is the first intranucleotide resonance assignment of a moderate size RNA based solely on ssNMR experiments. Riedel et al.[9] attempted the first RNA resonance assignment of the $(GUC)_{97}$ RNA using TEDOR (transferred echo double resonance). In another work, the ^{13}C resonances of a 14mer RNA[12] were assigned by comparison of ssNMR chemical shifts with those obtained from solution-state NMR spectroscopy. The RNA resonances could be detected at high-resolution in this study and ^{13}C, ^{13}C RFDR (radio-frequency-driven dipolar recoupling) experiments yielded direct coherence transfer peaks. In a recent study, Huang et al. reported a ^{13}C line width of 1 ppm for PEG- (polyethylene glycol) precipitated TAR-RNA (transactivation response element-RNA) and performed $^{13}C,^{13}C$ proton-driven spin diffusion (PDSD) spectra with an RNA sample containing only three selectively labeled nucleotides.[17]

Inspired by experimental schemes routinely applied for ssNMR studies of proteins,[25–29] we developed a combination of several SPECIFIC-CP-type experiments[25,28,29] to facilitate C→N→C and N→C→C magnetization transfer. In addition, we also performed a combined TEDOR-PDSD experiment, following the concept of heteronuclear magnetization transfer pioneered by Hing et al[30] and Michal et al[31] and further developed by others.[9,32,33] Application of selective labeling schemes together with the combination of CNC and TEDOR experiments yielded intranucleotide resonance assignment for 63 % of carbon (81 % of C1′) and 80 % of nitrogen atoms in the 26mer box C/D RNA in complex with L7Ae.

In a first attempt, we prepared the L7Ae—box C/D RNA complex using uniformly labeled $^{13}C,^{15}N$ box C/D RNA (u-RNA). The resolution obtained in the ^{13}C CP (cross polarization) experiment, as well as in the $^{13}C,^{13}C$ PDSD experiment, was poor because of strong resonance overlap (Figure S1a), an observation that revealed the need for nucleotide-type selective labeling. Figure 2 a shows the $^{13}C,^{13}C$ PDSD spectrum measured at 100 ms mixing time for the complex assembled with $^{13}C,^{15}N$ adenosine labeled RNA (A^{lab}-RNA). The nucleotides are annotated with roman numbers (I, II, III, etc.), starting from the one with the lowest C1′ chemical shift. PDSD was chosen for the homonuclear $^{13}C,^{13}C$ mixing step, as it is less effected by the dipolar

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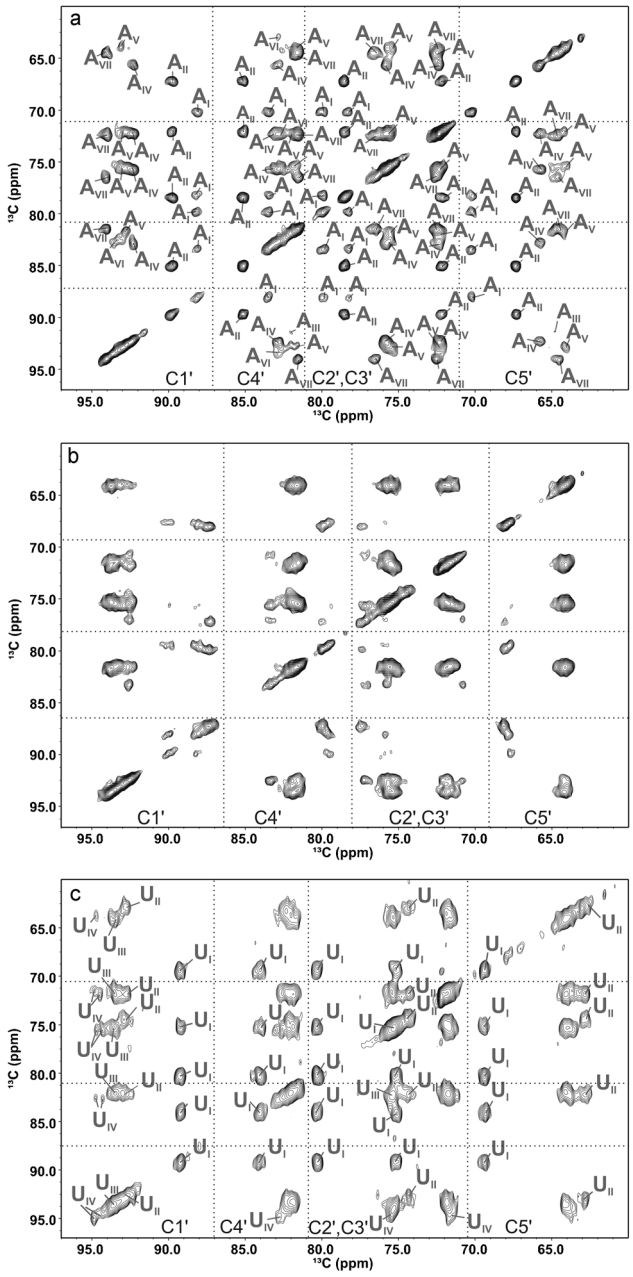

Figure 2. ^{13}C,^{13}C PDSD spectra of a) A^{lab}-RNA, b) (G,C)^{lab}-RNA, and c) U^{lab}-RNA in the L7Ae—box C/D RNA complex measured at a mixing time of 100 ms. The ^{13}C carrier was placed at 100 ppm. The acquisition times were equal to 29 ms and 5–7 ms in the direct and indirect dimension, respectively. The ribose region of the spectrum is shown together with the intranucleotide resonance assignment for A^{lab}-RNA and U^{lab}-RNA. The assignment assumes that the chemical shifts for ribose carbon atoms can be ranked as follows: $\delta(C1') > \delta(C4') > \delta(C2') > \delta(C3') > \delta(C5')$. This is an excellent assumption with the exception of $\delta(C2') > \delta(C3')$, which can sometimes be inverted. Ambiguity in the assignment of C2′ and C3′ resonances can be lifted by examination of ^{13}C,^{13}C RFDR spectra acquired at short mixing times, where directly connected C1′ and C2′ carbon atoms deliver stronger cross peaks than C1′ and C3′ carbon atoms. The nucleotide numbering is not related to the position in the RNA sequence; nucleotides were numbered starting from the one with the lowest C1′ chemical shift in each nucleotide type.

truncation than RDFR,[34] even if at the expenses of sensitivity.[35] This experiment achieves an almost complete set of intraribose ^{13}C–^{13}C correlations and partial ribose-base correlations (see Figure S1b in the Supporting Information). Increasing the mixing time to 500 ms delivers a complete set of intranucleotide correlations, with losses in sensitivity with respect to shorter mixing times (Figure S2a). Nevertheless, because of the good dispersion of the C1′ resonances of the A^{lab}-RNA and of the sharp ^{13}C line width (0.3–0.4 ppm), the intranucleotide assignment of the ribose carbon atoms of A^{lab}-RNA can be achieved on the basis of ^{13}C,^{13}C PDSD spectra only, acquired at different mixing times. The ribose spin systems of seven out of ten different adenosines were identified, with adenosine A_{III} lacking the C2′ and C3′ assignments.

The shape of all peaks is complex, probably because of the large $^1J_{CC}$ couplings between ^{13}C spin pairs in the ribose ring (about 40 Hz) and bases (up to 75 Hz).[36] Despite this source of line-broadening, analysis of the PDSD spectrum at 500 ms mixing time allows the almost complete assignment of both base and ribose ^{13}C resonances for A_I, A_{II}, A_{IV}, A_V, and A_{VII}, while the assignment of the C6/C2 atoms was incomplete for A_{III} and A_{VI}. This is probably because of the similarity in the chemical shifts of these atoms and because of the broadening of the C6 resonances as a consequence of the large $^1J_{CC}$ coupling (about 75 Hz for the C5–C6 spin pair in adenosines).[36,37]

Unlike for A^{lab}-RNA, the ^{13}C,^{13}C PDSD spectra do not allow the complete assignment of the ^{13}C resonances of ^{13}C,^{15}N G,C-labeled RNA ((G,C)^{lab}-RNA, Figure 2b.) Although several intraribose and ribose-base correlations are visible, the spin systems severely overlap (Figure S1c). The lower resolution of the spectra of the (G,C)^{lab}-RNA as compared to the A^{lab}-RNA is expected from the secondary structure of the 26mer box C/D RNA: guanosines and cytidines are located mainly in canonical A-form regions, whereas adenosines are present in loops and internal bulges, which usually display a larger chemical shift dispersion. From the ^{13}C,^{13}C PDSD spectrum, only three sets of resonances can be identified. The CNC and TEDOR experiments discussed later assign them to guanosines G_I, G_{II}, and G_{VI}.

The ^{13}C,^{13}C PDSD spectrum of ^{13}C,^{15}N U-labeled RNA (U^{lab}-RNA), measured at 100 ms mixing time (Figure 2c) shows good resonance dispersion. All four uridines can be identified, with one separate spin system (U_I) probably corresponding to U20, in close contact with the L7Ae protein (Figure 1a). A complete set of intraribose and C_{ribose}–$C2_{base}$ correlations is obtained at 100 ms mixing (Figure 2c and Figure S1d). The PDSD spectrum acquired at 500 ms mixing time delivers a full set of intranucleotide ribose-base ^{13}C,^{13}C correlations (Figure S2c). The C4 and C5 lines are broadened because of large $^1J_{C5C6}$ and $^1J_{C4C5}$ couplings,[37] whereas the ^{13}C resonances of U_{IV} display additional site-specific line-broadening.

The analysis of the ^{13}C,^{13}C PDSD spectra of nucleotide-selective labeled 26mer box C/D RNAs bound to L7Ae indicates that a complete assignment of intranucleotide resonances by homonuclear correlation experiments is only possible for well-dispersed spectral regions. For nucleotides

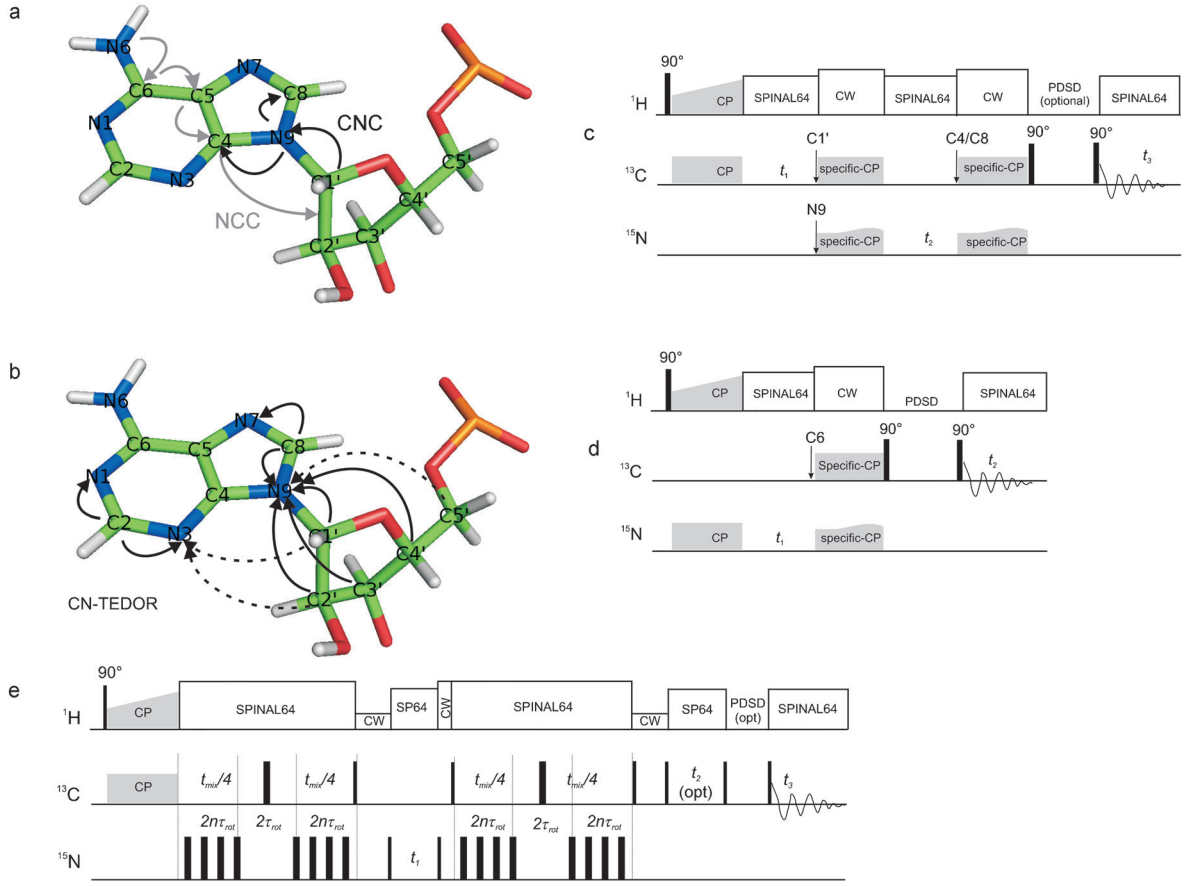

Figure 3. Magnetization transfer in the a) CNC (black) and NCC (gray) experiments, b) *z*-filtered CN-TEDOR experiment, both shown on the example of an adenosine. In (b) solid and dashed lines indicate short- and long-range correlations, respectively. Pulse sequences for c) the 3D CNC experiment, d) the 2D NCC experiment, and e) the *z*-filtered TEDOR[32] (*z*-filtered TEDOR-PDSD) experiments. Additional experimental details are included in the respective figure captions.

located in A-form helices, where resonances are notoriously overlapped, homonuclear correlations reach their limit. To facilitate complete intranucleotide resonance assignment we make use of the additional dispersion of ^{15}N resonances observed in heteronuclear correlation spectra.

Following the idea of the CANCO experiemnt in proteins,[27,29,38] we developed a 3D $C_{ribose}\rightarrow N\rightarrow C_{base}$ experiment that correlates spin systems of the ribose and base through the connecting N1(N9) atom (Figure 3a). This experiment uses a magnetization transfer pathway similar to that in the HCNCH experiment in solution-state NMR spectroscopy;[39] ^{13}C1′ magnetization is evolved during t_1 and then transferred by a SPECIFIC-CP step[25] to either N1 (pyrimidines) or N9 (purines), where ^{15}N chemical shifts evolve during t_2. In a second SPECIFIC-CP step, magnetization is transferred from N1 to C2,C6 (N9 to C4,C8) and detected during t_3. An optional DARR/PDSD[40,41] step can be added after the N1→C2,C6 (N9→C4,C8) SPECIFIC-CP transfer, allowing magnetization to be spread further in the spin system of the base (Figure 3a and Figure 3c); this has the advantage of improving the dispersion of overlapping resonances, albeit at the cost of sensitivity.

Figure 4 shows 2D spectra of the C(N)C correlation experiments performed on a) A^{lab}-RNA, b) $(G,C)^{lab}$-RNA, and c) U^{lab}-RNA in the L7Ae—box C/D RNA complex. Using 3D CNC experiments, six out of ten adenosines, three out of five cytidines, all seven guanosines, and all four uridines can be identified. Ribose C1′ and base C4/C8 or C2/C6 spin systems of the $(G,C)^{lab}$-RNA were assigned, which could not be resolved in the ^{13}C,^{13}C PDSD correlations. The lack of spin systems of adenosines and cytidines can be partially explained invoking significant motional flexibility at the 5′ and 3′ termini of the RNA, where one cytidine and two adenosines are located.

To obtain long-range ^{13}C–^{15}N correlations and to complete or confirm the assignment of base nitrogen and carbon resonances, we employed a ^{13}C–^{15}N TEDOR experiment (Figure 3b and 3e), which delivers correlations similar to those obtained from the HCN experiment in solution-state NMR spectroscopy.[42] In the ^{13}C–^{15}N *z*-filtered TEDOR,[30,32] ^{13}C magnetization is prepared by standard ^{1}H–^{13}C cross-polarization with a short mixing time (200 μs). The following TEDOR sequence transfers magnetization from ^{13}C to ^{15}N by the ^{13}C–^{15}N dipolar coupling reintroduced during the variable mixing time (1.8–4.8 ms).[32] After evolving ^{15}N chemical shifts in t_1, the magnetization is transferred back to ^{13}C, where it is either directly measured during t_2 (*z*-filtered TEDOR experiment)[32] or first spread over the ribose and base spin systems during PDSD (or DARR)[43] and then detected (*z*-filtered TEDOR-PDSD experiment). Optionally, the TEDOR-

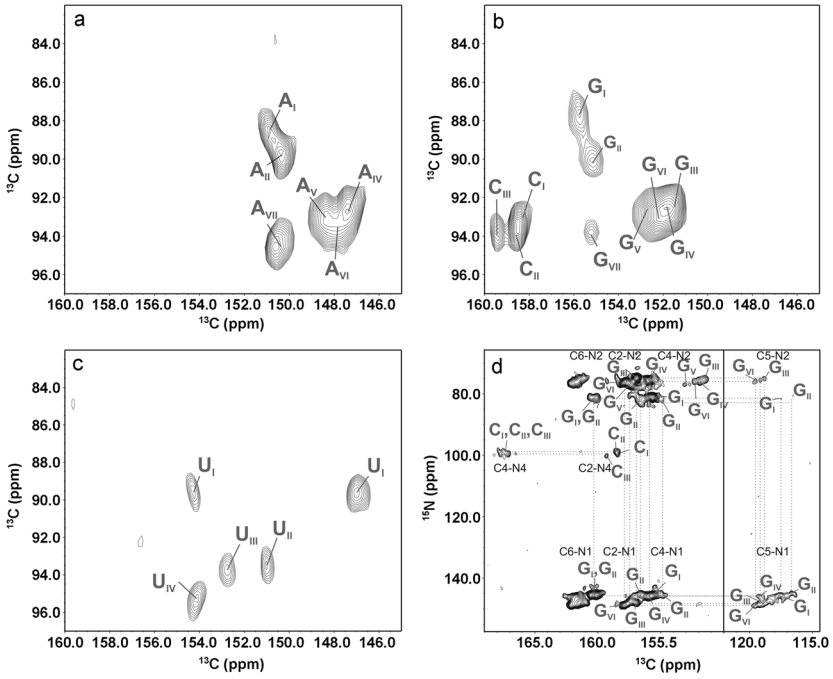

Figure 4. 2D CNC experiment for the box C/D RNA in complex with L7Ae for a) A^{lab}-RNA, b) $(G,C)^{lab}$-RNA and c) U^{lab}-RNA. The ^{13}C carrier was placed at 88 ppm during the C–N transfer and at 145 ppm during the N–C transfer, the ^{15}N carrier was placed at 168 ppm for A^{lab}-RNA, at 158 ppm for $(G,C)^{lab}$-RNA and at 148 ppm for U^{lab}-RNA. Acquisition times were equal to 9.8 and 2.3 ms in the direct and indirect dimension, respectively. In the 3D CNC experiment the acquisition time in the indirect ^{15}N dimension was equal to 7.0 ms. C1′–C2 and C1′–C4 correlations for pyrimidines and purines are shown, respectively. In (c) also the C1′–C6 cross-peaks are visible for U^{lab}-RNA. d) Example of a 2D NCC experiment for $(G,C)^{lab}$-RNA. The $^{13}C/^{15}N$ carrier was placed at 160/145 ppm during the N–C transfer. Acquisition times were equal to 20 and 5 ms in the ^{13}C and ^{15}N dimension, respectively. Intranucleotide resonance assignment was made using TEDOR-PDSD, NCC, and a 3D version of the CNC experiment. The numbering of resonances is not related to the position of the nucleotides in the RNA sequence.

PDSD experiment could be performed in a 3D version, analogously to recently reported 3D TEDOR-RFDR[33] or 3D TEDOR-DARR[43] experiments. The range of the ^{15}N–^{13}C magnetization transfer depends on the length of the TEDOR mixing period. At short mixing times, the experiment gives similar information to that obtained from the 3D CNC experiment, albeit with better efficiency for the C6(C8)→N1(N9) transfer and can be used to validate resonance assignment; at longer mixing times it delivers long-range intranucleotide correlations. Figure 5 shows 2D ^{13}C–^{15}N TEDOR spectra performed at the intermediate mixing time of 3.6 ms for a) A^{lab}-RNA, b) $(G,C)^{lab}$-RNA, and c) U^{lab}-RNA in the L7Ae—box C/D RNA complex. At this mixing time N1 (N9) is not only correlated to C1′, but also to the more distant C2′, C3′ and to some of the C4′ carbons.

In the ^{13}C–^{15}N *z*-filtered TEDOR spectrum of A^{lab}-RNA, seven out of ten adenosines were identified. The line width of separate resonances amounts to 0.4/0.6 ppm in the $^{13}C/^{15}N$ dimension.

For the $(G,C)^{lab}$-RNA sample, introduction of the ^{15}N dimension allows immediate differentiation between cytidines (pyrimidines) and guanosines (purines), because of the very different chemical shifts of the N1 and N9 atoms. Combining CNC and TEDOR experiments, the spin systems of three out of five cytidines and all seven guanosines were identified and assigned. The dispersion of the C6–N1 (C8–N9) resonances is good and allows the identification of all visible nucleotides. On the other hand, the dispersion of the C1′–N1 (C1′–N9) resonances is poor; assignment in this region was not possible based on the CN TEDOR experiment only but had to rely on the CNC experiment as well. In addition to the TEDOR experiment, a *z*-filtered TEDOR-PDSD experiment with a 2.4 ms TEDOR mixing step and 100 ms PDSD mixing time was performed for the $(G,C)^{lab}$-RNA sample (data not shown), attempting the complete assignment of carbon resonances in ribose and base. For cytidines, the chemical shifts of two C4′, three C2′, and three C3′ carbon atoms were assigned in this way; for guanosines nearly complete assignment of carbon resonances could be obtained for G_I, G_{II}, and G_{VI} whereas the C2′ and C5 carbon atoms were identified for G_{IV}. Assignment of ribose carbon atoms other than C1′ in G_{III}, G_V and G_{VII} was not possible because of strong resonance overlap (Table S1).

For the U^{lab}-RNA complex, all four uridines could be identified (Figure 5c); the C6–N1 cross peak of U_{IV} was not found in the spectrum whereas the C1′–N1 peak is significantly broader than those of U_I–U_{III}, probably indicating higher motional flexibility of this nucleotide.

Assignment of N1, N3 and N7 nitrogen atoms in adenosines and N7 nitrogen atoms in guanosines could be achieved by analysis of the C2–N1, C2–N3 and C8–N7 peaks in the broadband *z*-filtered CN-TEDOR spectrum; however, this assignment has not been performed in this study.

Last, we designed an experiment to assign the amino and imino nitrogen atoms in nucleobases. Inspired by the NCACX experiment used in ssNMR studies of proteins,[3,27–29,44] we developed the NCC experiment (Figure 3a and 3d), which is similar to the HNC-TOCSY-CH experiment in solution-state NMR spectroscopy.[45,46] A short initial ^1H–^{15}N magnetization transfer (200 μs) is followed by a SPECIFIC-CP transfer from ^{15}N to ^{13}C and by a PDSD[40] step of 150 ms length to spread the magnetization in the bases up to the ribose carbon atoms (Figure 3a and 3d). Figure 4d shows an example of the NCC spectrum measured for the $(G,C)^{lab}$-RNA sample in aqueous buffer. After the specific N4→C4 and N1→C6,C2/N2→C2 transfers in cytidines and guanosines, respectively, the magnetization is spread during the following ^{13}C,^{13}C PDSD mixing step by C4–C5 or C5–C6 contacts up to the ribose carbons. The assignment of the C6 carbon atoms of guanosines and C4 and C5 carbon atoms of cytidines was only partially possible because of overlapping resonances and line broadening because of the large $^1J_{CC}$ couplings in the bases.

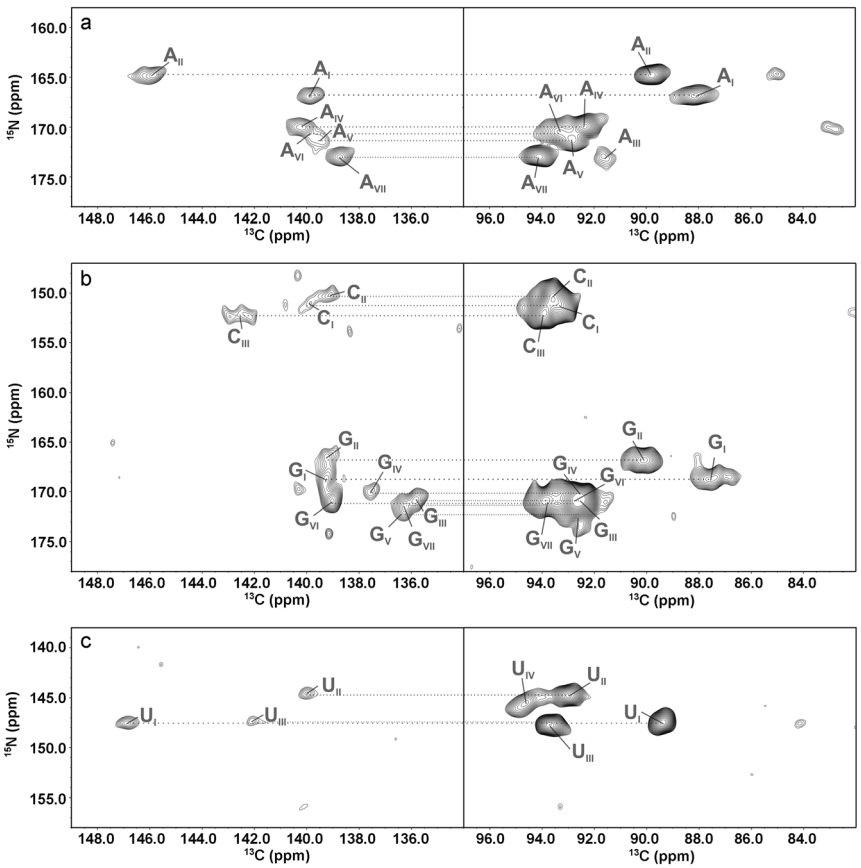

Figure 5. *z*-Filtered ^{13}C–^{15}N TEDOR experiment performed on a) A^{lab}-RNA, b) (G,C)^{lab}-RNA, and c) U^{lab}-RNA in the L7Ae—box C/D RNA complex. The ^{13}C carrier was placed at 100 ppm, the ^{15}N carrier was placed at 168 ppm for A^{lab}-RNA, 158 ppm for (G,C)^{lab}-RNA, and 148 ppm for U^{lab}-RNA. Acquisition times were equal to 15 and 6–9 ms in the direct and indirect dimension, respectively. Left and right panels show regions of C6–N1 cross-peaks (C8–N9 for purines) and C1′–N1 cross-peaks (C1′–N9 for purines), respectively.

In summary in this work we propose an experimental strategy for RNA resonance assignment by ssNMR spectroscopy. To improve the spectral resolution, selective-nucleotide labeling is used during RNA preparation. In particular, it is important to separate the two purine- and the two pyrimidine-spin systems, to avoid resonance crowding that cannot be resolved in heteronuclear experiments. Our strategy starts with the acquisition of homonuclear ^{13}C,^{13}C PDSD experiments at different mixing times, followed by heteronuclear correlation experiments. The selective CNC experiment delivers a unique set of C1′, C2, C6, N1 and C1′, C4, C8, N9 chemical shifts for pyrimidine and purine spin systems, respectively. A *z*-filtered CN-TEDOR experiment validates the chemical shift assignment obtained from the CNC experiment, while the CN-TEDOR-PDSD, in combination with the previously acquired ^{13}C,^{13}C PDSD experiment, is used to complete and confirm the assignment of ribose and base carbon atoms. Finally, the NCC experiment delivers the assignment of amino and imino nitrogen atoms.

Chemical shifts contain valuable information about RNA secondary structure. In particular, chemical shifts of base carbon atoms located in noncanonical regions are significantly different from those located in base-paired regions.[47] The analysis of the chemical shifts obtained from the ssNMR study of the 26mer box C/D RNA in complex with L7Ae suggests that adenosines A_I, A_{II}, A_{VII}, guanosines G_I, G_{II}, G_{VI}, and uridine U_I may be located in noncanonical structural regions.

Following intranucleotide resonance assignment, spin systems of different nucleotides should be connected with each other to perform sequential assignment. In solution-state NMR spectroscopy, RNA sequential assignment is performed by NOE experiments leveraging on the H6/H8 to H2′ cross-peaks or by HCP and HCP-TOCSY experiments.[36,48] In ssNMR spectroscopy sequential walk should imply long-range magnetization transfer experiments and selective labeling schemes of neighboring nucleotides pairs. Sequential RNA resonance assignment strategies, as well as new methodologies for the measurement of structural constraints by ssNMR spectroscopy, are active areas of research in our laboratory.

Experimental Section

Sample preparation: The L7Ae protein from *P. furiosus* was expressed and purified as described in Li and Ye.[49] The 26mer box C/D RNAs with nucleotide-selective labeling were prepared as described elsewhere.[13] Several L7Ae—box C/D RNA complexes with differently labeled RNA were assembled and micro-crystallized according to the published protocol[13,14] (see the Supporting Information for details). The microcrystalline precipitate was packed into a 3.2 mm ssNMR rotor by centrifugation.

ssNMR experiments: The ssNMR experiments were acquired using a Bruker Biospin Avance 700 MHz spectrometer equipped with a commercial 3.2 mm H/C/N triple resonance probe. ^{13}C,^{13}C homonuclear correlation experiments (PDSD) at different mixing times and heteronuclear correlation experiments (CNC, NCC, CN TEDOR and CN TEDOR-PDSD) were acquired. Details of the experimental parameters are given in the figure legends and in the Supporting Information.

Chemical shifts were referenced as described by Morcombe and Zilm.[50] Intranucleotide resonance assignment was performed by comparison of experimental chemical shifts with those reported in the BMRB[51] and assuming that the chemical shifts for the ribose carbon atoms are such that $\delta(C1') > \delta(C4') > \delta(C2') > \delta(C3') > \delta(C5')$. Chemical shifts for the base carbon atoms are assumed to be such that $\delta(C4) > \delta(C2) > \delta(C6) > \delta(C5)$ and $\delta(C6) > \delta(C2) > \delta(C4) > \delta(C8) > \delta(C5)$ for pyrimidines and purines, respectively.[51] The spectra were processed by NMRPipe[52] and visualized by NMRviewJ.[53]

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