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^{59}Co Solid-State NMR as a New Probe for Elucidating Metal Binding in Polynucleotides

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Abstract: Although magnesium fulfills several essential biochemical roles, direct studies on this ion are complicated by its unfavorable spectroscopic characteristics. This contribution explores the possibility of monitoring magnesium−nucleic acid binding via a combination of $[Co(NH_3)_6]^{3+}$ as surrogate for $[Mg(H_2O)_6]^{2+}$, and of high-resolution solid-state ^{59}Co NMR as a spectroscopic probe. Such strategy quenches fast cationic exchanges between bound and free states, while exploiting the superior NMR properties of the ^{59}Co spin. Experiments on relatively small amounts of tRNA can then discern resonances corresponding to different metal binding environments. These characterizations were assisted by studies on model compounds and by multinuclear ^{31}P−^{59}Co recoupling experiments.

Introduction

Cations have profound effects in defining the structure and function of nucleic acids.[1−3] In particular divalent metals such as magnesium are known to be essential for the proper physiological folding of polynucleotides, while also being involved directly in the catalytic activities of certain RNAs.[1−6] Interactions between Mg^{2+} and RNAs can be broadly categorized into nonspecific electrostatic attractions resulting from the anionic character of the polynucleotide, and specific coordinations arising between hydrated metals and pockets involving negatively charged phosphate groups as well as inner-sphere ligands derived from RNA's nucleotides.[7] Whereas nonspecific metal binding will always be present, specific metal−RNA interactions are of particular interest in understanding the structure and enzymatic chemistry of RNA. Arguably, the best established method for analyzing these ionic coordinations is X-ray crystallography. Yet even for nucleic acids that lend themselves to successful crystallization the spatial resolution of these structures can be sometimes insufficient to unambiguously characterize the metal binding sites. These complications may arise from incomplete occupancy numbers, from dynamic disorder of the metal sites themselves or of hydration water molecules, or as a result of Na^+, Mg^{2+} and H_2O having the same number of electrons. Spectroscopic strategies are therefore often used for facilitating the characterization of these complexes. One such approach involves replacing Mg^{2+} with its analogue Mn^{2+}, whose binding can be studied via paramagnetic NMR. Another approach involves titrating nucleic acids with either Mg^{2+} or other cations, and then monitoring the changes arising in ^{1}H, ^{13}C, ^{31}P or ^{15}N NMR spectra in the hope that these will reveal the location of the binding sites.[8−10] Out of the various magnesium analogues which bind to nucleic acids one of the most thoroughly explored is $[Co(NH_3)_6]^{3+}$, a species that size- and shape-wise is very similar to hexaaquomagnesium.[11] This complex exhibits added advantages in its favorable diffraction properties and the potential to simplify solution NMR spectra thanks to the use of labeled $[Co(^{15}NH_3)_6]^{3+}$;[12−16] extrapolation of hexamminecobalt data actually serves as one of the main sources for the location of putative hexaaquomagnesium binding sites in a variety of nucleic acids.

Despite their proven usefulness, these analytical strategies may sometimes lead to ambiguous or even conflicting pictures about the nature of metal−RNA binding. Such conflicts could

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be eased if a spectroscopic signature could be extracted *from the bound ions themselves*. In fact most of the metals that bind to nucleic acids are diamagnetic and possess significantly abundant isotopes that are NMR-active,[8,17] making them potential targets of this spectroscopy. Unfortunately the majority of these biologically significant isotopes also involve half-integer quadrupolar nuclei (^{7}Li, ^{23}Na, ^{25}Mg, ^{39}K, ^{43}Ca, $^{63/65}$Cu, ^{67}Zn) possessing spin numbers $S = {}^{3}/_{2}, {}^{5}/_{2}, {}^{7}/_{2}$. When placed in solution under physiological conditions the NMR spectra of these nuclides then tend to convey limited information as a result of metals exchanging between the bound sites and bulk solution, an exchange that masks potential chemical shift differences, and due to the efficient nature of a quadrupole relaxation that significantly and irreversibly broadens the NMR spectral lines.[9] These problems could be circumvented by carrying out the NMR experiments in the solid phase, where the exchange averaging caused by cationic migration will be stopped and relaxation broadening quenched by the absence of rapid reorientations. Spurred by ongoing methodological advancements in the field of half-integer solid-state quadrupolar NMR this potential has recently been demonstrated on studies of zinc bound to metalloproteins, of sodium sites in DNA quadruplexes, and of magnesium in ternary nucleotide complexes.[18−20]

On attempting to extend solid-state ^{25}Mg NMR as a direct probe for monitoring this metal's coordination to nucleic acids, we were faced with a number of fundamental sensitivity issues. These stem from the effective dilution imparted by macromolecular systems on the ^{25}Mg, compounded by the relatively poor NMR signal-to-noise and long spin relaxation times of this low-γ metal. Similar problems have been recently noted for ^{67}Zn NMR, and solutions were proposed based on combining static cryogenic procedures with high-field and multiple-pulse NMR.[20] Yet in an effort to preserve a high-resolution nature we investigate here an alternative, based on solid-phase ^{59}Co NMR observations of the hexamminecobalt(III) ion interacting with tRNA. This approach takes advantage of the unique magnetic properties of the ^{59}Co nuclide, a 100% naturally abundant isotope of simple observation thanks to a receptivity exceeding that of ^{13}C by 3 orders of magnitude.[21] Although its spin number is $^{7}/_{2}$, ^{59}Co possesses a moderate quadrupole moment ($Q = 0.42 \times 10^{-24}$ cm^2), which endows it with conveniently short relaxation times but induces negligible second-order broadenings when considering a highly symmetric octahedral complex such as hexamminecobalt. Most residual quadrupolar, shielding, or dipolar broadenings can then be removed from its solid-phase spectrum with the aid of magic-angle-spinning (MAS), which if sufficiently rapid will yield a single sharp peak for the central $-{}^{1}/_{2} \leftrightarrow +{}^{1}/_{2}$ spin resonance flanked by resolved spinning sidebands arising from the remaining satellite transitions. Furthermore ^{59}Co has one of the largest NMR shielding dispersions reported for any element (≈18 000 ppm), and therefore significant changes in the chemical shift of its central transition peak can be expected from even slight variations in the complex's coordination environment. The following sections summarize some of the protocols employed and results observed on applying this strategy based on the observation of solid-state ^{59}Co MAS NMR spectra to investigations of metal binding to nucleic acids.

Experimental Section

All the chemicals employed in this work were purchased from Sigma. Both the hexamminecobalt(III) and the mononucleotides that were studied were received as powders, and suitably recrystallized prior to investigation. A variety of protocols were also assayed for preparing the cobalt−tRNA samples. Eventually, the following one was adopted: ≈20 mg of mixed lyophilized tRNA from *E. Coli* was purified from potential divalent metals by dialysis against a buffer containing 5 mM phosphate (pH 6.7), 200 mM NaCl, and 0.1 mM EDTA (including a 5′ annealing period at 60 °C), followed by repeated sedimentation (2000 G, 30′, 0 °C). NMR samples were prepared by dialyzing and annealing the resulting tRNA solutions against phosphate buffers containing varying molar ratios of hexamminecobalt(III) chloride, NaCl (100 mM), and magnesium chloride. tRNA solutions were then concentrated using a 30 kD cutoff filter (Centricon) by centrifugation at 9000 G (10′, 4 °C), resuspended in 5 mL of buffer, reprecipitated with 100% ethanol, and centrifuged a final time into a solid pellet which was dried in a slight stream of nitrogen gas to a paste-like consistency. Four millimeter magic-angle-spinning (MAS) rotors equipped with suitable spacers were then loaded with ≤10 mg of samples thus prepared.

NMR spectra were collected on a variety of nucleotidic- and tRNA-based samples at one of two different magnetic fields strengths, 11.8 and 7.1 T, using double- and triple-resonance probeheads, respectively. The higher field was used for the ^{59}Co NMR tRNA acquisitions, whereas the lower field was used for acquiring ^{1}H-decoupled ^{59}Co/^{31}P MAS NMR data. ^{59}Co acquisitions were carried out using spinning rates in the 10−15 kHz range, while ^{31}P CPMAS NMR data were collected at rates of ca. 3−5 kHz. Chemical shifts were determined prior to the acquisition of the spectra using aqueous $[Co(NH_3)_6]Cl_3$ and triphenylphosphine as external references, and are considered accurate within ±1 ppm. Further acquisition details are presented in the corresponding figure captions.

Results and Discussion

^{59}Co NMR has actually been used before as a probe to study the binding of $[Co(NH_3)_6]^{3+}$ to DNAs dissolved in solutions, via relaxation measures of the single line that is then observed.[9,22] Figure 1 illustrates the much richer information that becomes available from solid-state NMR, with conventional single-pulse ^{59}Co MAS acquisitions showing the hexamminecobalt resonance dispersed over a wide range of chemical shifts depending on the details of its second-sphere coordination. Almost 400 ppm separate the multiple sites in crystalline hexamminecobalt chloride from the resonance of $[Co(NH_3)_6]^{3+}$ in either aqueous or water−ethanol solutions (Figure 1A,B). On coordinating hexamminecobalt to tRNA and preparing a solid pellet, different MAS line shapes can be observed depending on the protocol used to prepare the cobalt−tRNA complexes. This is not altogether surprising, given the strong dependence on the metal's environment known to characterize ^{59}Co NMR. For instance, NMR samples that were prepared from solutions that lacked sufficient ionic strength (low sodium and low cobalt concentrations) or from pellets that were excessively dried after the ethanol precipitation step resulted in a substantial broadening—almost a disappearance—of the metal's resonances. This is indicative of the binding site heterogeneity that can be

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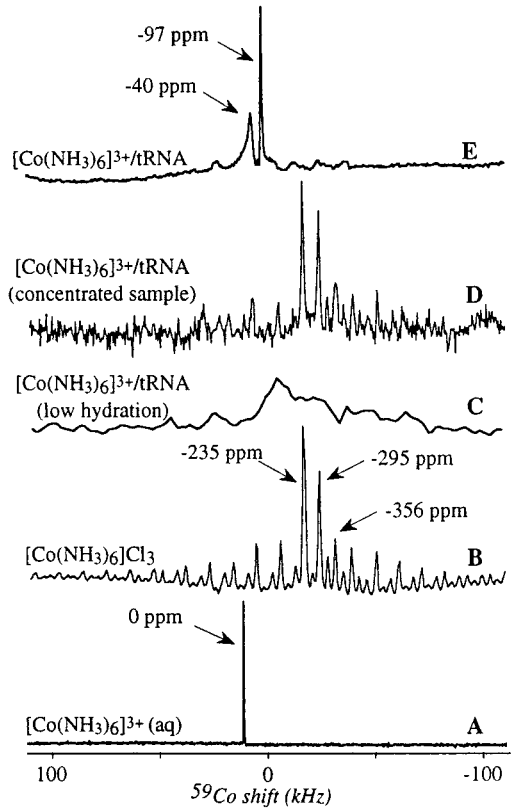

Figure 1. ^{59}Co NMR spectra acquired at 11.75 T on the following samples: (A) a 3/1 water/ethanol hexamminecobalt(III) chloride solution; (B) a polycrystalline hexamminecobalt(III) chloride powder; (C) a low-water tRNA/hexamminecobalt sample, precipitated into a pellet by addition of ethanol and subsequently denatured by excessive dehydration; (D) a low-water tRNA/hexamminecobalt sample, prepared into a pellet by continuous Centricon centrifugation at 9000 G for 4 h at 2 °C; and (E) a sample obtained by using the same procedure as in (C), but suitably hydrated. All the NMR data were collected by using a single 0.8 μs excitation pulse ($\approx$20° pulse angle), $\pm$250 kHz spectral widths, and 100 ms recycling delays; the carrier (zero) frequency was centered at 119.8 MHz, yet shift values are indicated in ppm relative to the resonance of (A). Except for this solution set, all acquisitions involved MAS at 11 kHz and 250000−500000 repetition scans. No substantive effects where visible upon introducing ^{1}H decoupling, whose use was therefore discontinued.

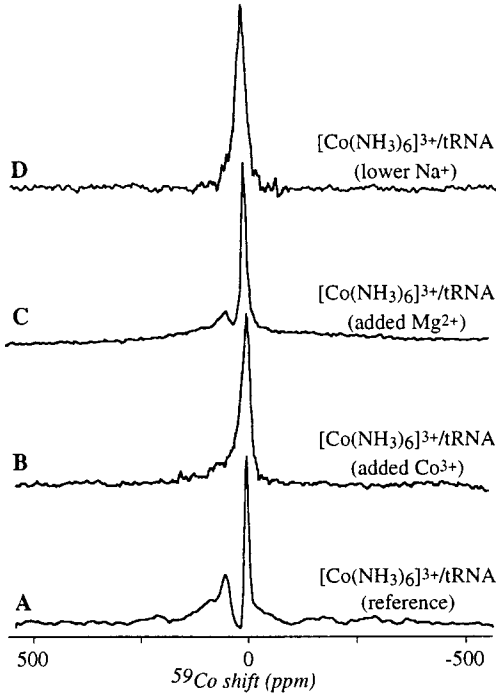

Figure 2. ^{59}Co MAS NMR spectra collected on mixed Co(III)/tRNA pellets prepared as described for Figure 1E, from solutions with the following relative [tRNA]:[$Co(NH_3)_6^{3+}$]:[Mg^{2+}]:[Na^{+}] concentrations: (A, reference sample) 1:10:0:1800; (B, high-cobalt sample) 1:20:0:1800; (C, added-magnesium sample) 1:10:10:1800; and (D, low-sodium sample) 1:10:0:90. The folding integrity of all samples was verified by thermal melts recorded on a Hewlett-Packard single-beam spectrophotometer with Peltiar temperature control. The NMR acquisition conditions used were as described in Figure 1.

expected to arise upon denaturing the tRNA strands (Figure 1C). Excessive sample moisture on the other hand led to ^{59}Co spectra which closely resembled those that can be acquired from aqueous $[Co(NH_3)_6]^{3+}$ solutions, showing a single sharp resonance that was only slightly upfield-shifted from the latter. Furthermore, experiments on samples where the ethanol precipitation step was replaced by a Centricon-only centrifugation and concentration of the dissolved sample into a solid pellet showed a systematic contamination with small amounts of hexamminecobalt(III) chloride (Figure 1D). Yet between these various extremes, a window of opportunity arose in which two qualitatively different kinds of cobalt sites bound to the tRNA could be spectrally resolved. A representative trace collected on such samples is illustrated in Figure 1E, showing a narrower ^{59}Co resonance appearing at approximately −95 ppm from aqueous $[Co(NH_3)_6]^{3+}$ and a broader peak resonating at approximately −40 ppm. The first of these resonances lacks a spinning sideband structure and is of the type that could be expected for nonspecifically bound cations undergoing liquidlike reorientations; motions of either a local or a delocalized nature that occur fast in the NMR time scale defined by the cobalt first-order quadrupole interaction. Despite its liquidlike character it is important to notice that this resonance exhibits a substantial chemical shift from its counterpart in highly hydrated or in dissolved tRNA samples, indicating that the dynamics of the cations that originate it—even if fast on the NMR time scale—are still taking place within different average environments than those characterizing the binding of hexamminecobalt in solutions. The second ^{59}Co peak observed in the MAS NMR spectrum differs from this sharp resonance both in its shift and in its structure: it exhibits a solidlike spinning sideband manifold of the kind that could be expected from hexamminecobalt ions bound in specific, tight interactions with the precipitated tRNA. Also interesting is the distinctive fine structure of this peak, suggestive of contributions arising from multiple and slightly inequivalent specific metal binding sites.

Although the conditions for which such distinctive MAS NMR features were observed had to be fine-tuned (hence explaining the mixed nature of the tRNAs used in the present analysis), further systematic changes in the positions, widths, and intensities of the two main ^{59}Co peaks could be observed upon varying the ionic conditions used to prepare the individual samples. Taking a natured solid sample prepared with a 10:1 $[Co(NH_3)_6]^{3+}$:tRNA molar ratio as an arbitrary standard (Figure 2A), experiments suggest that the relative intensity from the ^{59}Co NMR resonance that was attributed to the nonspecifically bound hexamminecobalt(III) ions could be enhanced by preparing samples with either increased relative cobalt concentrations, decreased overall concentrations of sodium, or addition of certain divalent cations such as magnesium (Figure 2B−D).

These changes can be rationalized in terms of the site identities ascribed in the preceding paragraph. For instance once all specific tRNA metal binding sites are nearly saturated, increasing the overall amounts of hexamminecobalt will increase mainly the ^{59}Co resonance associated with nonspecifically bound ions, as illustrated in Figure 2B. Similar increases should result upon withdrawing the nonspecific binding cation Na^+ (Figure 2C). And an increase in the relative intensity of this resonance is also to be observed when $[Co(NH_3)_6]^{3+}$ competes for the specific metal-binding tRNA sites with $[Mg(H_2O)_6]^{2+}$, which in comparison with Co(III) can be expected to have comparable binding affinities for the more specific metal binding sites yet lower affinities for the nonspecific binding environments of tRNA. Yet it should also be pointed out that the interpretation of these spectral variations should be exercised with care, as tRNA is a complex polybasic system known to possess a multitude of metal binding sites. The chelating affinities of these sites can be expected to vary greatly and in a quasi-continuous fashion, and both the number as well as the affinity of such binding sites is also liable to change upon varying the actual ionic conditions used to prepare a sample for study.[7,23,24] In fact these effects are likely responsible for at least part of the changes observed in the fine structure of the ^{59}Co NMR spectra shown in Figure 2, upon varying the ionic ratios used to prepare the samples. Indeed some of the peaks throughout this series will sometimes exhibit an additional inhomogeneous broadening, which we believe reflects the population of a variety of binding sites that are not necessarily found under all buffer/ionic conditions.

To further evaluate the chemical nature of the resonances arising from the $[Co(NH_3)_6]^{3+}$−tRNA complexes, a series of simpler complexes involving nucleotide-5′-monophosphates and hexamminecobalt(III) were also prepared and analyzed. Such complexes can form ordered polymeric adducts, where the outer sphere of the $[Co(NH_3)_6]^{3+}$ binds tightly to oxygen and nitrogen sites of the aromatic rings as well as to phosphate groups of the nucleotides.[25−27] Solid-state ^{59}Co and ^{31}P MAS NMR confirms the ordered arrangements adopted by these hexamminecobalt(III) complexes, which yield relatively sharp resonances on properly crystallized GMP-, AMP-, and CMP-based powders. Furthermore, the ^{59}Co MAS NMR resonances arising from these various solid spectra are similar among themselves (Figure 3), as well as to the site previously ascribed to $[Co(NH_3)_6]^{3+}$ specifically bound to tRNA (e.g., Figure 2A): they also possess featured centerbands in the −10 to −140 ppm region, some of them with comparable quadrupole couplings as judged by the intensities of their spinning sideband manifolds. Previous work identified outer-sphere interactions of hexamminocobalt complexes in yeast $tRNA^{phe}$ as involving hydrogen bonds with purine N7 and O6 sites along with phosphate oxygen interactions of neighboring polynucleotide strands;[12] these coordination patterns are similar to the ones suggested for the nucleotideMP−$[Co(NH_3)_6]^{3+}$ complexes,[25,26] thus explaining the similarities observed between the ^{59}Co NMR of $[Co(NH_3)_6]^{3+}$ groups in the nucleotidic and tRNA samples.

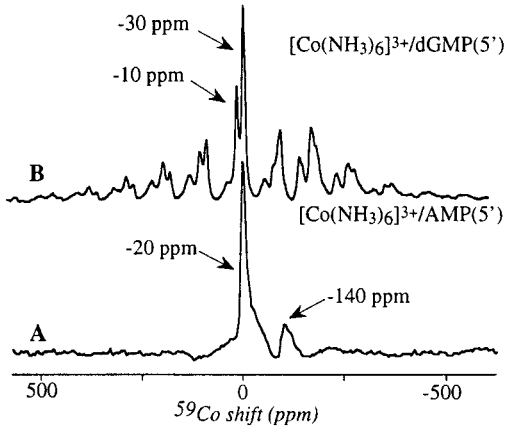

Figure 3. ^{59}Co MAS NMR spectra collected on polycrystalline $[Co(NH_3)_6^{3+}]$:AMP (A) and $[Co(NH_3)_6^{3+}]$:dGMP (B) complexes, showing the positions of the main centerbands with respect to aqueous hexamminecobalt. Spectra were acquired as indicated in Figure 1 but on a 7.1 T spectrometer on samples prepared by slow evaporation of 1:1 sodium mononucleotide: hexamminecobalt(III) chloride solutions at pH 7.1; similar spectra were obtained from other mononucleotidic complexes.

Additional insight about the specificity of the interaction between cobalt sites and phosphate groups can be extracted from multiple resonance NMR recoupling experiments on the nucleotidic complexes. Such methods employ rotor-synchronous radio frequency irradiation for preventing the averaging that MAS will otherwise impart on the internuclear dipolar couplings, and enable one to monitor spin−spin couplings along a second spectral time dimension.[28] From these data it is then possible to extract internuclear distances and eventually structures, even if a degree of modeling may be required when involving multiple spin systems and/or higher spin numbers. The presence of specific metal−ligand interactions on the nucleotidic complexes was evaluated by implementing such double resonance experiments between ^{59}Co and ^{31}P, using a modified version of the rotor-echo adiabatic-passage experiment (REAPDOR[29]) that we designed to minimize the extent of homonuclear ^{31}P−^{31}P dipolar recoupling. Results of these investigations on an adenosine-5′-monophosphate sample cocrystallized with hexamminecobalt(III) are shown in Figure 4. Just like its ^{59}Co counterpart, the ^{31}P MAS NMR spectrum of this complex shows two centerbands arising from slightly inequivalent sites. Although the chemical origin of these resonances remains to be completely elucidated, analyses of the REAPDOR curves demonstrate contacts of 5.0−6.0 Å between the phosphorus atoms that originate them and the cobalt. This range is again in good agreement with estimates arising from a model based on the specific hexamminecobalt(III) binding structure mentioned above.

Conclusions

The present work demonstrates the use of solid-phase ^{59}Co MAS NMR for studying the binding of $[Co(NH_3)_6]^{3+}$ to macromolecular nucleic acids. Attractive features of this approach are the narrow central transition resonances arising from the cations, coupled to the unique potential to discern inequivalent binding sites arising from cobalt's wide chemical shift scale. Favorable NMR properties of the ^{59}Co spin also allow for data

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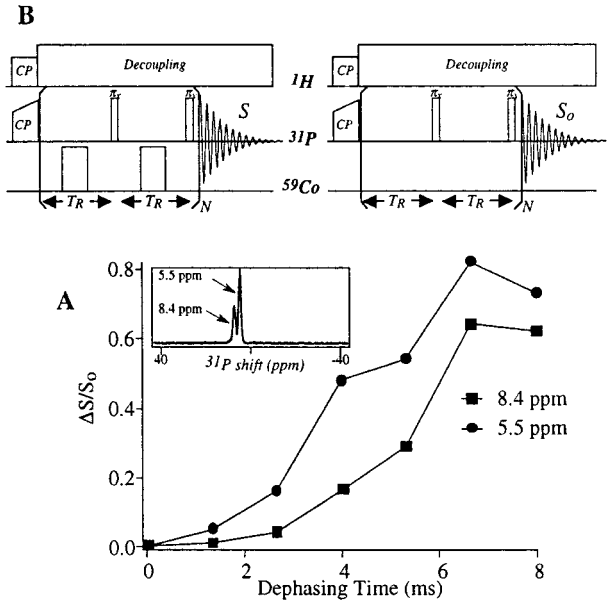

Figure 4. (A)^{31}P-observe/^{59}Co-dephase REAPDOR curves obtained for the two phosphorus sites of $[Co(NH_3)_6]^{3+}$:AMP (inset), collected at a field of 7.1 T. Data were acquired at 3.0 kHz MAS using 2 ms ramped ^{1}H cross polarization, 100 kHz ^{1}H decoupling, and 40 kHz ^{59}Co recoupling pulses that were 1/4 of a rotor period long. The actual pulse sequence employed in these recoupling experiments is depicted in part (B), and was adapted from the basic REAPDOR sequence to minimize the extent of the otherwise significant homonuclear ^{31}P−^{31}P dipolar recoupling. ^{59}Co-recoupled (S) and -decoupled (S_o) ^{31}P signals were acquired in consecutive scans for each of the assayed dephasing periods, while XY-4 phase cycling of the pulses was applied on the ^{31}P channel.

to be collected on relatively small amounts of RNAs or DNA (≈0.1 μmol in the present study), and to do so within times compatible with extensions to multidimensional NMR acquisitions involving peak assignments or distance measurements. We explored here this latter alternative with preliminary ^{59}Co−^{31}P dipolar measurements. Although extending this strategy to the ^{31}P MAS NMR spectrum arising from a complex biopolymer such as tRNA might prove problematic, opportunities may arise from the introduction of other specific labels into the nucleic acid. One such possibility could rely on the incorporation of ^{13}C in the relatively few methyl sites present in tRNA structures, which would enable the implementation of "triangulation" experiments based on the localization of the bound ligand via the observation of the cobalt-induced dipolar dephasing on several nucleotidic sites. This strategy would in turn be facilitated by the high spin number, symmetric environment, and 100% natural abundance of the ^{59}Co nucleus.[30] In addition to this structural potential one could also exploit the well-defined local anisotropies arising from the first-order quadrupole interaction—spectrally identified by the spinning sideband manifolds—to begin monitoring uncharted issues related to the dynamics characterizing RNA's metal binding sites. These studies could also be extended to the observation of additional NMR targets potentially available in $[Co(NH_3)_6]^{3+}$, such as ^{2}H and ^{15}N nuclei.

A basic tenet of the present study is that hexamminecobalt(III) can act as a surrogate for certain physiological metal complexes such as the hexaaquomagnesium ion. It has likewise been postulated that penta- and tetraamminecobalt(III) complexes bind to nucleic acids with specificities akin to penta- and tetraaquomagnesium,[27] thus opening a route for exploring the specific binding of such speciae to tRNA via ^{59}Co NMR. Although preliminary investigations indicate that spectral resolution in such studies will be much lower than in those involving hexamminecobalt(III), the sensitivity, dipolar recoupling, and dynamic potential described above will still apply upon studying these complexes. Besides its usefulness for investigating the binding of magnesium, solid-state ^{59}Co NMR can also be expected to enhance our understanding on the roles played by amminecobalt(III) complexes in their own right. As mentioned earlier $[Co(NH_3)_6]^{3+}$ has been observed numerous times in several structural contacts with nucleic acids, while hexamminecobalt in combination with divalent or monovalent ions has been shown to activate a number of ribozymes.[31,32] We trust that the advent of the simple yet powerful analytical methodology described in this study will assist in furthering such investigations.

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