A Selective Na^+ Aptamer Dissected by Sensitized Tb^{3+} Luminescence

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Abstract

A previous comparative study between two RNA-cleaving DNAzymes named NaA43 and Ce13d revealed the possibility of a common Na^+ aptamer in these two DNAzymes. Since Na^+ binding by DNA is a very fundamental biochemical problem, herein the interaction between Ce13d and Na^+ is studied in detail using sensitized Tb^{3+} luminescence spectroscopy. Na^+ displaces Tb^{3+} from the DNAzyme, and thus quenches the emission from Tb^{3+}. The overall requirement for Na^+ binding includes the hairpin and the highly conserved 16-nucleotide loop in the enzyme strand along with a few unpaired nucleotides in the substrate. Mutations studies indicate a good correlation between Na^+ binding and the DNAzyme cleavage activity, suggesting the critical role of Na^+ binding for the enzyme activity. Ce13d displays a K_d of ~20 mM Na^+, while other monovalent cations are in the ~40-60 mM range. The measured K_d for other metal ions are mainly due to non-specific competition. With a single nucleotide mutation, the specific Na^+ binding is lost, while another mutant improved the K_d to 8 mM Na^+. This study has demonstrated a Na^+ aptamer with important biological implications and analytical applications. It has also defined the structural requirements for Na^+ binding and produced an improved mutant.

Keywords: sodium, aptamers, terbium, luminescence, sensors

Introduction

Metal binding is a key topic in biochemistry with applications in analytical chemistry and medicine.[1-4] In the past two decades, significant progress was made in DNA-based metal binding.[5] First, certain nucleotides and DNA sequences can selectively bind metals, such as thymine-rich DNA for Hg^{2+},[6] cytosine-rich DNA for Ag^{+},[7] and guanine-rich DNA for Pb^{2+}, K^{+}, and Tl^{+}.[8,9] However, most other metals cannot be specifically recognized by such rationally designed sequences. The second method relies on aptamer selection. However limited success was made on this front and few high quality metal aptamers were reported so far.[10,11] Finally, metal ions serve as cofactors in DNAzyme catalysis.[12] DNAzymes are DNA-based catalysts isolated from in vitro selection. By using specific metal ions during selections, many DNAzymes were obtained with high specificity for Na^{+},[13,14] Pb^{2+},[15,16] Zn^{2+},[17] Cu^{2+},[18,19] UO_2^{2+},[20] Cd^{2+},[21] Hg^{2+},[22] and trivalent lanthanides.[23-27] While in principle such metal specificity is associated with a metal binding site, it is quite challenging to extract aptamer motifs from DNAzymes.

Sodium is one of the most abundant physiological metal ions influencing many biological reactions, processes, and cell signaling.[13,28] For example, elevated sodium results in water retention and high blood pressure.[29,30] Therefore, it is important to develop Na^{+} sensors. Current Na^{+} sensors have low binding affinity and specificity.[31,32] Using DNA for Na^{+} sensing might be a useful alternative, if a Na^{+} aptamer can be obtained. In DNA chemistry, Na^{+} has been conceived as a general salt ion without considering its specific binding.[33] Early work in using Na^{+} for DNAzyme catalysis was set to test whether DNAzymes can work in the absence of divalent metal ions.[34-36] Many ribozymes are active in a few molar of monovalent salts without much metal specificity.[37] Recently, a few well-defined Na^{+}-specific RNA-cleaving DNAzymes were reported.[13,14,25] In particular, a DNAzyme reported by Lu and co-workers named NaA43

has a rate of ~0.1 min^{-1} with Na^+ alone.[13] Interestingly, it has a stretch of 16 nucleotides identical to a lanthanide-dependent DNAzyme (named Ce13d) we reported previously.[25] Further studies indicate that Ce13d requires both Na^+ and a lanthanide ion for activity.[38,39] In Ce13d, those identical nucleotides are highly important for the cleavage activity,[40] and they are responsible for the sodium specificity.[39]

While the full-length DNAzyme apparently binds Na^+, it is interesting to test whether mutations that change the DNAzyme activity can also influence Na^+ binding. Among the various methods to study metal binding, we chose sensitized Tb^{3+} luminescence here due to its simplicity and high sensitivity. Tb^{3+} is a widely used probe for nucleic acids.[41,42] Free Tb^{3+} ions have a low extinction coefficient and poor luminescence. After binding with DNA, the emission is significantly enhanced due to the DNA antenna transferring energy to Tb^{3+}. Guanine is the most effective nucleotide in enhancing Tb^{3+} luminescence,[43-45] and Ce13d is rich in guanine in the enzyme loop. Therefore, Tb^{3+} is an ideal probe for this system. Herein, we report our findings in correlating the activity of Ce13d with its Na^+ binding property, and our efforts in identifying the structural motifs for Na^+ binding.

Experimental Section

Chemicals. All the DNA samples were from Eurofin Genomics. See Table S1 for the DNA sequences and modifications. The metal salts were obtained from Sigma-Aldrich (St. Louis), and dissolved in Milli-Q water. Buffers were purchased from Mandel Scientific (Guelph, ON) and prepared using Milli-Q water. Denaturing gel loading dye was obtained from New England BioLabs and the other the gel running reagents were from Bio Basics Inc.

Sensitized Tb^{3+} luminescence. The DNAzyme complexes were prepared by annealing the uncleavable substrate (all DNA analog, named Sub-dA) and an enzyme strand (1 μM each) in Tris buffer (50 mM, pH 7.5 with a monovalent salt, 25 mM). Annealing was performed by heating the samples at 95 °C for 1 min and then slowly cooling down to room temperature over 30 min. Then, the DNAzyme complex (200 μL) was mixed with Tb^{3+} (final concentration of 5 μM). The sensitized Tb^{3+} emission was measured in a quartz cuvette using a fluorometer (FluoroMax-4, Horiba Scientific) by exciting at 290 nm. Alternatively, the DNAzyme complex (2 μM) was similarly annealed in the Tris buffer containing Li^+ or Na^+ (25 mM). Afterwards, Tb^{3+} (5 μM) was added and the luminescence intensity at 543 nm was monitored in a 96-well plate with a microplate reader (M3, SpectraMax). The luminescence intensity at 543 nm in Li^+ and Na^+ was compared. The k_d value was calculated based on the equation: $F = F_0 - \frac{a \cdot [M^+]}{k_d + [M^+]}$, where F and F_0 are the luminescence intensity before and after adding metal, respectively; and $[M^+]$ is metal concentration.

Activity assays. The Ce13d activity assay was carried out in MES buffer (50 mM, pH 6.0) with NaCl (25 mM) and Ce^{3+} (10 μM). The DNAzyme complex was formed using enzyme strand (1.5 μM) and FAM-labeled substrate (1 μM, the cleavable Sub-FAM substrate). At designated time points, a small aliquot was transferred to urea (8 M) to quench the reaction and the samples were separated by denaturing polyacrylamide gel electrophoresis (15%) and analyzed using a ChemiDoc MP imaging system (Bio-Rad).

Results and Discussion

Probing Na^+ binding using Tb^{3+} luminescence. The secondary structures of the Ce13d and NaA43 DNAzymes are shown in Figure 1A, and B, respectively. Each DNAzyme complex contains a substrate stand and an enzyme strand. The substrate has a single RNA linkage (rA, ribo-adenine) serving as the cleavage site. The structure of Ce13d was based on both Mfold prediction and biochemical characterization,[25] while the NaA43 structure was directly adapted from its original paper.[13] It needs to be pointed out that current information is insufficient to fully support either structure. The nucleotides in red are identical in these two enzymes, and our previous work indicated they are responsible for Na^+ binding.[39] Both DNAzymes require Na^+ for activity, while the Ce13d requires an additional lanthanide ion.[38,39] These nucleotides are rich in guanine (guanine content = 50%) and these unpaired guanines can tightly bind Tb^{3+} to enhance its emission.[43-45] To study Na^+ binding, we chose Ce13d since it is smaller. In addition, all the unpaired guanines in Ce13d are in the Na^+ binding loop, while NaA43 has quite a few extra free guanines in other positions. Since Tb^{3+} luminescence mainly probes guanine,[43-45] Ce13d should provide more direct Na^+ binding information in this case.

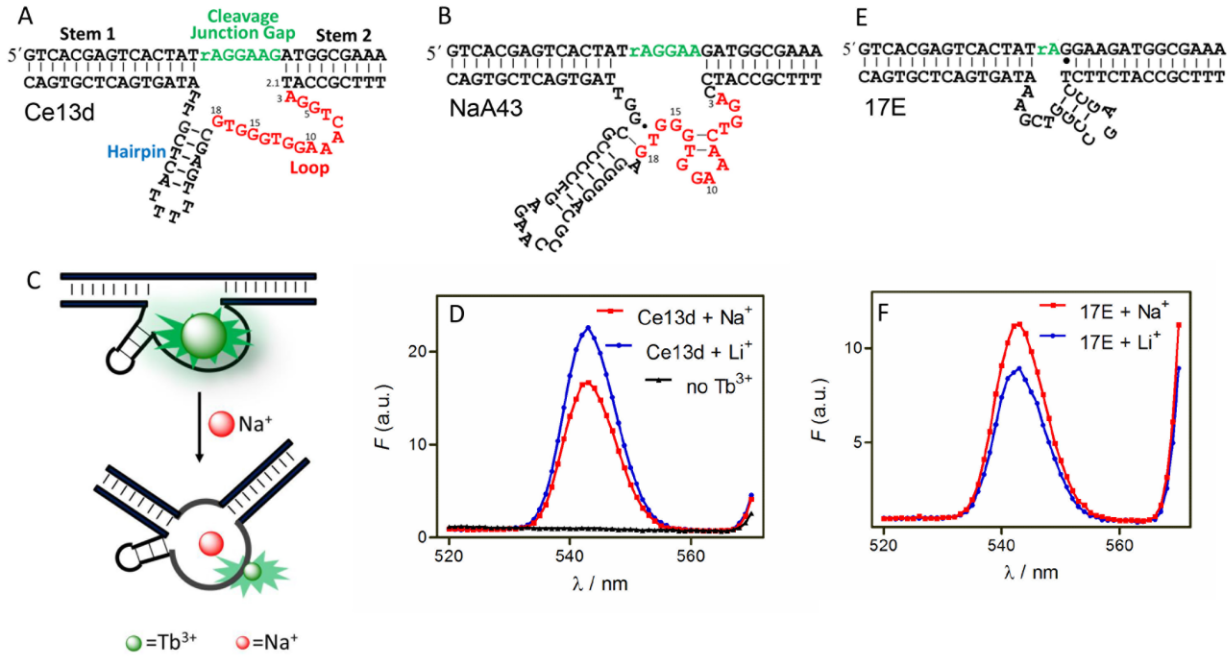

Figure 1. The secondary structures of (A) the Ce13d and (B) the NaA43 DNAzymes. The identical nucleotides in the loop regions are highlight in red. Ce13d contains two double-stranded substrate binding arms (Stems 1 and 2), a hairpin, a highly conserved loop, and an unpaired substrate cleavage junction region, each is marked in (A). (C) A scheme illustrating Na^+-induced specific folding of Ce13d, resulting in decreased Tb^{3+} luminescence. (D) Tb^{3+} luminescence spectra of Ce13d in 25 mM Na^+ or Li^+. (E) The secondary structure of 17E. (F) Tb^{3+} luminescence spectra of Ce13d in 25 mM Na^+ or Li^+. The concentration of the DNAzymes is 1 μM with a final concentration of 5 μM Tb^{3+}.

The scheme of our measurement is shown in Figure 1C. In the presence of Na^+, specific Na^+ binding folds the DNAzyme into a compact structure, thus weakening Tb^{3+} binding and decreasing its emission intensity. In the presence of other metals (such as Li^+), binding is non-specific and the drop of emission might be less. To demonstrate this, we prepared the Ce13d complex in either a Na^+ or Li^+ buffer, and Tb^{3+} was then added. To avoid cleavage, the RNA

base in the substrate was replaced by its DNA counterpart (named Sub-dA, see Table S1). As expected, the Ce13d/Tb^{3+} in Na^+ emitted less than that in Li^+ (Figure 1D). We also tested the 17E DNAzyme (Figure 1E), which is independent of Na^+.[36] In this case, the opposite was observed, with the Li^+ intensity being lower than that of Na^+ (Figure 1F). This is attributed to the higher charge density of Li^+, making 17E more compact and less accessible to Tb^{3+}.[46,47] Since 17E is much smaller than Ce13d, we also did the same experiment with a larger DNAzyme named Pb-C30,[16] and observed also lower emission in Li^+ (Figure S1). Therefore, Tb^{3+} is a good probe for Na^+ binding by Ce13d,[39] and it was used for the rest of this study.

Structural motifs in Ce13d for Na^+ binding. To understand the core sequences for Na^+ binding, we defined the following motifs in Ce13d (Figure 1A): 1) the two double-stranded substrate binding arms; 2) a hairpin that can be replaced by other hairpin sequences without affecting activity;[25] 3) a conserved loop known to be important;[40] and 4) the unpaired nucleotides in the substrate near the cleavage junction. The first two motifs are unlikely to be important for specific Na^+ binding, and we focus on the last two.

We started by investigating whether the 16-nucleotide loop alone (without the substrate or the rest of the enzyme) can bind Na^+. A cartoon showing this motif is presented at the top of Figure 2A. This 16-mer oligo was annealed in the Na^+ or Li^+ buffer, and then Tb^{3+} was added. The emission was higher in the Na^+ buffer (Figure 2A, the red trace on top), suggesting that Na^+ binding to the loop was weaker than Li^+. Therefore, the loop alone cannot form the Na^+ aptamer. Next we expanded the loop to the entire Ce13d (but without the substrate strand), and Na^+ still showed higher emission (Figure 2B), suggesting the substrate strand is required.

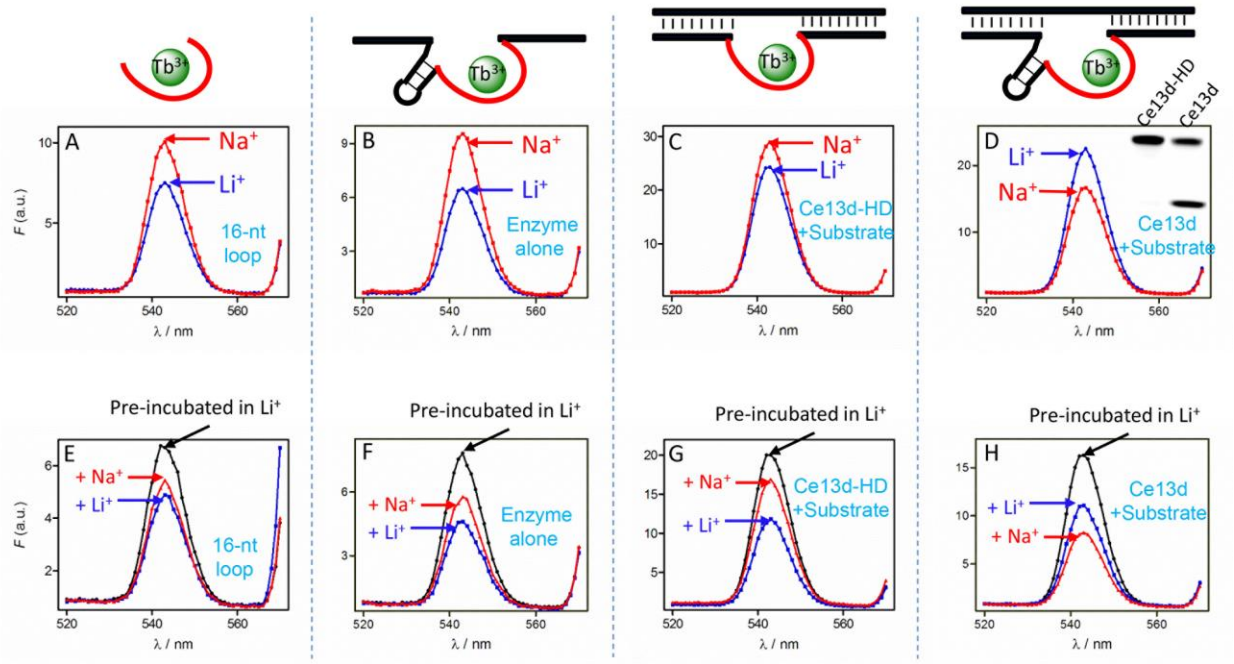

Figure 2. Tb^{3+} sensitized luminescence spectra in the presence of the 16-nucleotide loop (A, E), the Ce13d enzyme strand alone (B, F), Ce13d-HD (the hairpin removed enzyme hybridized with the substrate) (C, G), and the wild-type Ce13d DNAzyme complex (D, H). The cartoons of each sample are also shown. For the upper four spectral panels, the samples were annealed in 25 mM Na^+ or Li^+, prior to monitoring the Tb^{3+} luminescence. For the lower panels, the samples were annealed in 25 mM Li^+ first, and 25 mM additional Na^+ or Li^+ was added before monitoring their Tb^{3+} luminescence. Inset of (D): gel images showing the activity of Ce13d-HD and Ce13d in presence of 25 mM Na^+ plus 5 μM Ce^{3+}. For all the luminescence experiments, the final DNA concentration was 1 μM with 5 μM Tb^{3+}.

We then annealed the substrate and enzyme to form the complex, but the hairpin was truncated from the enzyme (denoted Ce13d-HD). This truncated complex also yielded a higher intensity in Na^+, indicating that Na^+ binding was still disrupted (Figure 2C). Finally, we tested the full Ce13d complex, and lower emission with Na^+ was achieved (Figure 2D, the blue trace on

top). To correlate Na^+ binding with cleavage activity, we also used the RNA substrate to measure cleavage in the last two systems. Only the wild-type Ce13d showed substantial cleavage, while the hairpin deleted Ce13d-HD was nearly inactive (Figure 2D, inset). Therefore, the hairpin is importance both for Na^+ binding and cleavage activity.

To further test metal binding, another set of experiments were performed by first forming the DNA complexes in the Li^+ buffer (black traces, Figure 2E-H). Then, additional 25 mM Na^+ or Li^+ was added. Tb^{3+} was added at last before measuring emission intensity. Here we aim to test whether Na^+ binding can still occur in the presence of Li^+. In each case (Figure 2E-H), we obtained the same result as that in Figure 2A-D. For the experiment in Figure 2H, we also tried other monovalent metals (Figure S2), and reached the same conclusion. Therefore, Na^+ can still form the aptamer binding complex even in the presence of other monovalent metal ions.

Probing the gap in the substrate strand. So far, we have focused on the enzyme strand. We next moved our attention to the substrate. While most of the substrate nucleotides are paired with the enzyme, Ce13d has six unpaired nucleotides near the cleavage junction forming a long gap (Figure 1A, in green). The wild-type Ce13d displays a stronger emission peak in Li^+ than in Na^+, with a luminescence ratio of ~1.35 (Figure 3A, see Figure 3E for quantification). To test the importance of the gap for Na^+ binding, we gradually sealed it by extending the enzyme strand. The mutated DNAzyme complexes are shown in Figure 3B-D as insets. By extending a single C base, the gap was shorted from six to five. Although the emission in Na^+ was still lower (Figure 3B), the ratio decreased from 1.35 to 1.20 suggesting slightly weakened Na^+ binding. This luminescence difference further diminished with the 4-nucleotide gap (Figure 3C), and even inversed with the 3-nucleotide gap (Figure 3D).

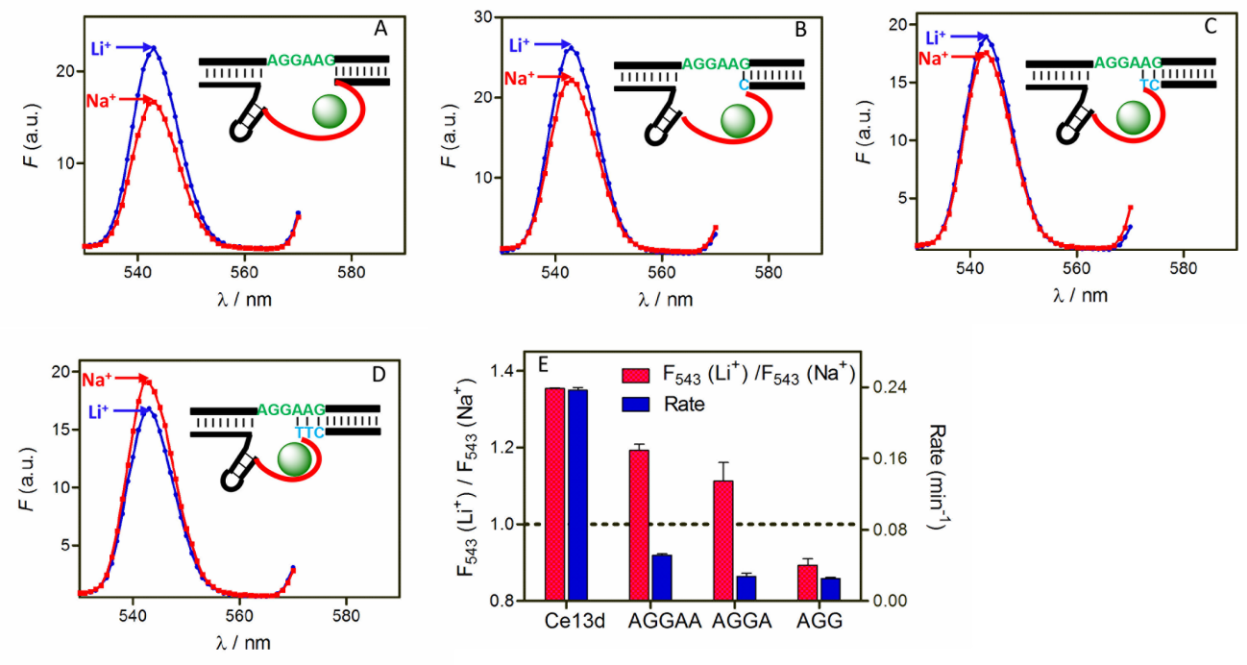

Figure 3. Tb^{3+} luminescence spectra of (A) the wild-type Ce13d, and mutants with (B) AGGAA, (C) AGGA, and (D) AGG as the substrate gap nucleotides near the cleavage junction in the Li^+ or in Na^+ buffer. The gaps are sealed by gradually extending the enzyme strand while maintaining the same substrate sequence (insets). (E) Tb^{3+} luminescence intensity ratio at 543 nm in Li^+ and in Na^+ of these samples. A dashed line at the ratio of 1 is drawn. Red bars above this line indicates specific Na^+ binding. Their cleavage activities are also plotted (blue bars using the right side *y*-axis).

For a quantitative understanding, we plotted the luminescence intensity ratio at 543 nm in Li^+ and in Na^+ (F_{Li}/F_{Na}, Figure 3E, red bars). If the ratio is higher than 1, specific Na^+ binding is maintained. Conversely, the Na^+ aptamer is abrogated if the ratio is below 1. This conservative threshold is set based on the principle that Li^+ is more efficient than Na^+ in condensing DNA. While this threshold is somewhat arbitrary, it is a convenient way to evaluate specific Na^+ binding.

To relate Na^+ binding with cleavage activity, these extended DNAzymes were also hybridized with the cleavable substrates (Figure 3E, blue bars). Among the different gap lengths, the original Ce13d has the highest activity with a rate of ~0.24 min^{-1}. As this gap gradually sealed, the cleavage activity also dropped. Again, there is a good correlation between Na^+ binding and activity for this part. Note that Na^+ binding was plotted as a ratio using Li^+ as a reference, while the activity was measured as absolute values. Thus, these two are not quantitatively compared, and we only discuss the general trend. Overall, the substrate with the six-nucleotide gap is optimal for Na^+ binding and activity. There are two possible roles for this gap. First, some nucleotides in this gap might involve in Na^+ binding. Alternatively, Na^+ binding may need a large binding cavity and this gap serves as a simple spacer (thus the nucleotide sequence is unimportant). Our previously studies showed that a single mutation on this gap (still maintaining the total nucleotide number of 6) abolished the Na^+ binding.[39] Therefore, we reason that the gap nucleotides contribute directly to metal binding instead of being a simple spacer.

Systematic mutation of the conserved nucleotides. Since the conserved nucleotides in the enzyme loop (Figure 1A, the red nucleotides) are the most important for activity and Na^+ binding, we next studied this part carefully. The data in Figure 2A indicate that these 16 nucleotides alone cannot work, and it has to be placed in the whole DNAzyme scaffold. Based on the wild-type Ce13d, we systematically mutated each nucleotide in this loop to the other three, yielding a total of 45 mutants.

For each mutant, we measured their Tb^{3+} luminescence in Li^+ over that in Na^+ (Figure 4A). Note that the absolute fluorescence intensity is slightly different for different mutants. Therefore, we only compared the relative luminescence of the same DNA in the presence of Li^+

or Na^+. The physical meaning of this ratio is whether a DNA *specifically* binds Na^+ or not. Li^+ has stronger non-specific binding, yielding a ratio smaller than 1. For the samples with the ratio greater than 1, we conclude specific Na^+ binding. The wild-type Ce13d has a ratio of ~1.8. This value is larger than that in Figure 3E, since we used 2 μM DNAzyme and a plate reader here (instead of 1 μM DNAzyme and a fluorometer for Figure 3E). Most of the mutants cannot bind Na^+ specifically, except for the A3G, A8G, A10C, G14A, and G14T mutants. Among these, the G14T mutant (ratio = 2.2) appears even better than the wild-type Ce13d.

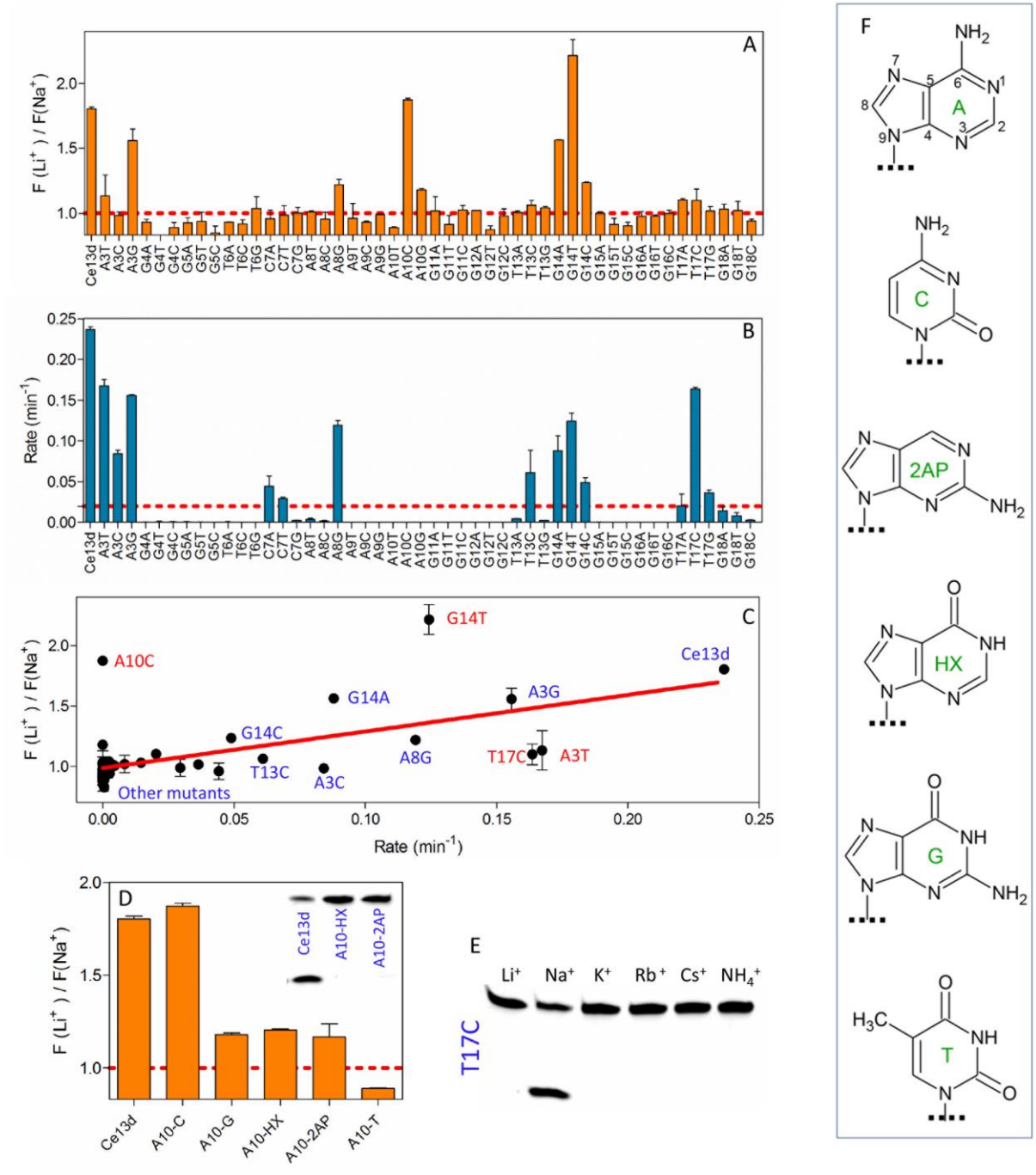

Figure 4. (A) Tb^{3+} luminescence intensity ratio in Li^+ over in Na^+ of the forty-five Ce13d mutants. The DNAzymes (2 μM) were annealed in 25 mM Li^+ or Na^+, and then 5 μM Tb^{3+} was added. (B) Cleavage activity of the mutants in 25 mM Na^+ with 10 μM Ce^{3+} added to initiate cleavage. This is re-plotted from reference.[40] (C) A plot correlating the Na^+ binding data and

cleavage activity data of Ce13d and its mutants. A few mutations showing inconsistent results are highlighted in red. (D) Other mutants tested for the A10 position. (E) Cleavage activity of the T17C mutant in 100 mM of various monovalent salts with addition of 10 μM Ce^{3+}. (F) The structures of the nucleobases involved in the discussion.

We then compared the Na^+ binding data with catalytic activity (Figure 4B).[40] In this case, the cleavable substrate was tested in the Na^+ buffer. Most of the mutations significant slowed or even fully abolished the DNAzyme activity. Three nucleotides (A3, G14 and T17) are quite tolerable to mutations, since most of their mutants still exhibited relatively fast cleavage rates (e.g. >0.02 min^{-1}). In addition, the C7A, A8G, and T13C mutants are also quite active. By plotting the Na^+ binding data and enzyme activity data together, we observed a good correlation among them in general (Figure 4C), suggesting that Na^+ binding is a major factor for the activity.

There are still a few cases of inconsistency (marked in red in Figure 4C). For example, the A10C mutation completely abolished the DNAzyme activity, but it showed good Na^+ binding similar to the wild-type. Comparing the structures of A and C, they both have an exocyclic amine group at the C6 position (Figure 4F), and they are the only ones do among the four DNA bases. Since the A10C retained Na^+ binding, we reason this amine contributes to Na^+ binding. To further test this, we mutated A10 to 2-aminopurine (2AP), which inhibited the activity and also decreased Na^+ binding (Figure 4D and its inset). Therefore, the amino group has to be at the C6 position to be functional. Changing this A to hypoxanthine (HX) also inhibited activity and binding, suggesting that the amino group is likely to be a hydrogen bonding donor in forming the Na^+ binding pocket.

For all the A10 mutants, good Na^+ binding appears to be a pre-requisite for activity. Binding Na^+ does not guarantee activity (e.g. the A10C mutant), but without Na^+ binding, the

activity is all lost (e.g. the rest of the mutants). Compared to cytosine, adenine has an additional N7 site that may serve a functional role. Since A10C has good binding to Na^+, the N7 site is unlikely to directly coordinate with Na^+, while it may form a hydrogen bond with other nucleotides to fulfill a structural stabilization role. For example, in the Diels–Alderase ribozyme, the A18 hydrogen bond with U8 to form a reverse Hoogsteen pair through N7 and exocyclic amine groups, which is important for its activity.[48] Further studies are needed to fully understand the role of A10, and this experiment has pointed out some directions.

In another case, the T17C mutant is quite active for cleavage, but it seems to have no Na^+ binding ability. To confirm this, we measured its activity in the presence of all the monovalent cations (Figure 4E), but Na^+ is still required for activity. This specific Na^+ requirement however is not reflected by Tb^{3+} luminescence. Another mutant, A3C also showed a similar performance, where the Na^+ specificity was observed by gel-based activity (Figure S3), but not Tb^{3+} luminescence. The exact reasons for such inconsistency are unclear at this moment, and it might be due to alternative folding of the mutated DNAzyme in the presence of Tb^{3+}. Other than these few, the rest of the mutants (over 40) showed a good correlation between Na^+ binding and cleavage activity.

Since NaA43 contains the same Na^+ binding motif, a parallel study might also offer insights into Na^+ binding. As shown in Figure S4A, under the same condition, Ce13d showed a F_{Li}/F_{Na} ratio of 1.8, and NaA43 only has a ratio of 1.1. While this experiment also supports Na^+ binding by NaA43, the range of signal change is very small, and Tb^{3+} luminescence is not the best probe for Na^+ binding in this original NaA43 (signaling can be improved by shortening the hairpin structure).[39] For the two special mutants: A10C and T17C, we also tested their cleavage activity in the NaA43 in the presence of monovalent ions (no Ce^{3+} added). None of them

produced cleavage even in presence of Na^+ (Figure S4B), suggesting that the requirement for activity in NaA43 might be more stringent than that in Ce13d. For example, the T17C mutant is active in Ce13d although it failed to show Na^+ binding as probed by Tb^{3+} luminescence.

Na^+ binding affinity comparison. Having tested the structural motifs in Ce13d for Na^+ binding, we next quantitatively measured metal binding affinity. With increasing salt concentration, a progressive decrease of Tb^{3+} luminescence of the wild-type Ce13d was observed for all the tested metal (Figure 5A), which is attributable to the charge screening effect weakening the interaction between the DNA and Tb^{3+}. Among these cations, Na^+ decreased the emission most significantly, with apparent dissociation constant (K_d) to be 20 ± 1 mM Na^+, which is lower than the other metals and indicates its specific and strong binding to the DNAzyme. Aside from Na^+, the K_d of the rest metal ions appeared to increase slightly with increasing the atomic number (Figure 5D). This is explained by the lower charge density of the larger cations and thus weaker interactions with the DNAzyme. It is also interesting to compare Ce13d with other Na^+ sensors reported, many of which having K_d's above 100 mM.[49-51] Therefore, DNA can achieve selective Na^+ binding with good Na^+ affinity. While our Tb^{3+} method is quite simple, the information obtained needs to be carefully interpreted. Besides the specific Na^+ binding by the DNA aptamer, the K_d obtained also contains information about non-specific Na^+ binding and competition from the Tb^{3+} probe. Therefore, the measured K_d is called observed or apparent K_d instead of the true K_d. It is likely that the true K_d is smaller than the apparent K_d, and the difference between Na^+ and other ions should be even larger.

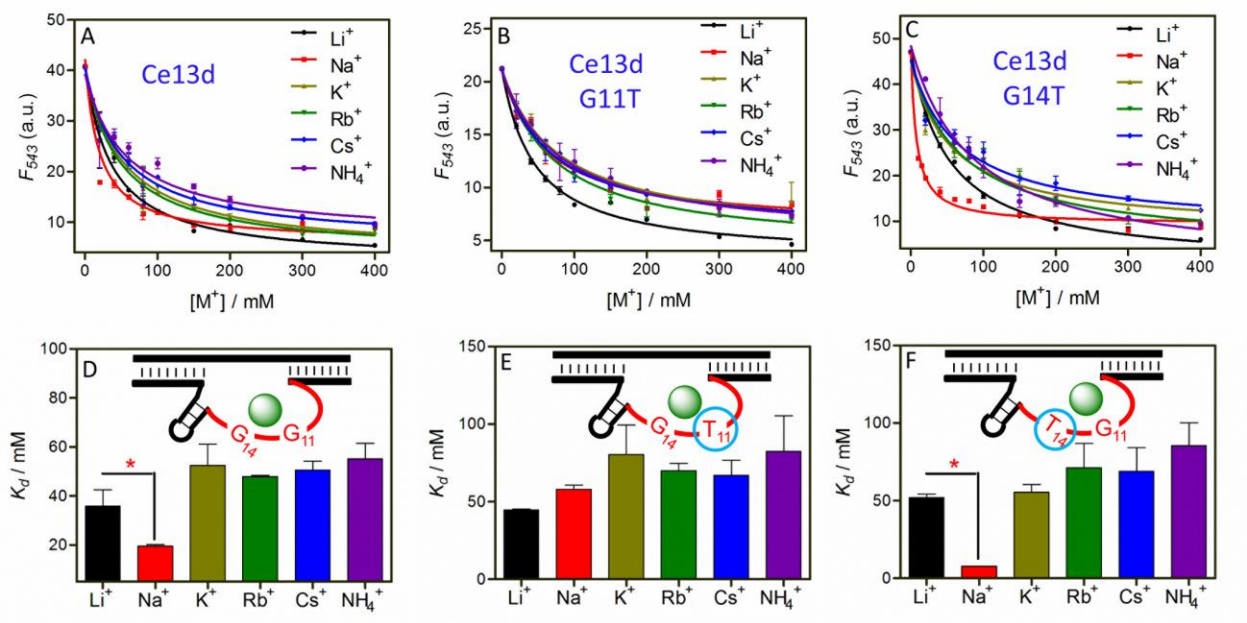

Figure 5. Tb^{3+}-sensitized luminescence intensity at 543 nm of (A) the Ce13d DNAzyme, (B) the inactive G11T mutant, and (C) the G14T mutant (a tighter Na^+ binder) with increasing concentrations of various monovalent metal ions. Dissociation constants for (D) the Ce13d DNAzyme, (E) the G11T mutant, and (F) the G14T mutants with each metal ion. Insets are the schematics of the DNAzymes tested with the mutation positions circled. (n=3, $*P < 0.05$, Student's t test).

As a control, we also titrated the inactive G11T mutant (Figure 5B). With this single nucleotide change, the response of Li^+ became the strongest due to its highest charge density (Figure 5E). Therefore, the abnormal luminescence decrease from the wild-type Ce13d with Na^+ strongly supports the Na^+ aptamer binding. The striking difference from this single point mutation also indicates a well-defined aptamer instead of non-specific interactions.

A mutant with even higher Na^+ binding affinity. Since the G14T mutant showed even better Na^+ binding property (Figure 4A), we also measured its metal concentration dependent emission (Figure 5C). The difference between Na^+ and other monovalent metals is indeed larger with a K_d of just 8 mM Na^+ (Figure 5F). This affinity is 2.5-fold tighter than that for the wild-type Ce13d, even though its cleavage rate is only about half of Ce13d. At the same time, the G14T mutant has a K_d of 51 mM for Li^+. Therefore it has much better specificity for Na^+ binding (e.g. its K_d ratio (Li^+/Na^+) is ~6 compared to ~2 for the wild-type Ce13d). Ce13d-G14T might be a better choice for the further biosensor development.

There is a crossover in the titration curves in Figure 5C for Na^+ and Li^+ (also in Figure 5A). The luminescence intensity was initially lower with Na^+, but at high metal concentrations, the intensity became lower in Li^+. This suggests that at high metal concentrations, non-specific interactions can gradually dominate. These titration experiments proceeded only up to 400 mM salt. To investigate whether these salts can fully mask the interaction between Tb^{3+} and DNA, we tested up to 2 M salt (Figure S5), where the luminescence decreased to close to the background. This is due to the strong screening effect of concentrated salts around the DNA, and thus hindering Tb^{3+} binding. Again, since this screening effect depends on the metal charge density. Li^+ is more efficient in this regard, resulting in lower luminescence intensity.

The K_d values for other monovalent metal ions in Figure 5 also need to be interpreted with care. The K_d for the binding of Na^+ is mainly from specific Na^+/DNA interactions, and this K_d value agrees with those obtained from other techniques, including DMS footprinting, and cleavage activity assay.[39] However, none of the other metal ions have any measurable binding based on the latter two techniques.[39] Therefore, their K_d's in the Tb^{3+} luminescence measurement here are a reflection of non-specific electrostatic interactions with the polyanion

DNA. Throughout this paper, we have mainly concluded specific Na^+ binding based on the Tb^{3+} luminescence intensity in comparison with Li^+. The fundamental reason for such specificity should be from a specific Na^+ aptamer in this DNAzyme. A single nucleotide mutation can abolish Na^+ binding, while such mutations do not affect the non-specific interaction with other metal ions.

Conclusions.

In this study, sensitized Tb^{3+} luminescence was employed to characterize the Na^+ aptamer in the Ce13d DNAzyme. In presence of Na^+, Ce13d folds into a compact structure, weakening Tb^{3+} binding and decreasing luminescence intensity. This offers a facile way to rationally understand the Na^+ aptamer in Ce13d. In the enzyme strand, the highly conserved 16-nucleotide loop is particularly important for Na^+ binding, as indicated by both Tb^{3+} luminescence studies and enzymatic activity results. However, this loop alone cannot bind Na^+ and it has to be placed in the DNAzyme scaffold. In addition, the catalytic core of the enzyme contains a hairpin. The hairpin structure is also necessary for Na^+ binding, although it does not directly participate in catalysis. The role of substrate strand was studied and the original 6-nucleotide cleavage junction gap is optimal and required for Na^+ binding. Therefore, it seems difficult to truncate any structural motifs in Ce13d without losing Na^+ binding. Finally, binding affinity of the Ce13d was determined for Na^+ along with various other monovalent metal ions. Among them, Na^+ has the tightest K_d of ~20 mM. With a single point mutation, the specific Na^+ binding is lost. We identified a mutant (G14T) that gives higher binding affinity (K_d = 8 mM) and better specificity (with K_d 6-fold lower in Na^+ than in Li^+) compared to the wild-type, which might be useful for further biosensor development. This study has explicitly demonstrated a Na^+ aptamer motif from

the recently reported RNA-cleaving DNAzymes. The implication of this aptamer in biology and application in analytical chemistry will be the topics of further studies.

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Supporting Information

Supporting information for this article is given via a link at the end of the document.

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