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Exploring Metal Ion Coordination to Nucleic Acids by NMR

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Abstract: Metal ions play a crucial role in charge compensation, folding and stabilization of tertiary structures of large nucleic acids. In addition, they may be directly involved in the catalytic mechanism of ribozymes. Most metal ions applied in the context of nucleic acids *in vivo* and *in vitro* bind in a kinetically labile fashion. Hence, the detection of metal ion binding sites, not to mention the elucidation of the specific coordination sphere, still poses largely unresolved problems. Here we describe the different strategies applied and the progress made over the last years to characterize metal ion coordination to large nucleic acids by NMR.

Keywords: DNA · Metal ions · NMR · Ribozymes · RNA

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1. Introduction

Nucleotides and nucleic acids do not only carry out a multitude of functions in living organisms[1] but are at the same time also inevitably linked to metal ions.[2] The most simple cases are mononucleotides like the energy carriers and neurotransmitters ATP^{4-} and GTP^{4-}.[3,4] Both of them are believed to carry two Mg^{2+} ions in solution, one of them being rather tightly bound.[4] In the case of larger nucleic acids, like catalytic RNAs (ribozymes), also positively charged proteins and polyamines play an important role. However, their accumulated intrinsic negative charge of the bridging phosphates cannot be compensated for solely by positively charged amino acid side chains or polyamines, but in addition high amounts of monovalent and divalent metal ions are needed. In living systems, the natural metal ion cofactors usually considered to be involved with nucleic acids are Na^+ and Mg^{2+}.[5] The vast majority of these ions is used for gross charge compensation, *i.e.* is diffusely bound, allowing large nucleic acids to fold to a compact three-dimensional architecture. Such diffuse metal ion–nucleic acid interactions are often described with a nonlinear Poisson-Boltzmann equation.[6,7] Much more interesting in terms of local structure and possible function are those metal ions that are site-specifically bound and account for the compensation of about 10% of the negative charge.[5,8] These ions occupy key sites in the three-dimensional architecture and play a direct role in, *e.g.* domain assembly by coordinating to the domain junctions and stabilizing local structures like a strong kink in the phosphate sugar backbone.[9] In addition, they can be directly involved in the catalytic mechanism of DNAzymes and ribozymes. Thereby it should be taken into account that such a 'directly' involved metal ion can also affect catalysis from a distance, *i.e.* in a structural or an electrostatic manner.[2]

Evidently, it is of great interest to localize such metal ions and to characterize their binding sites, as only with this knowledge can we understand the functioning of catalytic RNAs and DNAs. This is already a challenging task, because Mg^{2+} is not only spectroscopically silent, but also displays a rather high ligand exchange rate, *i.e.* is kinetically labile. What makes this story even more interesting (and at the same time also more challenging) is that many more metal ions than just Na^+ and Mg^{2+} can be involved with nucleic acids:

i) Redox active metal ions are used in combination with specific sequences or modified oligonucleotides to build up electronic molecular devices for nanotechnology.[10–12]
ii) Other metal ions than Mg^{2+} are used for *in vitro* studies of, *e.g.* ribozymes, to localize binding sites and elucidate the functional roles of these ions.[13–17]
iii) DNAzymes are known that function with a whole battery of different divalent ions, although being usually most active with one specific kind.[18,19]

It is obvious that the different coordination properties of metal ions will be the basis for their discriminating effect on structure and function of nucleic acids. Very recently, the first hints started to evolve that also naturally occurring ribozymes might control their activity by switching the metal ion. The two most prominent examples are the hammerhead ribozyme, which is accelerated by many transition metal ions,[20,21] and the self-splicing group II intron ribozyme Sc.ai5γ, that is severely hampered by small concentrations of Ca^{2+}.[22,23]

In this review, we will now briefly discuss the different NMR methods applied over the past years to investigate the interaction of metal ions with nucleic acids. As will be evident to the reader, we are only at the very beginning of understanding the manifold ways of these interactions and certainly many unprecedented and fascinating results will evolve in the future.

2. General Aspects of Metal Ion Binding to Nucleic Acids

2.1 *Metal Ion Binding in Natural Systems*

Nucleic acids and their building blocks offer a multitude of donor atoms to coordinate metal ions.[2,5] The phosphate chain of 5'-nucleoside mono-, di- and triphosphates is well known to bind one, if not two Mg^{2+} ions in solution (Fig. 1A).[4,24–26] Along the same lines, also in the case of longer nucleic acids, the most prominent sites for metal ions are the two non-bridging oxygen atoms of the negatively charged phosphate-sugar backbone (Fig. 1A). However, aside from these negatively charged residues, also the nucleoside moieties offer coordinating atoms: The ring nitrogens, the carbonyl oxygens and in some cases even the sugar oxygens. The purine N(7) position is thereby certainly the most prominent one due to the well-known coordination of *cis-platin* to this site.[28]

Metal ions associated with nucleic acids in natural systems are usually considered the alkaline and earth alkaline ions Na^+ and Mg^{2+}, and potentially also K^+ and Ca^{2+}, which partly can occur in high millimolar amounts in living organisms (Table).[5] All of these metal ions show a higher affinity towards oxygen compared to nitrogen ligands. Nevertheless, also Mg^{2+} binding to N(7) is well known, although it is weaker than phosphate coordination. In natural systems, metal ion coordination takes place *via* several interactions, as only such a strong enough binding can be achieved to hold these ions in place. Metal ions associated with RNA and DNA molecules are usually kinetically labile, meaning that their ligand exchange rate is fast, ranging in the order of 10^5 (Mg^{2+}) to 10^9 s^{-1} (Ca^{2+}).[5] At the same time, these divalent ions are present in their solvated form in water, *i.e.* water ligands have to be replaced in order for the metal ion to coordinate directly to the nucleic acids: a so-called inner sphere coordination takes place (Fig. 1C–E). However, interactions between M^{n+} ions and the nucleic acid can also take place mediated through the coordinated water molecules, *i.e.* in an outer sphere fashion (Fig. 1B). Such interactions are by no means weaker than inner sphere, as each of such a hydrogen bond accounts for about 20 $kJmol^{-1}$. As a consequence, one can imagine that site-specifically bound Mg^{2+} ions are held tightly in place through an extensive network of inner- and outer sphere coordinations.

2.2 *The Use of Mimics*

The high ligand exchange rate of the metal ions commonly associated with nucleic acids makes it difficult to investigate these ions in solution.[5] This task is further hampered by the fact that alkaline and earth

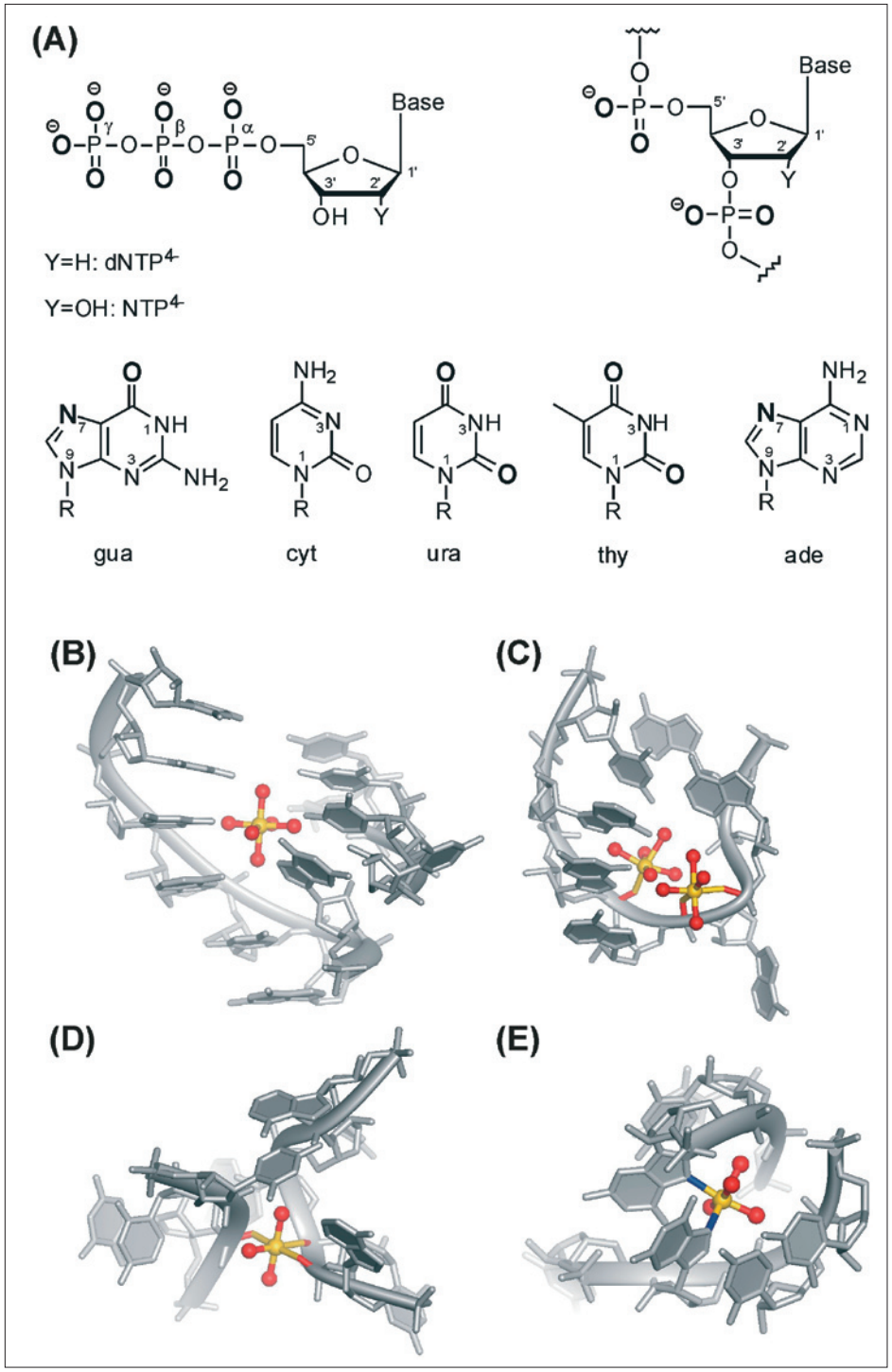

Fig. 1. Nucleotides and their metal ion coordinating atoms (in bold). **(A)** Chemical structures and numbering schemes of the most common nucleobases in RNA and DNA. The structure of a (d) NTP^{4-} is shown at the top left. On the top right a nucleotide within a DNA or RNA strand is depicted together with the bridging phosphate groups. At the bottom the nucleobases guanine, cytosine, uracil (only in RNA), thymine (only in DNA) and adenine are shown from left to right. **(B)** Outer sphere binding of a hexahydrated Mg^{2+} ion to RNA. **(C)** Two Mg^{2+}-ions coordinating in a partially inner sphere manner to either one (left) or two (right) phosphate oxygens. Additionally the Mg^{2+} ions exhibit outer sphere contacts to purine N(7) and O(6) positions. **(D)** Three inner sphere contacts of a half-dehydrated Mg^{2+} ion to phosphate oxygens bridging two strands are shown. **(E)** *Cis*-inner sphere coordination of a Mg^{2+} ion to two N(7) guanine atoms of two different strands. Panels **(B)**–**(E)** have been prepared with MOLMOL[27] based on the PDB file 1S72 of the large ribosomal subunit of *H.marismortui*.[8]

Table. Ion concentrations inside and outside a typical mammalian cell.[29] The concentrations of M^{2+} ions total about 20 mM, but most of these ions are bound to proteins, nucleic acids and other molecules. Ca^{2+} is mostly stored in various organelles.[30,31] The intracellular chloride concentration is much lower than the total metal ion concentration: Other anions, like phosphate and carboxylate groups of nucleic acids and proteins as wells as further functional groups make up for the difference to reach charge neutrality.

Ion	$Conc_{intracell.}$ [mM]	$Conc_{extracell.}$ [mM]
Na^+	5–15	145
K^+	140	5
Mg^{2+}	0.5	1–2
Ca^{2+}	10^{-4}	1–2
Cl^-	5– 15	110

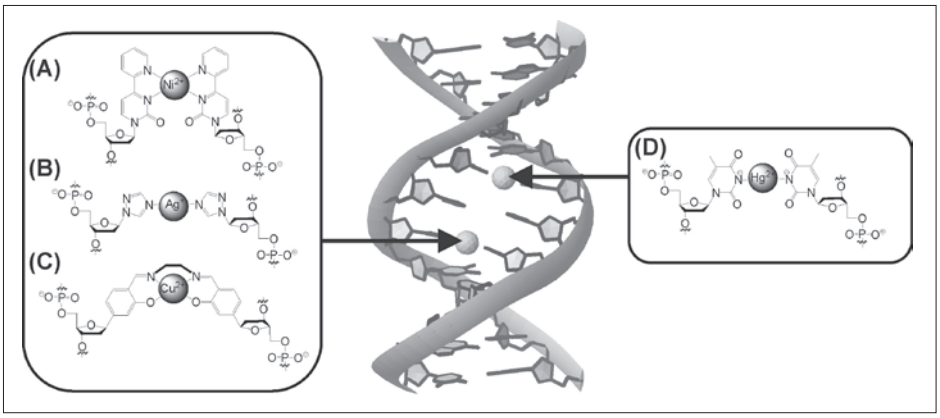

Fig. 2. Metal-modified base pairs in a DNA double helix. **(A)** A cytosine-derived base pair selectively binds Ni^{2+} compared to other divalent ions.[48] **(B)** Artificial triazole nucleosides complex linearly coordinated Ag^+ ions with high affinity.[49] **(C)** An ethylenediamine-crosslinked salen base pair chelates a Cu^{2+} ion.[50] **(D)** Two thymines of a T–T mismatch base pair are deprotonated at the N(3) position thus coordinating a Hg^{2+} ion in between. The stability of this Hg^{2+} mediated base pair is comparable to a normal Watson-Crick base pair.[51]

alkaline metal ions are basically spectroscopically silent. Hence, other metal ions are commonly applied to mimic, *e.g.* Mg^{2+}. These ions are either directly detectable by NMR or other spectroscopic methods, or have a higher affinity towards nucleic acids. For example, inner sphere binding of Mg^{2+} towards oxygen ligands can be studied using Cd^{2+} in so-called thio-rescue studies:[13,32] Thereby, one or more specific carbonyl or phosphate oxygen atoms in a nucleotide are replaced with sulphur atoms. Mg^{2+} has an intrinsic lower affinity towards sulphur compared to oxygen ligands and will thus bind weaker. In contrast, metal ion binding can be 'rescued' by applying a more thiophilic ion like Cd^{2+} or Zn^{2+}.[33–36] The introduction of a sulphur atom has the positive side effect that the chemical shift of the attached carbon or phosphorous atom is strongly affected: *e.g.* in a thiophosphate group, the ^{31}P resonance is shifted downfield to about 55 ppm, compared to about 0 ppm of a regular phosphodiester bridge in nucleic acids, making such a position well distinguishable.[37]

A further mimic for the diamagnetic Mg^{2+} is the paramagnetic Mn^{2+} ion. The latter is used for EPR[38,39] and also NMR studies, where the resonances are severely broadened upon binding of Mn^{2+} in the vicinity.[26,40,41] Outer sphere binding of Mg^{2+} can be probed with $[Co(NH_3)_6]^{3+}$ (see also Section 3). The ammonia ligands in this kinetically inert complex do not exchange with solvent molecules, allowing them to mimic the $[Mg(H_2O)_6]^{2+}$ ion. Binding of this cobalt(III) complex can then be observed by NOE cross peaks between the ammonia and protons of the nucleic acids[42–44] or even by solid state ^{59}Co NMR experiments.[45]

Also mimics for monovalent metal ions, in particular K^+, are known. Potassium binding to nucleic acids can be mimicked and observed by applying NH_4^+, allowing the detection of NOE cross peaks similar to the situation with $[Co(NH_3)_6]^{3+}$.[46] Another mimic specific for K^+ is the Tl^+ ion, which has a similar ionic radius and metal–ligand bond length, but is very toxic already at low concentrations. Nevertheless, direct detection is possible using ^{205}Tl NMR.[47]

2.3 *Artificial Metal Ions–Nucleic Acids Systems*

The self-assembling properties of DNA have prompted the development and studies of DNA duplexes of specific sequences or containing modified nucleobases that are able to tightly bind metal ions in the inner core of the helix. The base pairing properties of DNA can be altered by incorporating metal ions in the center of two opposite nucleobases, allowing the double strand to implement non-natural base pairs held together through coordinative forces rather than hydrogen bonds.[10,11] For example a thymine–thymine (T–T) mismatch base pair is stabilized by addition of Hg^{2+}, which replaces the H(3) protons of the two opposite pyrimidine bases (Fig. 2D). Incorporation of ^{15}N labeled thymine nucleobases allowed the first direct spectroscopic proof of such T–Hg^{2+}–T base pairs: ^{15}N NMR studies revealed a ^{15}N–^{15}N *J*-coupling across the Hg^{2+} ion (see Section 5).[51] A highly selective oligodeoxyribonucleotide Hg^{2+} sensor could be created based on this specific interaction.[52,53] Recent studies showed that also an RNA duplex synthesized by *in vitro* transcription can incorporate Hg^{2+} ions to form uracil–Hg^{2+}–uracil base pairs.[12] *In vitro* transcription thereby has the advantage that large amounts of nucleic acid (RNA) can be made easily. Another pyrimidine mismatch pair was stabilized by an Ag^+ ion. The cytosine–Ag^+–cytosine (C–Ag^+–C) base pair was reported recently to be stabilizing C–C mismatched DNA duplexes.[54]

The possibilities to incorporate metal-mediated base pairs into DNA are dramatically increased by the use of artificial nucleosides.[11] New nucleobase analogues can not only be designed to specifically bind certain metal ions, but the modified DNA can also serve as a scaffold for one-dimensional arrays of metal ions by the incorporation of several consecutive artificial base pairs. These arrays can then be used as self-assembling nanowires or nanomagnets.[55] There are also examples where catalysis of enantioselective reactions is realized through the metal ions of base-modified DNA helices.[56] Fig. 2A–C shows a few examples of artificial nucleosides within a DNA helix that exhibit specific binding properties towards different metal ions. The base pairs can also be covalently linked, as for example in a metal-salen base pair (Fig. 2C). With these artificial nucleosides a metal-modified DNA double helix with ten consecutive Mn^{3+}-salen base pairs was synthesized.[50] Constructs like this will allow the synthesis of more complex DNA molecules containing different metal binding sites in specific positions.

This expansion beyond the genetic four letter code by the introduction of new metal-modified artificial base pairs leads to the development of new metal ion sensors, novel molecular electronics, or possible multi-metal enzyme analogues.

3. NMR Strategies to Investigate M^{n+} Binding to Nucleic Acids

Various NMR methods are known to study metal ion binding to nucleic acids

using different probes and mimics. These methods range from the observation of chemical shift changes, which can be done with basically any metal ion, over paramagnetic line broadening studies, to the most direct way of the detection of NOE contacts between the nucleic acid and probes like cobalt(III)hexammine or the ammonium ion. The most important experiments are discussed in more detail below.

3.1 *Magnesium(II) Coordination*

The detection of the metal ions that are naturally associated with nucleic acids (*i.e.* Na^+, K^+ and Mg^{2+}), is a challenging task as they are spectroscopically silent and can thus not be observed directly in an NMR experiment. Mg^{2+} binding is kinetically labile and therefore the metal ion does not necessarily have only one fixed coordination site within the nucleic acid structure.[5] Furthermore, the interaction with the nucleic acid is not always direct (inner sphere coordination) but can also be mediated through the water ligands of the solvated metal ion (outer sphere coordination) (Fig. 1). However, it is possible to observe Mg^{2+} coordination by NMR because the chemical shifts as well as the resonance line widths of the nucleic acid protons are affected by a coordinating Mg^{2+}. A change of the chemical shift can be due to either a M^{n+}-coordination in close vicinity of the observed proton or to a long-range structural change that is induced by the metal ion. The broadening of resonance lines is observed due to the relatively fast ligand exchange rate of Mg^{2+}, which is in the order of the NMR time scale.[34] This effect is limited specifically to the binding site thus allowing a more precise localization of the metal ion within the structure. [^{1}H,^{1}H]-NOESY titrations of RNA hairpins with $MgCl_2$ recorded in D_2O reveal chemical shift changes and line broadening at specific sugar and nucleobase residues (Fig. 3A).[26,41,57,58] For example, a large chemical shift change is observed for the $U19_{H1'}$–$A20_{H8}$ crosspeak within domain 6 of a group II intron ribozyme but the line width is unvaried, arguing against a direct coordination of Mg^{2+} at the A20 N7 position.[26,60] At the $G1_{DP}$, on the other hand, the chemical shift change is accompanied by a significant broadening effect indicating a direct binding of Mg^{2+} at the 5'-terminal diphosphate (DP) group.[41] The combination of chemical shift mapping and line broadening studies with Mg^{2+} thus yields a rather detailed picture about Mg^{2+} binding and accompanying structural changes of the nucleic acid. However, to precisely localize the binding sites as well as the liganding atoms and to determine the geometry of the coordination by NMR, much more information is necessary. To the best of our knowledge, at the moment, no study exists where this has been accomplished for kinetically labile metal ions. The closest system to achieve this goal is the use

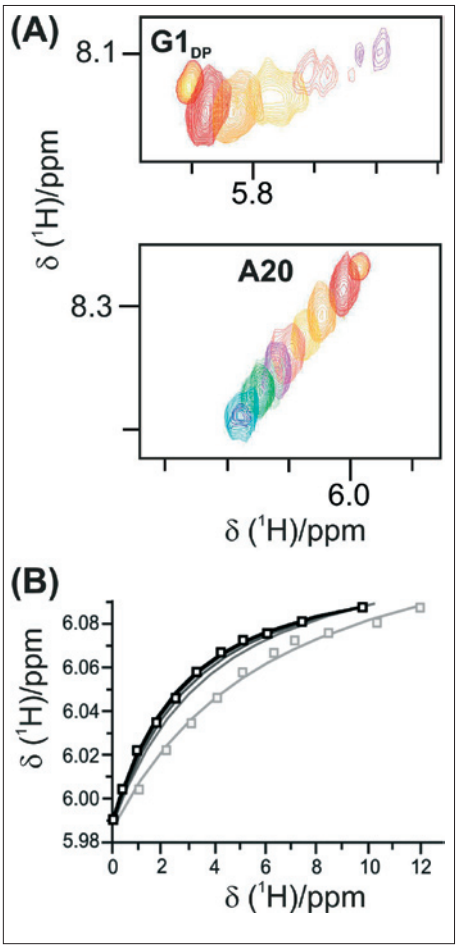

Fig. 3. **(A)** Section of the [^{1}H,^{1}H]-NOESY spectrum of a 27-nucleotide long RNA hairpin (D6-27) upon addition of Mg^{2+} (0–12 mM, red – blue) (adapted from ref. [59]). Both crosspeaks shift upon addition of Mg^{2+} indicating metal ion binding. In the case of the $G1_{DP}$ resonance, significant line broadening is additionally observed, suggesting a direct binding of Mg^{2+} at the 5'-terminal diphosphate group. **(B)** Plot of the change in chemical shift of a ^{1}H resonance in D6-27 *versus* [Mg^{2+}] and fit with a non-linear least-squares fit according to Eqn. (1). By taking the other binding sites within D6-27 into account, the available [Mg^{2+}] for this binding site gets smaller, and the log*K* values increase with every iteration round (grey to black). A significant improvement of the fit is observed from the application of the iterative procedure.

of a inert complex like $[Co(NH_3)_6]^{3+}$, which mimics the hexahydrated Mg^{2+}. By recording NOE contacts between the amine and nucleic acid protons direct distance information can be obtained.

3.2 *Cobalt(III)hexammine: A Probe for Outer Sphere Coordination*

Cobalt(III)hexammine is used as a probe for outer sphere complexation by $[Mg(H_2O)_6]^{2+}$ with RNA. The reason is mainly based on geometric similarities but this complex has also been shown to activate magnesium(II)-dependent enzymes.[61,62] Due to its higher charge, $[Co(NH_3)_6]^{3+}$ interactions with the nucleic acid are about ten times stronger than is observed for $[Mg(H_2O)_6]^{2+}$.[63] With respect to DNA, $[Co(NH_3)_6]^{3+}$ has been shown to stabilize Z form DNA, as well as to promote the conversion from B to A form, which is also driven by naturally occurring cations and polyamines.[64,65]

$[Co(NH_3)_6]^{3+}$ coordination to nucleic acids is usually studied either by chemical shift mapping or by observation of NOE contacts between the ammonia protons of the Co(III) complex and the imino protons or aromatic protons of the nucleic acid. For example, in their studies on the stabilization of DNA isomers by $[Co(NH_3)_6]^{3+}$ and polyamines, Robinson and Wang used such NOEs between the ammonia ligands and guanosine H(8), imino, and cytosine amino protons of DNA to determine the exact geometry of the interaction.[65] Similarly Tinoco *et al.* employed $[Co(NH_3)_6]^{3+}$ as a mimic of outer sphere complexation of the hydrated magnesium(II) ion by RNA solving the solution structures of $[Co(NH_3)_6]^{3+}$ coordinated to a group I intron ribozyme domain (Fig. 4), a viral RNA pseudoknot, a GAAA tetraloop and the P4 element of the RNAse P ribozyme.[43,44,66,67] The interaction between $[Co(NH_3)_6]^{3+}$ and nucleic acids is highly dynamic:[44] Only one resonance is observed for all 18 ligand protons of $[Co(NH_3)_6]^{3+}$ at 3.65 ppm, arguing for a fast rotation of the complex in its binding pocket.

Despite all these above listed advantages, $[Co(NH_3)_6]^{3+}$ also has some caveats that

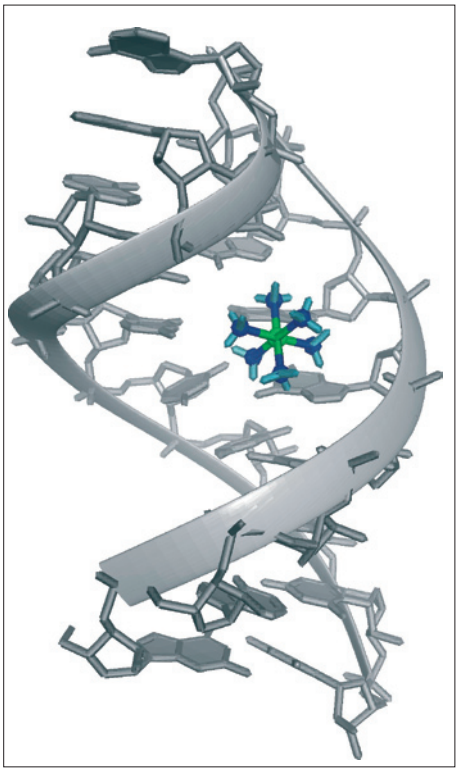

Fig. 4. NMR solution structure of the P5b stem-loop from a group I intron ribozyme with a bound $[Co(NH_3)_6]^{3+}$. The Fig. was prepared with MOLMOL[27] using the PDB coordinates 1AJF.[44]

one needs to be aware of. This Co(III) complex can only substitute for $[Mg(H_2O)_6]^{2+}$, but not for partially dehydrated Mg(II) complexes, *i.e.* inner sphere coordinated Mg^{2+}. A recent evaluation of Mg^{2+} binding within the large ribosomal subunit has shown that only nine out of 116 Mg^{2+} are bound in a completely outer sphere manner.[5,8] As a consequence the majority of Mg^{2+} ions is partially inner sphere bound and cannot be mimicked with $[Co(NH_3)_6]^{3+}$.

3.3 *Paramagnetic Metal Ions as Probes for Inner Sphere Coordination*

The paramagnetic Mn^{2+} ion is commonly used as a probe for inner sphere metal coordination to nucleic acids. Paramagnetic ions induce a more rapid relaxation of the nuclei in their vicinity, leading to line broadening of the respective resonance. The broadening effect is proportional to the distance between the paramagnetic ion and the nucleus with a r^{-6} dependence.[63] Mn^{2+} binding usually has a short lifetime and because of the fast exchange between bound and unbound state the resonances of the nuclei in close proximity to the binding site are selectively broadened already at very low Mn^{2+} concentrations. Paramagnetic line broadening induced by Mn^{2+} has been successfully used to localize divalent metal ion binding sites in selfsplicing ribozymes.[42,68,69]

3.4 *NH_4^+: A Probe for Monovalent Cation Binding*

The monovalent ions Na^+ and K^+, both commonly associated with nucleic acids *in vivo* and/or *in vitro*, are as difficult to observe as, *e.g.* Mg^{2+}. In addition to their spectroscopic silence, the M^+ ions usually bind even weaker than Mg^{2+}. Well known substitutes for K^+ are Tl^+ and NH_4^+. Tl^+ is one of the most sensitive NMR nuclei, but its high toxicity as well as redox activity limits its practical use in the laboratory. Ammonium instead is an often used substitute for K^+, which in the form of $^{15}NH_4^+$ is also observable by NMR. For example, the titration of a DNA quadruplex with $^{15}NH_4Cl$ allowed the first direct localization of a monovalent cation binding site by recording NOEs between the ammonium ion and the DNA protons.[46] The titration studies showed however, that NH_4^+ is coordinated preferentially over Na^+ and K^+ and its tetrahedral geometry as well as its potential to form hydrogen bonds with the nucleic acid makes it distinctly different from the two alkali ions.

4. Determination of Affinity Constants of M^{n+} Binding to RNA

As already described above chemical shift mapping and line broadening analysis can be used to determine metal ion binding sites in a qualitative way. As for large nucleic acid structures, chemical shift changes are easier to measure and with greater certainty than the line width, only the first effect will be discussed here in more detail. However, in all the formulas listed below, the chemical shift can be replaced by the line width to calculate binding constants.

To calculate affinity constant K_{Ai} of a metal ion towards a binding site *i* in a RNA quantitatively, the chemical shift changes δ_{obs} of the observed protons are plotted versus the M^{n+} concentration $[M^{n+}]_{tot}$. The experimental data is then fit to a 1:1 binding isoterm (Eqn. (1)) using for example a Levenberg-Marquardt nonlinear least-square regression.[58,70] $[RNA]_i$ corresponds to the concentration of the RNA, δ_{RNAi} to the chemical shift of the unbound binding site, and $\delta_{RNA\cdot M}$ to the one of the fully bound site.

Usually, more than only a single coordination site is present in nucleic acids. These are thus filled up in parallel if they have similar affinities towards the metal ions. As a consequence, a metal ion bound to one site is not available to bind to another site simultaneously. This means that the actual available $[M^{n+}]$ for each site is smaller than $[M^{n+}]_{tot}$, and that in order to calculate intrinsic M^{n+} affinities for a given coordination site, the binding of M^{n+} to all the other sites has to be taken into account. To automate this extensive calculation, we have recently developed ISTARv2.2, a MatLab script, that performs the iterative procedure to calculate the intrinsic metal ion affinity for each site K_{ai} from the NMR chemical shift change data.[26] This program runs in two steps: First, the affinity constants $K_{A,est}$ are estimated according to Eqn. (1) using $[M^{n+}]_{tot}$ and the chemical shift data of protons as obtained from NOESY titration experiments. Based on these $K_{A,est}$ values and additional line broadening data the individual binding sites are defined manually. The $K_{A,est}$ values obtained for each proton of a binding site are then averaged to give a first set of averaged affinity constants $K_{A,av1}$ for each individual binding site. The $K_{A,av1}$ values are then used to calculate the bound $[M^{n+}]_{bound,i}$ at each site by using Eqn. (2):

The M^{n+} concentration available for binding to a specific site *i*, is then given by Eqn. (3).

These corrected M^{n+} concentrations for each individual site $[M^{n+}]_{avail,i}$ are then used in the next iteration step, fitting the chemical shift change data again to a 1:1 binding model. The second set of affinity constants $K_{A,est2}$ for each evaluated proton are again averaged to obtain improved affinity constants $K_{A,av2}$ for each individual binding site. These corrected averaged affinity constants serve as a basis to calculate again the amount of bound M^{n+} to each binding site. The described procedure has to be repeated until no change of the $K_{A,av}$ values within the error limits is observed (Fig. 3B). The final affinity constants $K_{A,fin}$ are obtained by plotting the average values of each site *versus* the number of the iteration round and fitting the data to an asymptotic function.

Most importantly, the above described iteration procedure ISTARv2.2 is generally applicable to all systems that have multiple binding sites for the same ligand.

5. Artificial Systems

Besides the studies on metal ion interactions with DNA and RNA in naturally occurring systems, investigations of artificial metal-modified nucleic acids become increasingly important, as these molecules are promising candidates for nanodevices and can also be employed as tools in biotechnology. Although the development and investigation of such artificial systems is currently a popular research field,[11] only few NMR studies are known in which direct nucleic acid–metal contacts or metal-induced structural changes could be observed.[12,51]

$$\delta_{obs} = \delta_{RNA_i} + \left(\delta_{RNA\cdot M} - \delta_{RNA_i}\right)\frac{\left[M^{n+}\right]_{tot} + \left[RNA_i\right]_{tot} + \frac{1}{K_{Ai}} - \sqrt{\left(\left[M^{n+}\right]_{tot} + \left[RNA_i\right]_{tot} + \frac{1}{K_{Ai}}\right)^2 - 4\left[M^{n+}\right]_{tot}\left[RNA_i\right]_{tot}}}{2\left[RNA_i\right]_{tot}} \quad (1)$$

$$\left[M^{n+}\right]_{bound,i} = \frac{\left(K_{A,av1_i}\left[M^{n+}\right]_{tot} + K_{A,av1_i}\left[RNA_i\right]_{tot} + 1\right) - \sqrt{\left(-\left(K_{A,av1_i}\left[M^{n+}\right]_{tot} + K_{A,av1_i}\left[RNA_i\right]_{tot} + 1\right)\right)^2 - 4K_{A,av1_i}{}^2\left[M^{n+}\right]_{tot}\left[RNA_i\right]_{tot}}}{2K_{A_i}} \quad (2)$$

$$\left[M^{n+}\right]_{avail,i} = \left[M^{n+}\right]_{tot} - \sum\left[M^{n+}\right]_{bound} + \left[M^{n+}\right]_{bound,i} \quad (3)$$

One promising way to build-up metal-modified DNA is to use natural nucleotides combined with metal ions. Already in 1963 Katz proposed that Hg^{2+} ions bind specifically to thymine–thymine mismatches in a linear fashion to form metal-ion mediated base pairs. The N(3)-H protons of two opposite located thymine residues of the DNA double strand are replaced by one Hg^{2+} ion.[71] Just recently Tanaka *et. al* were able to prove the proposed bond formation between Hg^{2+} and the N(3)s of the two thymines by NMR.[51] They performed ^{15}N NMR experiments with DNA oligomers containing thymine–thymine mismatches in which either two labeled thymines (Fig. 5A and C) or one labeled and one unlabeled thymine were used (Fig. 5B). In ^{15}N experiments a splitting of the ^{15}N resonances of about 2.4 Hz was observed for the first case, which disappeared when a labeled thymine was base paired with an unlabeled one. This clearly shows that the splitting of the ^{15}N resonances is due to a ^{15}N–^{15}N J-coupling across Hg ($^2J_{NN}$) and is therefore a direct evidence for the formation of thymine–Hg^{2+}–thymine base pairs. Furthermore all N(3) resonances assigned from a [^1H,^{15}N]-HSQC spectrum show downfield shifts of about 30 ppm (~155 ppm to ~185 ppm) upon complexation with Hg^{2+} ions. Such large downfield shifts can only be explained by a proton–metal exchange and therefore also give evidence for the formation of the linear thymine–Hg^{2+}–thymine base pair.

DNA strands are usually made by chemical synthesis, making it difficult to synthesize very long strands as well as high amounts thereof. This is especially true if modified nucleotides are used. As RNA offers the same principles in terms of sequence specificity and structure, we have set up a system to use T7 RNA polymerase to obtain nucleic acids in defined length and sequence in a fast and highly efficient way by *in vitro* transcription:[72] Several RNA constructs containing stretches of 2, 6, 10 and 20 uracils in length[12] were obtained in relatively high yield. The incorporation of Hg^{2+} to form uracil–Hg^{2+}–uracil base pairs was investigated by NMR using diffusion-ordered spectroscopy (DOSY).[12] Three of the designed constructs were palindromic, thus the addition of Hg^{2+} ions induces a structure conversion from hairpin to double helix due to the uracil–Hg^{2+}–uracil base pair formation. Such a conversion should not only result in distinct changes of chemical shifts but also in the size of the construct. DOSY experiments were used to determine the hydrodynamic radius r_H of the molecules in absence and in presence of Hg^{2+} ions.[12] In addition, we compared the experimental values with the theoretical ones for the hairpin and duplex formation of the constructs. Depending on the theoretical ratio q (q = L/d) between the length L and the diameter d of the RNA sequences the molecules can be either modeled as a spherical particle ($q < 2$) or as a symmetrical cylinder ($2 < q < 30$).[73,74] The apparent hydrodynamic radius r_H can then be calculated according to Eqn. (4) and (5), respectively.

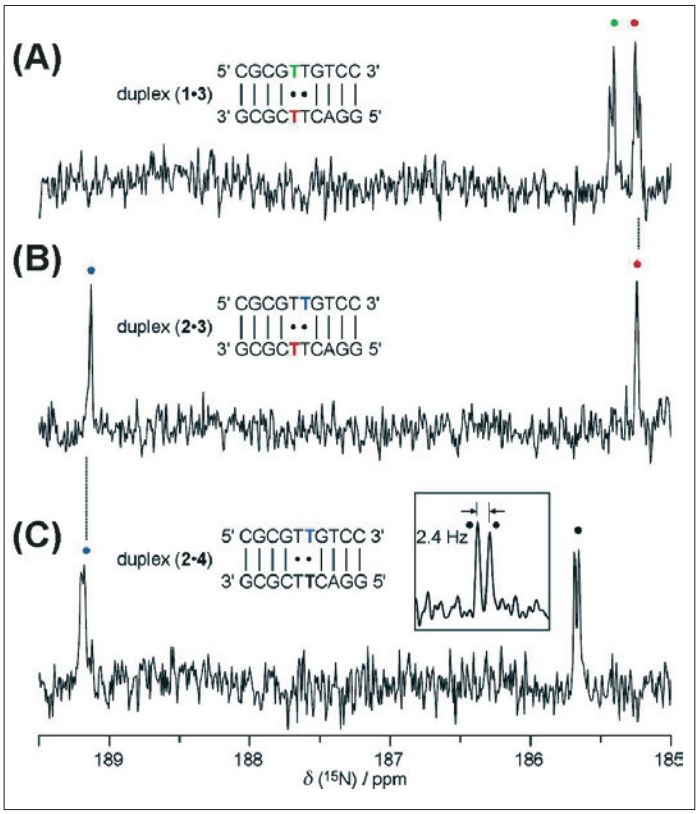

Fig. 5. ^{15}N-NMR spectra of DNA double-helices with two thymine–Hg^{2+}–thymine base pairs. In **(A)** and **(C)** two ^{15}N labeled thymines are directly base paired leading to a splitting of the ^{15}N resonances of about 2.4 Hz due to a ^{15}N–^{15}N J-coupling across Hg^{2+} ($^2J_{NN}$). No splitting is observed when a ^{15}N labeled thymine is directly base paired with an unlabeled one **(B)**. Reproduced with permission from Ref. [51]

$$r_H = \frac{L}{2} \quad (4)$$

$$r_H = \frac{L}{2\left(\ln q + 0.312 + 0.565q^{-1} - 0.1q^{-2}\right)} \quad (5)$$

For example, the palindromic construct with six uracil residues in-line clearly adopts a hairpin structure with a hydrodynamic radius of 1.6 nm in the absence of Hg^{2+} ions. Upon addition of Hg^{2+} a 30%-increase to 2.1 nm is observed unambiguously arising from a hairpin-duplex transition. The experimental and the theoretical data are thereby in good agreement.[12]

The two examples above nicely illustrate that NMR is a powerful tool to explore metal-ion binding sites and metal-induced structural changes within artificial oligonucleotide systems.

6. Concluding Remarks

The manifold interactions of metal ions with natural and artificial nucleic acids is a highly fascinating and diverse world, ranging in research fields from classical coordination chemistry, *via* bioinorganic chemistry to biochemistry and nanotechnology. It is well known that metal ions are crucial for structure and function of catalytically active RNAs and DNAs, *i.e.* ribozymes and DNAzymes, and hence it is somewhat surprising to realize how little is known on these relations. However, aside from the many coordinating atoms in nucleic acids, the lability of the ligands of the applied and used metal ions make the elucidation of structural and thermodynamic properties highly challenging. Here we have shortly summarized the current state of solution NMR experiments to address these questions. It will be fascinating to see what novel experiments will be developed in the near future and what unprecedented metal ion binding pockets will be discovered and described.

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