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Communication

Nucleotide coordination with 14 lanthanides studied by isothermal titration calorimetry

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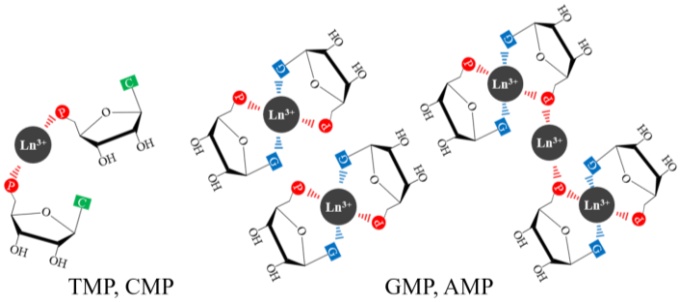
Graphical Abstract



ITC reveals the increasingly importance of entropy for heavier lanthanides binding to nucleotides. The phosphate group forming chelating effect with purine bases but not with pyrimidines.

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ABSTRACT

With their hydrolytic, optical and magnetic properties, lanthanide ions (Ln^{3+}) are versatile probes for nucleic acids. In addition, nucleotide-coordinated Ln^{3+} ions form useful nanoparticles. However, the thermodynamic basis of their interaction is still lacking. In this work, isothermal titration calorimetry (ITC) is used to study the binding between nucleotides and 14 different Ln^{3+} ions. Ln^{3+} interacts mainly with the phosphate of cytidine and thymidine monophosphate (CMP and TMP), while the nucleobases in adenosine and guanosine monophosphate (AMP and GMP) are also involved. Phosphate binding is fully entropy driven since the reactions absorb heat. Nucleosides alone do not bind Ln^{3+} and the purines need the phosphate for chelation. With increasing atomic number of Ln^{3+}, the binding reaction with GMP goes from exothermic to endothermic. The entropy contribution starts to increase from Gd^{3+}, explaining the 'gadolinium break' observed in many Ln^{3+}-mediated RNA cleavage reactions. This study provides fundamental insights into the Ln^{3+}/nucleotide interactions, and it is useful for understanding related biosensors, nanomaterials, catalysts, and for lanthanide separation.

Lanthanides refer to a group of 15 elements from La to Lu. While they have been researched mostly for electronic, optical, and magnetic applications [1], lanthanide ions (Ln^{3+}) are also useful tools in biochemistry. For example, nucleic acids enhance the luminescence of Tb^{3+} [2-4], which has been used for probing metal binding and designing biosensors [5]. Ln^{3+} can cleave RNA at high metal concentrations [6,7]. Since cleavage activity is sensitive to RNA secondary structure, this is also useful for structural probing [8]. In addition, Ln^{3+} ions were used as metal cofactors in ribozymes and DNAzymes [3,9-17]. DNAzymes are DNA-based catalysts with promising applications in biosensor

development [18,19], gene silencing [20-23], nanotechnology [24], and bioinorganic chemistry and catalysis [25,26]. Finally, nucleotide-coordinated Ln^{3+} makes useful nanomaterials for enzyme encapsulation and sensing [27-32].

Based on their atomic number, lanthanides are divided into the light (La to Gd) and heavy (from Tb to Lu) groups, a useful distinction in describing their interaction with nucleic acids. A few studies have demonstrated the group trend of Ln^{3+} for interacting with nucleic acids. For example, the heavy lanthanides are much more active in cleaving a dinucleotide RNA junction [33]. Similar observations were also made in some RNA-cleaving DNAzymes: the GR5 [13], and Tm7 [16]; both are only active with the heavy lanthanides. Apparently, some properties change around Gd^{3+}, and this type of 'gadolinium break' has been often observed [13]. A few other nucleic acid enzymes (*e.g.* the Ce13d and leadzyme), however, have quite similar activities across the lanthanide series [12,14,15]. Finally, we recently used Tb^{3+} luminescence spectroscopy to study Ln^{3+} binding to DNA [34]. Most of these studies however, do not provide quantitative thermodynamic information.

Nucleic acids provide an interesting scaffold for lanthanide binding. They contain a negatively charged polyphosphate backbone that can strongly interact with lanthanides *via* electrostatic and Lewis acid/base interactions. At the same time, the four nucleobases with both oxygen and nitrogen based ligands may allow fine tuning of more specific coordination [35]. However, few previous works systematically studied their binding thermodynamics [36-41]. Isothermal titration calorimetry (ITC) is a powerful technique to study biomolecular binding, and a few studies reported on Ln^{3+} binding to nucleic acids [4,42]. Herein, we employ ITC to examine Ln^{3+} binding to nucleotides and nucleosides for the whole lanthanide series, revealing important thermodynamic trends and insights of their coordination.

We chose to use nucleoside monophosphates in this work to avoid complications related to folding and denaturation of nucleic acids by Ln^{3+}. In addition, we studied the basic structural units of nucleic acids: the nucleosides and phosphate. The molecules used in this study are shown in Fig. S1 (Supporting information). The ITC experiments were performed at pH 6 with 25 mmol/L NaCl. This condition is relevant to biochemical studies of Ln^{3+} interacting with nucleic acids. The light Ln^{3+} ions coordinate to nine water molecules, while the heavy ones are smaller and they coordinate to eight [43]. The change in coordination number occurs at Gd^{3+}. The first pK_a of Ln^{3+} ranges from 9.33 (La^{3+}) to 8.17 (Lu^{3+}) [44]. Therefore, at pH 6, Ln^{3+} remains largely as free non-hydrolyzed ions.

Since guanine is the most important nucleotide for sensitized Ln^{3+} luminescence [5,34], and for binding Ln^{3+} [45-47], we first studied guanosine monophosphate (GMP). By optimizing the experimental conditions, we found the background is lower by titrating lanthanides into nucleotides, instead of the other way (Fig. S2 in Supporting information). By integrating the heat, we obtained the binding curve, from which we can extract all the thermodynamic constants, including ΔH, ΔS, the association constant (K) and ΔG.

After fixing the experimental conditions, a series of titrations were performed by injecting each respective Ln^{3+} into GMP (Fig. 1). Some interesting trends were observed. For example, binding of the light Ln^{3+} was accompanied with heat release, while the heavy ones resulted in heat absorption. The baselines are quite flat for each injection and the peaks are quite symmetric, indicating fast binding kinetics.

To have a complete understanding, we then repeated the experiment with other nucleotides. Instead of using all the 14 Ln^{3+}, we chose Nd^{3+} and Lu^{3+} to represent light and heavy Ln^{3+}, respectively. When Nd^{3+} was titrated into AMP (Fig. 2A), the trace was quite similar to that of Nd^{3+} into GMP. Initially, the amount of heat released was large, and a typical binding curve was observed. The ΔH of this reaction was calculated to be -1.97 ± 0.15 kcal/mol, which is slightly larger than that for Nd^{3+} into GMP (-1.40 ± 0.29 kcal/mol). When Lu^{3+} was titrated into AMP, the system showed a very small amount of heat response (Fig. 2F). However, we can at least conclude that the amount of heat released is much less (almost zero) compared to that of the Nd^{3+} titration. Interestingly, for TMP and CMP, the interactions were entropic, for both the light Nd^{3+} and the heavy Lu^{3+} (Figs. 2C, 2D, 2H, 2I). For both nucleotides, binding was much tighter to Lu^{3+} with more heat absorbed.

A nucleotide contains a phosphate and a nucleoside. To dissect the contribution of each component, we first probed the phosphate binding using ribose-5-phosphate (R5P, see Fig. S1B in Supporting information for structure), for which the base is removed. Phosphate is an important metal ligand in nucleic acids [48,49]. Its binding is endothermic (Figs. 2E and 2J), with the heavy Ln^{3+} absorbing much more heat (notice the different scale in the *y*-axis). This is quite similar to that of CMP and TMP. It is clear that purine and pyrimidine nucleotides have very different binding properties, and the pyrimidines rely on the phosphate for binding. For the purine nucleotides, binding also involves the bases.

After the phosphate, we then studied the nucleosides, and none of them gave much heat (Fig. S3 in Supporting information). This is not surprising since lanthanides are strong hard Lewis acids and the nucleosides are soft Lewis

bases. Therefore, without the phosphate, the interaction with the bases alone is weak, even for guanine. Taken together, the phosphate interaction is required for binding of Ln^{3+} to nucleotides. Without a base, the phosphate part can still bind, but not the other way around. However, the bases can influence phosphate binding, which explains the differences among the nucleotides. There might be some synergistic effects between the base and phosphate (*e.g.* chelating effect).

In the above results, we only described the data qualitatively. We then integrated the heat of each injection to obtain binding curves (Fig. 1 bottom panels), showing three types of possible coordination sites. An example illustrating these three sites is shown in Fig. S2F (Supporting information). For all the titrations, these three stages of binding were well separated. The affinity of stage 3 is drastically increased as the atomic number of Ln^{3+} increases. We calculated the thermodynamic parameters for all of the Ln^{3+} ions using a three-site model (Fig. 3, Table S1 in Supporting information).

The thermodynamic trends and values of site 1 (Figs. 3D, 3H, 3K) are very similar to those from site 2 (Figs. 3E, 3I, 3L). Therefore, we reason these two have the same chemical origin. The first site has a binding ratio of 0.05 ± 0.01 for all the Ln^{3+} (Fig. 3A), meaning each Ln^{3+} can bind to 20 GMP molecules. This has far exceeded the chemically possible coordination number of Ln^{3+}. For comparison, site 2 has a ratio of 0.5 ± 0.04 for all the Ln^{3+} (Fig. 3B). Therefore, site 1 is omitted from most of the subsequent discussion, due to its small binding ratio. It is likely that the binding of site 1 is due to the initial attraction of multiple GMP by each Ln^{3+} ion. As more Ln^{3+} was added, the initial complex broke to form the final binding complex. This process leads to an apparent two binding stages, but sites 1 and 2 likely have the same chemical origin. The second and third binding sites both have a Ln^{3+}-to-GMP ratio of 0.5 ± 0.06 (Figs. 3B and 3C). After finishing all the binding reactions, the final ratio was still 0.5 ± 0.06, indicating that these binding reactions took place simultaneously and each Ln^{3+} could participate in both sites 2 and 3.

For each binding site, we obtained ΔH and the association constant K. From K, the ΔG was calculated and then ΔS was calculated. For site 2, the enthalpy of binding goes from negative to positive and the point of transition is at Dy^{3+} (Fig. 3E). For the third binding site, the ΔH is positive for all the Ln^{3+} (Fig. 3F). Therefore, binding at site 3 is fully entropy driven. For the second site, the binding affinity between Ln^{3+} and GMP is quite similar for all of the Ln^{3+} (Fig. 3I) at ~10^5 L/mol (K_d = 10.2 ± 3.5 µmol/L). This binding affinity agrees with that from luminescence-based measurements [34]. Given the relatively small difference and the error bar size, we do not quantitatively describe the K_d trend here. Interestingly, for site 3, binding is very weak (K_d = 1.1 ± 0.3 mmol/L) for the first few light Ln^{3+}. Then, the binding affinity increased drastically reaching 3.8 ± 0.7 µmol/L for the last few heavy Ln^{3+} (Fig. 3J). The overall trend of entropy increased as the atomic number of Ln^{3+} increased for both sites 2 and 3 (Figs. 3L and 3M). Therefore, binding of the heavier Ln^{3+} is more entropy driven. It is interesting to note that the ΔS difference between Lu^{3+} and La^{3+} is much larger for the third binding site than the second one.

We then examined the trend of entropy change (Figs. 3K-M). By considering only the metal ion and GMP, a binding reaction should decrease the entropy. The fact that entropy is increased for each Ln^{3+} binding to GMP is attributed to the associated water molecules. Ln^{3+} ions are highly hydrated and the number of associated water molecules changes from 9 for the lighter Ln^{3+} ions to 8 for the heavier ones. The change of water coordination number is attributed to the size contraction. For entropy related contributions, it is likely that the bound waters are released from the Ln^{3+} and inner-sphere interactions are achieved with GMP. For the lighter Ln^{3+}, the number of released water molecules may be smaller or the interaction might be more of an outer-sphere nature, with more bound water molecules remaining associated with these lanthanides after binding to GMP.

The standard entropy of liquid water is 16.7 cal/mol K. The entropy of water bound to metal salts is around 10 cal/mol K, regardless of the metal species [50]. Therefore, the entropy gain from releasing a bound water molecule is ~6.7 cal/mol K. The entropy change of our system at its largest was 32.7 ± 1.5 cal/mol K (Fig. 3M), which corresponded to the release of 5 water molecules. For the smaller Ln^{3+}, the number of released water molecules was 2-3. Therefore, the difference appears to be that more water molecules are released from the heavier Ln^{3+}. In every case, the Ln^{3+} ions were not completely dehydrated after binding. We ignored the release of water from GMP and the change of entropy due to GMP-Ln^{3+} binding, but this description did provide a better physical picture of the difference between the light and heavy Ln^{3+}.

A question is why the larger Ln^{3+} ions tend to absorb heat. The enthalpy of hydration for Ln^{3+} becomes gradually more negative as the atomic number increases. For example, Lu^{3+} has the largest value of -898.3 kcal/mol, compared to the value of -784.6 kcal/mol for La^{3+} [44]. In addition, the number of coordinated water molecules decreases from 9 to 8 from La^{3+} to Lu^{3+}. Therefore, the energy cost of dehydration is nearly 30% more for Lu^{3+} than that for La^{3+}

(112.3 kcal/mol for Lu^{3+} *vs.* 87.1 kcal/mol for La^{3+}), which can explain the heat absorption required for binding heavier Ln^{3+}.

None of the nucleosides produced much heat with Ln^{3+} (Fig. S3 in Supporting information), indicating very weak interaction with the bases and the sugar ring. When a phosphate and nucleosides are linked to form nucleotides, new interactions can take place due to the chelation effect. Based on the heat profile from ITC titrations, TMP and CMP behave like R5P. In other words, their phosphate interaction dominates (true for both light and heavy Ln^{3+}). We also quantitatively fitted the four nucleotides plus R5P (Fig. 4, Table S2 in Supporting information). The three site-model was applied also to AMP. We did not show site 1 since it again had a very low ratio of <0.05. For CMP, TMP and R5P, we only used a two-site model (site 1 and 3) since site 2 could not be identified in their binding curves. For these three, the contribution of site 1 was also less than 0.05 and thus we also omitted them for discussion. Ln^{3+} binding to AMP and GMP is more influenced by the bases. For example, when the light Ln^{3+} interacted with AMP or GMP, there was an overall release of heat in the initial ITC profile.

By observing the ITC profiles of R5P, the enthalpy term is unfavorable for either light or heavy Ln^{3+}. Therefore, its binding of Ln^{3+} is fully entropy driven. As such, Ln^{3+} ions interact with the phosphate *via* inner-sphere coordination and the coordinated water molecules are lost (thus inner sphere). This is reasonable since phosphate is a strong ligand for Ln^{3+}. The interaction between Ln^{3+} and the purine base of GMP or AMP is likely outer-sphere coordination. This interaction resulted in a reduction in enthalpy (negative ΔH). A difference between light and heavy Ln^{3+} is that for light Ln^{3+}, the enthalpy contribution from the purine interaction is enough to overcome the effects of the phosphate interaction and the net result is a release of heat. For heavy Ln^{3+}, the heat release of the purine interaction is insufficient to overcome the larger heat absorbed in the phosphate interaction and the result is an overall enthalpically unfavourable process.

Via extensive spectroscopic studies [51-53], it was concluded that in acidic pH, Ln^{3+} undergoes inner-sphere coordination with the phosphate, and outer-sphere coordination with the N7 of guanine. We reason that the chemical origin of the second binding site is the chelation of Ln^{3+} by the phosphate and the guanine base, which might take place at the O6 and N7 positions [46,48,54]. Given the similarity of the ITC profiles between AMP and GMP and previous literature, we tend to believe that the N7 site is more important, which is common in these two nucleotides.

ITC also gives insight into the binding stoichiometry. For all of the lanthanides, a final binding ratio of 0.5 ± 0.06 is observed, meaning that every lanthanide ion binds to two GMP molecules, regardless of the size of the lanthanide. For GMP and AMP, binding site 2 originated from the chelation of phosphate and the purine base, and binding site 3 was purely from the remaining coordination power of the phosphate. Since the K_d of these two sites are similar for the heavy lanthanides, binding to these two sites are taking place simultaneously (Fig. 5B). For the light lanthanides, site 2 might be filled first since it has much higher affinity than site 3 (Fig. 5A). For CMP, TMP and R5P, the main interactions are with the phosphate part and this explains that we mainly see one type of site in Fig. 3 for them (site 3) (Fig. 5C). Note that these binding configurations are hypothesized based on our ITC data, and further confirmation requires more rigorous structural biology tools such as NMR and X-ray crystallography.

Our interest on the lanthanides is from *in vitro* selection of RNA-cleaving DNAzymes using Ln^{3+} as a cofactor [14-16,55-57]. Most of the DNAzymes showed a clear lanthanide size-dependent activity trend [55]. In every case, binding of Ln^{3+} to the scissile phosphate is critical since it can neutralize the negative charge in the transition state. So far, we have observed a few types of behavior in lanthanide trends for DNAzyme catalysis. For the Lu12 and Ce5 DNAzymes, their activity gradually decreased as the atomic number of Ln^{3+} increased [14,57]. This continuous trend agrees well with the thermodynamic trends reported here for the GMP interactions. Note that the scissile phosphate in our system is flanked immediately by a guanine and an adenine, both are purine nucleotides. The Ce13d DNAzyme binds only one Ln^{3+} ion and it has a very similar activity with all the other lanthanides [15,58]. This may suggest an outer-sphere interaction to some extent such that the charge of the metal is more important.

In summary, we performed systematic ITC experiments to study the interaction between four types of nucleotides and 14 lanthanide ions. Purine nucleotides interact with Ln^{3+} *via* both the phosphate and nucleobase, and there is a synergistic chelation effect between these two groups. Pyrimidine nucleotides interact with Ln^{3+} mainly *via* the phosphate group, with little contribution from the bases. With increasing atomic number of Ln^{3+}, the interactions with phosphate increase, explaining the better efficiency of heavy Ln^{3+} as a cofactor for some DNAzymes. From quantitative thermodynamic calculations, lanthanide binding is mainly driven by entropy from released water molecules during the binding reaction, especially for the heavy lanthanides. The release of water suggests inner sphere coordination, thus making it easier to distinguish heavy lanthanides than light ones. The thermodynamic

insights from this study can be used to explain the lack of nanoparticle formation with TMP, CMP, and Ln^{3+}. In addition, it rationalizes the simple and more complex trend of Ln^{3+} activity in various DNAzymes by changing the number of ions involved. Such insights are useful for guiding rational ligand design for better separation of these important metals.

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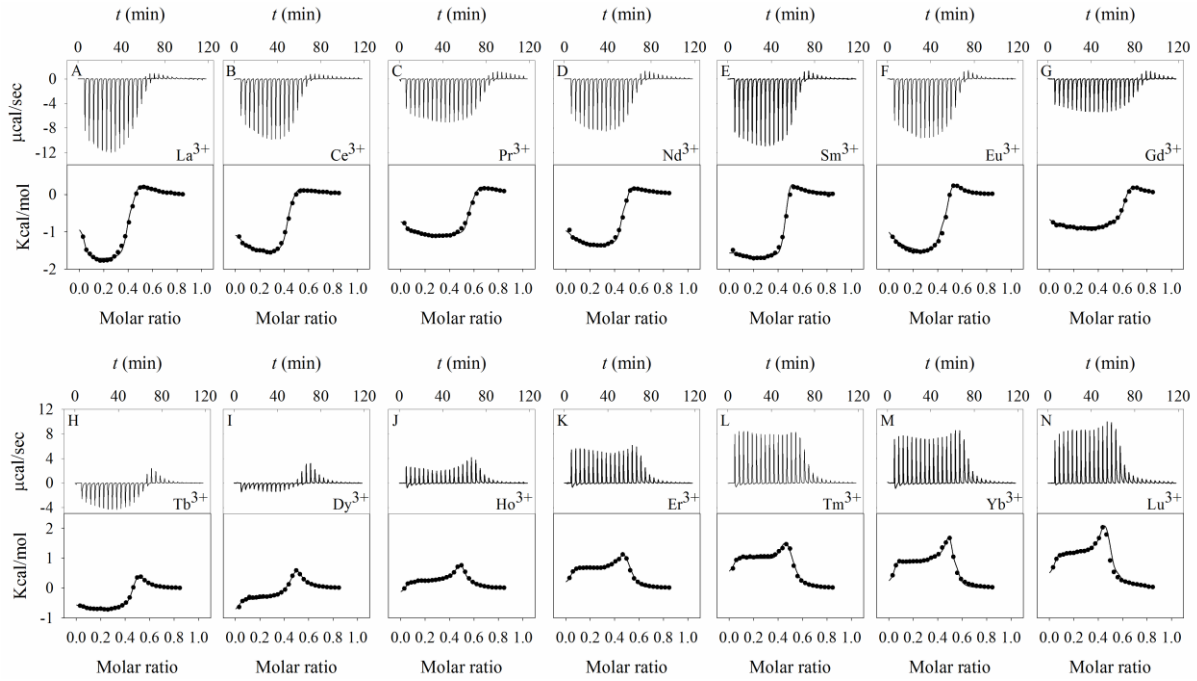

Fig. 1. The complete ITC titration traces of 14 Ln^{3+} ions into GMP in the buffer A at 297 K: (A) La^{3+}; (B) Ce^{3+}; (C) Pr^{3+}; (D) Nd^{3+}; (E) Sm^{3+}; (F) Eu^{3+}; (G) Gd^{3+}; (H) Tb^{3+}; (I) Dy^{3+}; (J) Ho^{3+}; (K) Er^{3+}; (L) Tm^{3+}; (M) Yb^{3+} and (N) Lu^{3+}. Each peak corresponds to an injection of 200 nmol of Ln^{3+} into a fixed 7.275 µmol of GMP. Background heat of titrating Ln^{3+} into buffer was subtracted. The integrated heat of each titration is also shown and the data points were fitted to extract thermodynamic parameters.

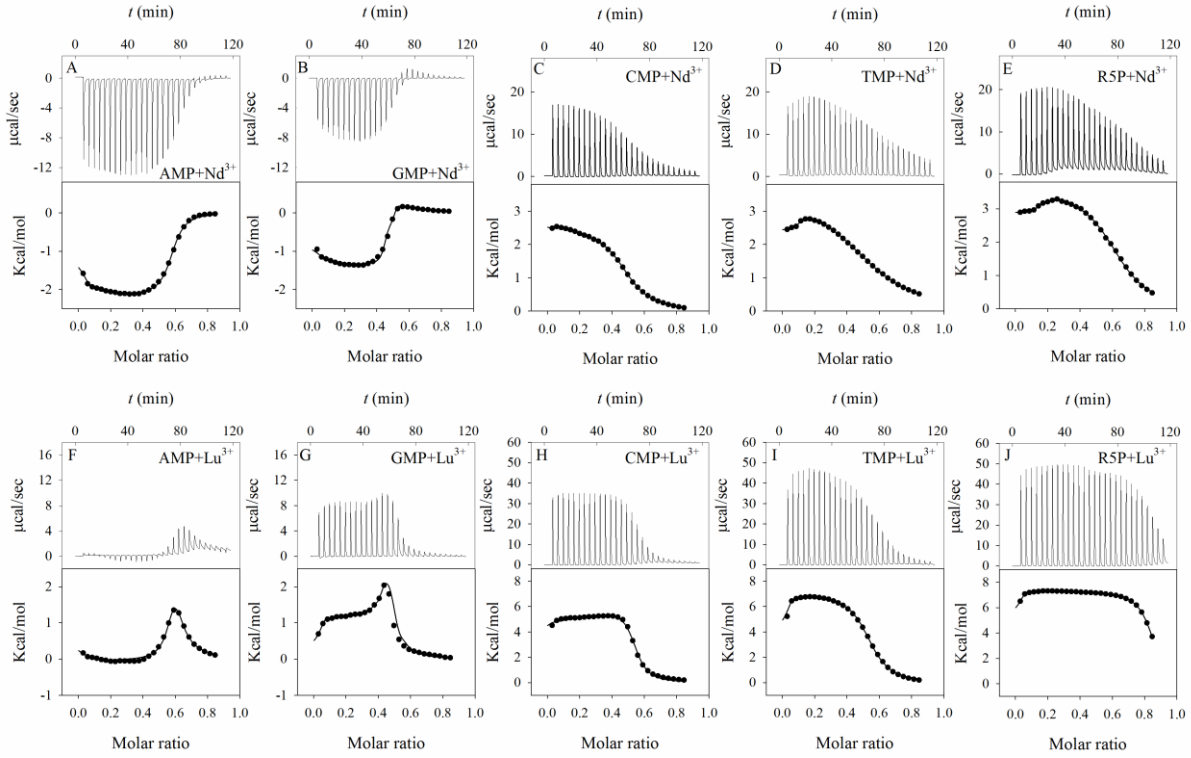

Fig. 2. ITC traces of Nd^{3+} into 5 mmol/L (A) AMP, (B) GMP, (C) CMP, (D) TMP and (E) ribose 5-phosphate (R5P). ITC traces of Lu^{3+} into 5 mmol/L (F) AMP, (G) GMP, (H) CMP, (I) TMP and (J) ribose 5-phosphate (R5P).

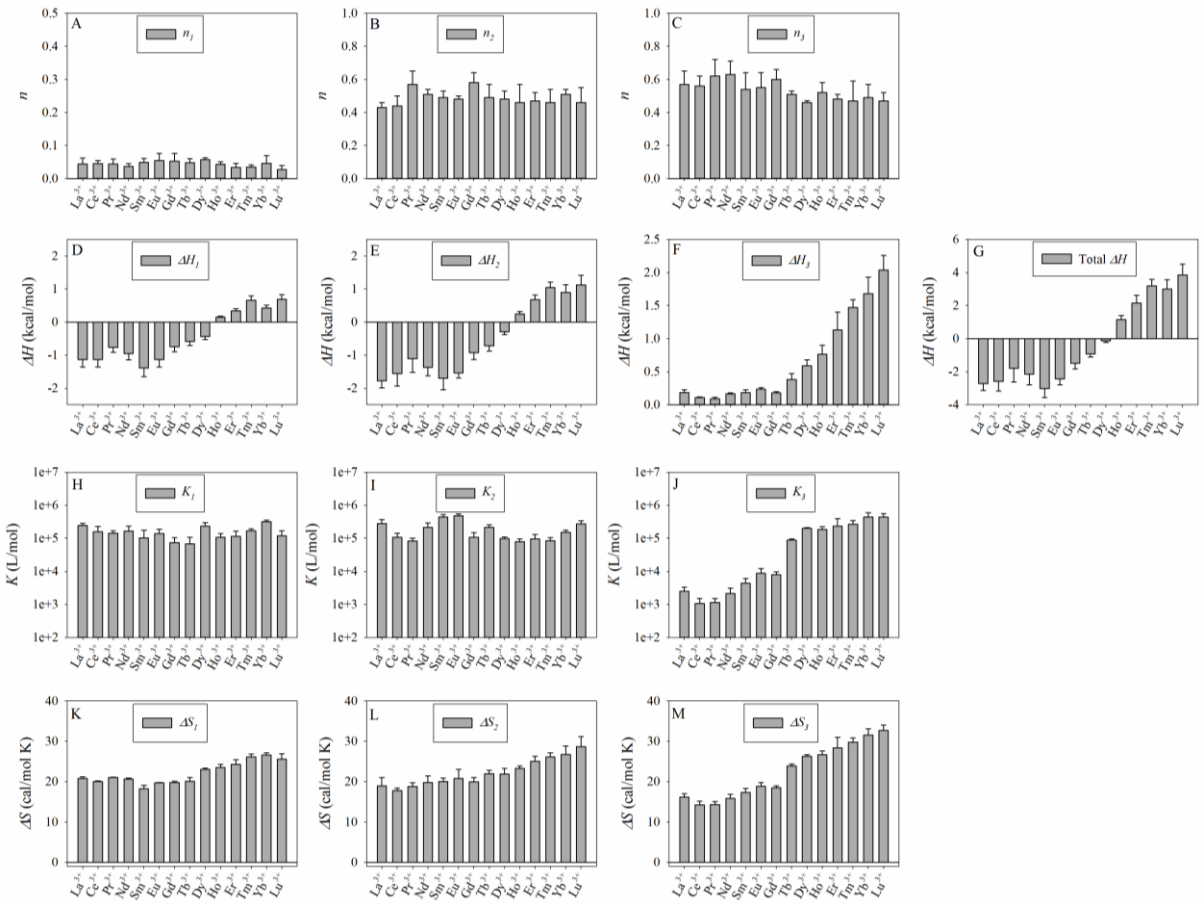

Fig. 3. The thermodynamic trends of Ln^{3+} titrated into GMP solution based on a three-site model: (A-C) the binding ratio, *n*, between Ln^{3+} and GMP; (D-G) reaction enthalpy; (H-J) binding constant; (K-M) reaction entropy. The total enthalpy change for all the three sites added together is presented in (G).

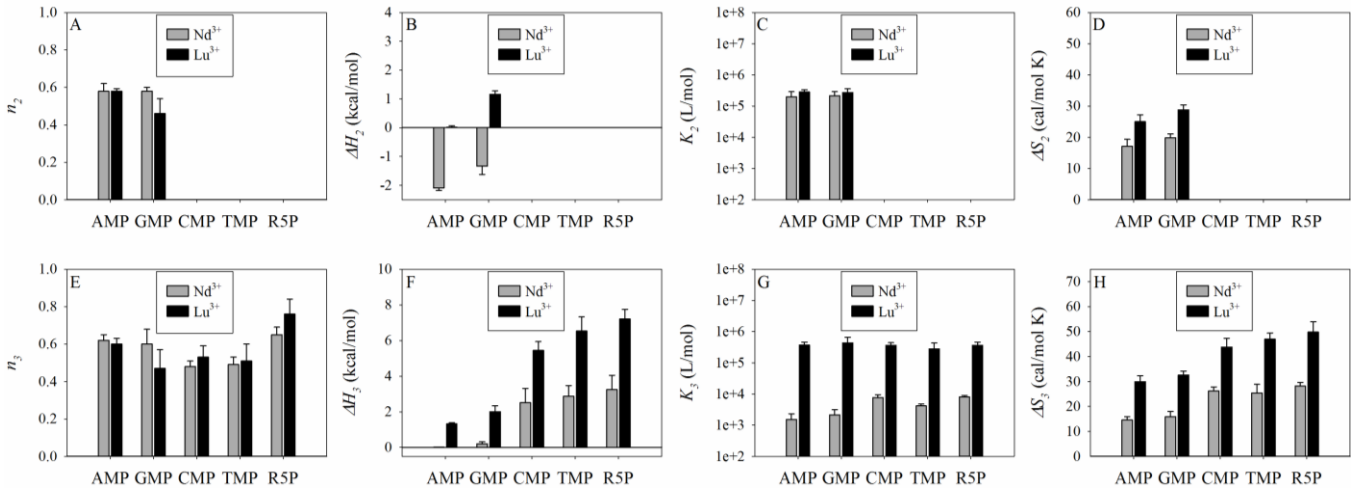

Fig. 4. Comparison of different nucleotides and R5P for binding Nd^{3+} and Lu^{3+} ions in terms of binding stoichiometry (A, E), enthalpy (B, F), binding constants (C, G) and entropy (D, H) for binding site 2 (A-D) and 3 (E-H). Data for site 1 are not plotted due to the very small binding ratio.

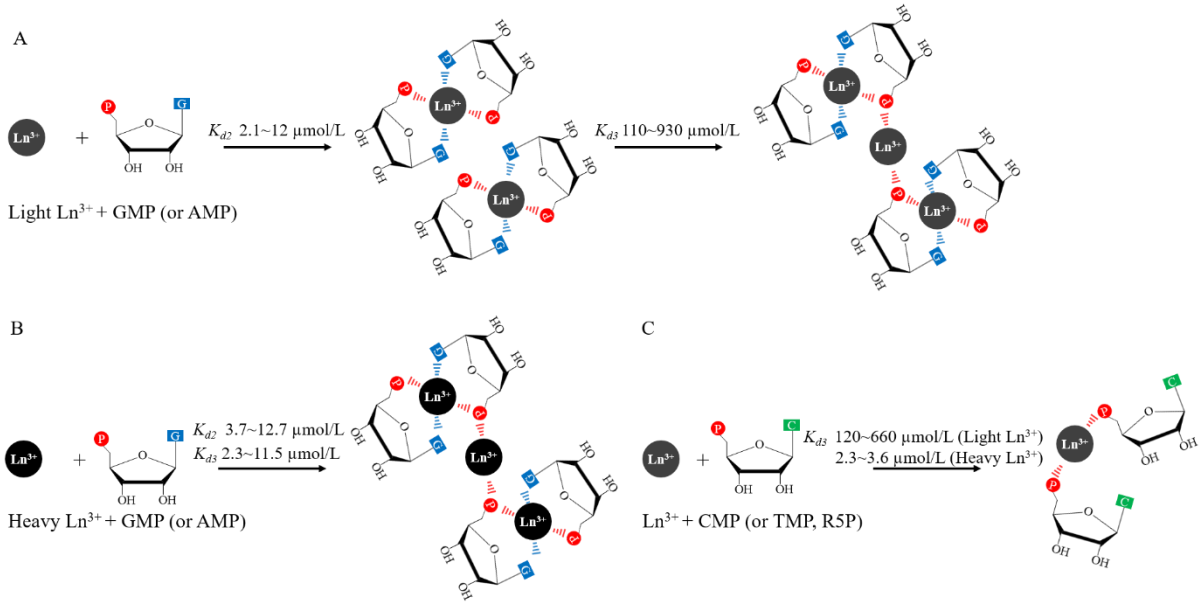

Fig. 5. A scheme showing the coordination interactions. (A) For GMP and AMP interacting with light Ln^{3+} (La to Gd), the phosphate and the base first chelate Ln^{3+}. With further additions of Ln^{3+}, the remaining coordination power of the phosphate is used. (B) For GMP and AMP interacting with heavy Ln^{3+} (Tb to Lu), the phosphate and the base bind nearly simultaneously (K_2 and K_3 ranges are nearly equal). Drawing is a simplified cartoon and does not represent the exact coordination number. For example, initially more than two GMP should be coordinated to each Ln^{3+} at low Ln^{3+} concentrations. (C) For CMP and TMP, the phosphate part is playing the main coordination role with little base involvement, similar to that of R5P.