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Splitting a DNAzyme enables a Na^+-dependent FRET signal from the embedded aptamer†

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Recently, a few Na^+-specific RNA-cleaving DNAzymes have been reported, and a Na^+ aptamer was identified from the NaA43 and Ce13d DNAzymes. These DNAzymes and the embedded aptamer have been used for Na^+ detection. In this work, we studied the Na^+-dependent folding of the Ce13d DNAzyme using fluorescence resonance energy transfer (FRET). When a FRET donor and an acceptor were respectively labeled at the ends of the DNAzyme, Na^+ failed to induce an obvious end-to-end distance change, suggesting a rigid global structure. To relax this rigidity, the Ce13d DNAzyme was systematically split at various sites on both the enzyme and the substrate strands. The Na^+ binding activity of the split structures was characterized by 2-aminopurine fluorescence, enzymatic activity, Tb^{3+}-sensitized luminescence, and DMS footprinting. Among the various constructs, the only one that retained Na^+ binding was the split at the cleavage site, and this construct was further labeled with two dyes near the split site. This FRET result showed Na^+-dependent folding with a K_d of 26 mM Na^+. This study provides important structural information related to Na^+ binding to this new aptamer, which might also be useful for future work in biosensor design.

Introduction

The interaction between metal ions and DNA is a very fundamental topic in nucleic acids research, with applications in analytical chemistry, biophysics, and physiology.[1] Na^+ is the most commonly used salt ion to adjust ionic strength, which can influence the stability of DNA duplexes. Most previous studies only focused on electrostatic interactions, while little attention was paid to their sequence-specific binding. Among the group 1A metals, both K^+ and Na^+ can stabilize G-quadruplex structures,[2] but Na^+ is often less effective than K^+.[3]

Recently, a few RNA-cleaving DNAzymes have been shown to be highly selective for Na^+ over K^+.[4] For example, the NaA43 DNAzyme reported by Lu and co-workers can cleave its substrate with Na^+ alone, but not with other metals.[4a] Its Na^+ binding sequence is identical to that of the Ce13d DNAzyme reported by us.[4b] It turns out that Ce13d also requires Na^+ for activity, in addition to a lanthanide ion.[5] With extensive biochemical studies, we have identified the important nucleotides for Na^+ binding and activity.[5b,6] Further studies using sensitized Tb^{3+} luminescence, 2-aminopurine (2AP) fluorescence, and DMS footprinting all confirmed a well-defined Na^+ aptamer in Ce13d.[5b,7] The K_d of the aptamer ranged from 20 to 40 mM Na^+ depending on the method of probing and the DNAzyme sequence. In particular, 2AP fluorescence indicated very interesting adaptive binding in the presence of Na^+.[4d,7a]

Since 2AP only probes local folding near the labeling site, to gain deeper insights we herein examined the global folding of Ce13d using fluorescence resonance energy transfer (FRET). FRET is a powerful tool for studying the structure and dynamics of biomolecules, and it has been used to dissect the folding of the 17E and 39E DNAzymes, and ribozymes, and relate their folding to activity.[8] While we did not find sodium-dependent global folding for Ce13d, a FRET signal was observed after splitting the Ce13d DNAzyme at the substrate cleavage site. Therefore, this study has provided relevant insights into this important DNAzyme and aptamer for Na^+ binding.

Materials and methods

Chemicals

All the DNA samples were obtained from Integrated DNA Technologies (Coralville, IA), and their sequences and modifications are shown in Table S1.† The metal salts, dimethyl sulfate (DMS), β-mercaptoethanol, and piperidine were purchased from Sigma-Aldrich. The buffers were sourced from

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Mandel Scientific Inc. (Guelph, Ontario, Canada) and all the solutions were prepared using Milli-Q water.

FRET titration

To monitor the FRET signal of the original Ce13d DNAzyme, the enzyme strand at a final concentration of 200 nM (Table S1,† Ce13d) and the dual-labeled substrate strand (Cy5-Sub-Cy3) at a final concentration of 100 nM were annealed in buffer A (100 mM LiCl, 50 mM Tris, pH 7.5) by heating the sample at 95 °C for 2 min and gradually cooling to 4 °C over 1 h. The fluorescence emission spectra from 530 nm to 700 nm were recorded using a fluorometer (FluoroMax-4, Horiba Scientific) by exciting the sample at 513 nm, while Na^+ was gradually titrated into the sample.

To study the FRET of the split Ce13d DNAzyme, 10 μM of the enzyme strand (Ce13d-long or Ce13d-G5C-long), 12 μM TAMRA and FAM labeled S3 split substrate fragments (Sub-S3L-TAMRA and Sub-S3R-FAM) were annealed in buffer A. The complex was purified by a 15% native polyacrylamide gel following a previously reported protocol.[8g] The purified sample was soaked in buffer A, and the final DNA concentration was estimated by measuring the fluorescence intensity at 526 nm (Ex = 485 nm) after denaturing the DNAzyme complex. The sample was diluted to 200 nM by buffer A (quantified as the Sub-S3R-FAM concentration), and small volumes of concentrated metal ions were added to the sample. The fluorescence emission spectra were recorded from 520 nm to 630 nm. The fluorescence emission ratio of the acceptor over donor (E_{580}/E_{524}) was calculated to quantify the FRET efficiency.

2-Aminopurine fluorescence

To probe Na^+ binding with a split enzyme strand, a 2-aminopurine (2AP) modified substrate was used (Sub-2AP). The split DNAzyme was formed by annealing 1 μM Sub-2AP and split enzyme fragments (each 2 μM) in buffer A. To measure the binding affinity of Na^+ and specificity of the S3 split structure, an A_8-2AP modified enzyme strand was employed (Ce13d-$A_8$2AP). The DNAzyme was formed with 1 μM Ce13d-$A_8$2AP and it split the substrates (each 2 μM) in buffer A. For each 100 μL sample, the fluorescence intensity was monitored immediately after the addition of a small volume of a monovalent metal ion. The fluorescence spectra were recorded from 360 nm to 450 nm (Ex = 310 nm), and a peak intensity at 370 nm was used for quantification.

DNAzyme cleavage activity

The DNAzyme complex was formed by annealing 1 μM FAM-labeled cleavable substrate (Sub-rA-FAM) and 2 μM intact DNAzyme or split enzyme fragments (each 2 μM) in buffer B (25 mM NaCl, 50 mM MES, pH 6.0). Then, a final concentration of 10 μM Ce^{3+} was added to initiate the cleavage reaction. At designated time points, the reaction was stopped by transferring 2.5 μL of the reaction solution to 14 μL of urea (8 M). Then, the cleaved products were separated on a 15% denaturing polyacrylamide gel electrophoresis (PAGE) and the results were analyzed by a ChemDoc MP imaging system (Bio-Rad).

Tb^{3+} luminescence

The split substrate pair was annealed with the enzyme strand (1 μM each) in buffers (50 mM Tris, pH 7.5, 25 mM Li^+/Na^+). Then, a final concentration of 5 μM Tb^{3+} was added to 200 μL of DNAzyme, and the sensitized Tb^{3+} emission spectrum from 520 nm to 570 nm was recorded in a quartz cuvette by exciting the mixture at 290 nm.

DMS footprinting

For this experiment, the DNAzyme structures were formed by annealing an intact substrate (10 μM, Sub-dA-FP) or the split substrate pairs (each 10 μM) with a FAM labeled enzyme strand (4 μM, Ce13d-FP-FAM) in buffers (400 mM Na^+/K^+, 50 mM Tris, pH 7.5). For all the samples, 1 μL of freshly prepared 4% DMS was added to 10 μL of a DNAzyme complex and incubated for 15 min to methylate the guanines. The reaction was stopped by adding 200 μL of quenching solution (1 M β-mercaptoethanol, 600 mM NaOAc, pH 5.2), followed by ethanol precipitation. The DNA was washed with 70% ethanol, dried, and then dissolved in 50 μL of freshly prepared piperidine (10%). The DNA was then heated at 90 °C for 30 min to cleave the methylated guanine sites, and piperidine was removed by vacuum drying at 60 °C for 30 min. The products were then analyzed using a 15% PAGE gel.[5b]

Results and discussion

Na^+ binding probed by FRET

The secondary structure of the Ce13d DNAzyme complex is shown in Fig. 1A. Its substrate strand has a single RNA linkage (rA denoting ribo-adenine) serving as the cleavage site. Its enzyme strand has a hairpin and a loop in the catalytic core. The cleavage activity of Ce13d requires both Na^+ and a trivalent lanthanide ion (*e.g.* Ce^{3+}).[5b] We previously established that the big loop in the enzyme strand and the single-strand gap in the substrate are directly involved in Na^+ binding (*e.g.* forming a Na^+ aptamer), while the role of the lanthanide ion is mainly to interact with the scissile phosphate.[5b,7b] When this RNA linkage is replaced by its DNA analog, Ce13d becomes a highly selective and stable Na^+ aptamer.[5a,7] The Na^+-dependent local folding of the aptamer motif was confirmed by 2AP fluorescence,[7a] and sensitized Tb^{3+} luminescence spectroscopy.[7b]

To study its global folding, we labeled Ce13d with Cy3 (FRET donor) and Cy5 (acceptor) at the ends of its substrate strand (Fig. 1B). The fluorescence was monitored from 530 nm to 700 nm by exciting the donor at 513 nm. Before adding Na^+, two obvious fluorescent peaks were observed at 560 nm and 660 nm due to Cy3 and Cy5, respectively (Fig. 1C). The peak intensity at 560 nm was significantly stronger than that at 660 nm, which is due to a relatively long distance between the two fluorophores. If this distance is reduced by folding, a

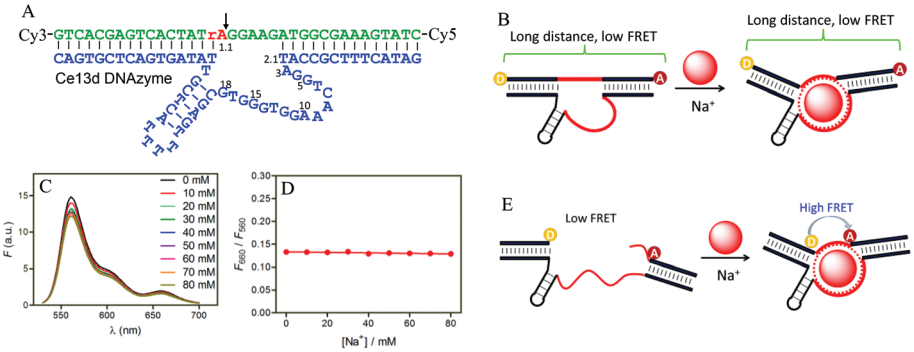

Fig. 1 (A) The secondary structure of the Ce13d DNAzyme with Cy5 and Cy3 fluorophores labeled at the two ends. The arrowhead indicates the cleavage site. For the FRET study, the cleavage site RNA (rA) is replaced by a DNA base analog. (B) A scheme showing the lack of end-to-end distance change upon Na^+ binding for the intact DNAzyme although local folding occurs. (C) Fluorescence spectra of the dual-labeled Ce13d in the presence of various Na^+ concentrations. (D) The acceptor-to-donor fluorescence intensity ratio (F_{660}/F_{560}) as a function of Na^+ concentration. (E) A scheme showing the FRET signal of the split Ce13d upon Na^+ binding.

stronger FRET efficiency is expected, resulting in a stronger acceptor fluorescence.

Upon the addition of Na^+, the peak intensities slightly decreased at both peaks (Fig. 1C). To quantify the effect of [Na^+], we plotted the fluorescence intensity ratio at 660 nm (acceptor) over 560 nm (donor) *versus* [Na^+] (Fig. 1D).[9] This ratio remained ~0.13 with [Na^+] ranging from 0–80 mM, which covered the dissociation constant of Na^+ with Ce13d (*i.e.*, <40 mM Na^+).[5b,7] Therefore, with 80 mM [Na^+], the binding of Na^+ with Ce13d should be close to saturation, but this binding was not reflected by FRET. In previous studies, Lu and co-workers also failed to observe a FRET signal change when they titrated the labeled 17E DNAzyme with Pb^{2+}. Then it is concluded that 17E follows a "lock-and-key" model for Pb^{2+} binding and a global folding was not required.[8e] In a later contact photo-crosslinking study, it was reported that 17E underwent local folding upon the addition of Pb^{2+}.[10] In our Ce13d, sufficient evidence supported Ce13d following an adaptive binding process for Na^+, in which a local conformational change was observed.[5b,7] Therefore, it is likely that the overall global structure of Ce13d is rather rigid, and Na^+ binding does not affect the end-to-end distance even though the aptamer motif folding occurs.

To probe Na^+ binding by FRET, we hypothesize that we need to relax the rigidity of the DNAzyme structure. One possible solution is to split the DNAzyme as shown in Fig. 1E. The initially more flexible structure might allow a large distance change upon Na^+ binding. If the FRET pair is labeled optimally, a Na^+-dependent signal might be observed. Splitting an aptamer has been reported previously. For example, the adenosine and cocaine aptamers can be split into two halves.[11] While splitting DNAzymes was also explored,[12] we herein aim to split Ce13d at critical positions (*e.g.* the cleavage site) for Na^+ binding and activity, which has never been previously demonstrated.

Splitting the enzyme strand

We need to find an appropriate split site for Ce13d to relax its rigidity yet still retain Na^+ binding. We first split the enzyme strand into two halves, since the enzyme strand is the main body for the Na^+ aptamer.[5b,7] Our previous biochemical assays indicated that most of the nucleotides in the single-stranded loop are highly conserved (Fig. 2A, nucleotides in red).[6] To minimize any potential adverse effects on Na^+ binding, we chose three split sites between the less conserved nucleotides (Fig. 2A, the split site indicated by the arrowheads), and they

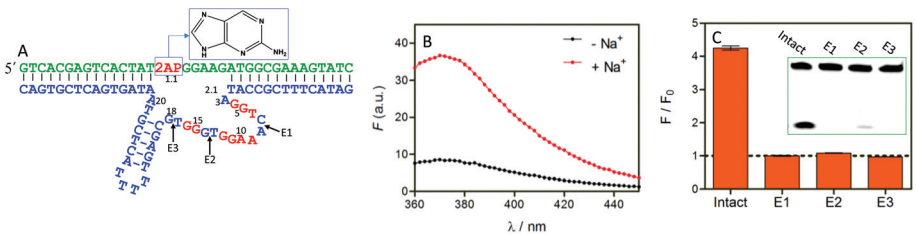

Fig. 2 (A) Splitting of the enzyme strand at three places indicated by the arrowheads. The 2AP-modified substrate is used to probe folding, and the structure of 2AP is also shown. To produce higher fluorescence enhancement, the T_{20} nucleotide in the original DNAzyme was mutated to an adenine (A_{20}) here without affecting its Na^+ binding or enzymatic activity.[7a] (B) The fluorescence spectra of 2AP-labeled intact Ce13d before and after adding 100 mM Na^+. (C) Normalized fluorescence increase of the intact enzyme and split enzymes. Inset: A gel image showing the cleavage activity of each DNAzyme construct.

were named E1, E2 and E3 (each construct contained both halves), respectively.

We first needed to confirm whether the split DNAzymes could still bind Na^+. For this purpose, we employed 2AP fluorescence spectroscopy. The structure of the 2AP-modified Ce13d is shown in Fig. 2A, where 2AP replaced adenine on the cleavage site ($A_{1.1}$). 2AP is a fluorescent adenine analogue, whose fluorescence is sensitive to its local environment. After being incorporated into DNA, its fluorescence is strongly quenched by base stacking and aromatic collision effects, and its local DNA folding may change its fluorescence.[13] We previously used 2AP to study the Na^+-dependent local folding of Ce13d.[7a]

For intact Ce13d, adding 100 mM Na^+ resulted in a strong enhancement of the 2AP fluorescence (Fig. 2B), which can be attributed to Na^+-induced folding alleviating the base stacking of 2AP.[7a] For a quantitative analysis, we plotted the normalized 2AP fluorescence at 370 nm (Fig. 2C), which increased over 300%. However, none of the split DNAzymes showed any obvious fluorescence enhancement, suggesting a full loss of Na^+ binding (Fig. 2C, see Fig. S1† for the spectra).

We then measured the catalytic activity using the cleavable substrate (2AP replaced by ribo-adenine). In the presence of both Ce^{3+} and Na^+, the original non-split enzyme produced a substantial cleavage, while only a trace amount of cleavage was observed for E2, and the remaining two constructs were completely inactive (Fig. 2C, inset). This was consistent with the 2AP probing result. Therefore, splitting the enzyme loop did not seem to be viable.

Splitting DNAzymes on the enzyme strand has been reported previously, and two well-known examples are called Maxizymes[12f] and MNAzymes.[12g] In these designs, the split junction was rigidly confined in proximity by a linker DNA, which can rescue the activity. Alternatively, aptamers were incorporated around the split site to assist active DNAzyme formation.[14] The 10–23,[12a,b] GR5 and 17E DNAzymes[12e] can tolerate certain splitting modifications in the enzyme strand. In contrast, none of the split Ce13d DNAzymes maintained their activity, suggesting the structural stringency of Ce13d in terms of metal binding.

Splitting the substrate strand

Since it appeared difficult to split the enzyme strand, we then moved our attention to the substrate strand. Our previous work has demonstrated that the single-stranded gap nucleotides in the substrate strand are conserved for Na^+ binding.[5b] Therefore, split sites were first chosen in the double-stranded arm region to minimize any adverse effects on Na^+ binding (Fig. 3A, see S1, S2 and S4 splitting sites). In addition, we also designed the S3 split site, the cleavage site for Ce13d.

We first screened these four split structures by a Tb^{3+} luminescence assay that we developed previously.[7b] Free Tb^{3+} has a very low extinction coefficient, and thus it is poorly luminescent. Upon binding to DNA (especially to a guanine base), energy transfer from the DNA to Tb^{3+} can significantly enhance Tb^{3+} emission.[15] Our previous work has indicated that Na^+ can competitively displace Tb^{3+} from Ce13d and thus decrease its emission, while the other group 1A metals such as Li^+ and K^+ are much less effective in this displacement reaction.[7b] Therefore, the Tb^{3+} luminescence sensitized by Ce13d is weaker in Na^+ than in Li^+ (Fig. S2A†). The peak intensity ratio (F_{Li}/F_{Na}) at 543 nm was plotted in Fig. 3B and the ratio was higher than 1, which indicates specific Na^+ binding.[7b] By using this method, we tested the Na^+ binding of each split structure (Fig. S2B–E†). Among them, only the S3 split showed a ratio higher than 1 (Fig. 3B). Note that for non-Na^+ binding DNA, we expect the ratio to be smaller than 1, since Li^+ has a higher charge density and thus has stronger electrostatic inter-

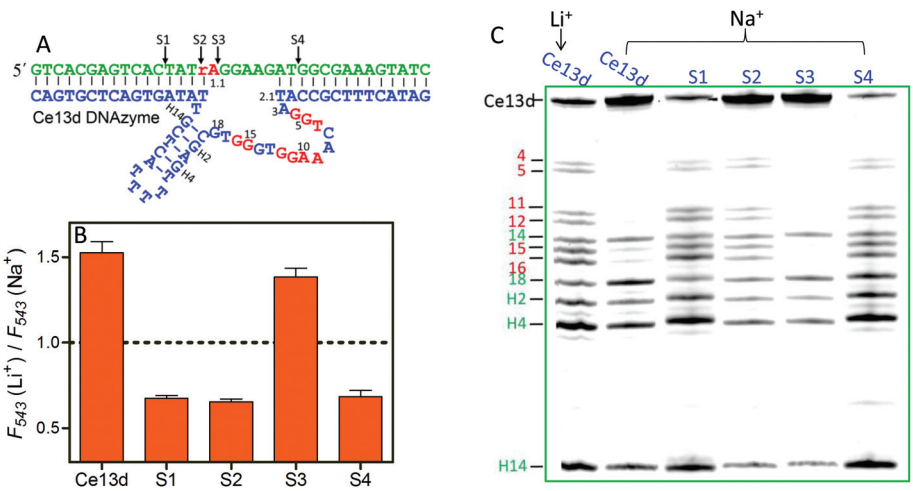

Fig. 3 (A) Splitting the Ce13d DNAzyme complex at the substrate strand. The arrowheads indicate the split sites, and the name of each split structure is shown on top of the arrowheads. For each split site, a pair of split substrate halves was used to assemble the DNAzyme complex. (B) The Tb^{3+} luminescence ratio in the presence of Li^+ over Na^+ for the Ce13d DNAzyme, and the different split constructs. A ratio greater than 1 suggests specific Na^+ binding. (C) DMS footprinting of Ce13d in Li^+ and Na^+, and the different split structures in Na^+. Each band is assigned to cleavage at a specific guanine in the enzyme strand.

actions with DNA. Therefore, the Na^+ binding ability might be maintained for the S3 split construct, while all the other ones are likely incapable of Na^+ binding.

Although Tb^{3+} luminescence is simple to perform, it is a relatively low resolution method. To further confirm the above observations, we performed a dimethyl sulfate (DMS) footprinting experiment, which specifically probed the guanines in Ce13d.[5b] We labeled the 3′-end of the enzyme strand with a FAM fluorophore. The guanines outside the catalytic core region were replaced by cytosine to simplify gel interpretations (see Fig. S3† for the DNAzyme structure for this experiment). We first tested the non-split DNAzyme. DMS can methylate guanines at the N7 position, allowing for depurination and subsequent cleavage of the DNA at the methylated guanine sites by piperidine (Fig. 3C, lane 1). In the presence of Na^+, some guanines in the aptamer core (*e.g.* G_4, G_5, G_{11}, G_{12}, G_{15} and G_{16}, see Fig. 1A for the numbering system) became less accessible to DMS, and therefore their cleavage was inhibited by Na^+ (Fig. 3C, lane 2, protected guanines marked in red). By observing the cleavage pattern, we can probe for specific Na^+ binding sites.

We then performed DMS footprinting for each split structure in the presence of Na^+. For S1, S2 and S4, each guanine site could be assigned to a cleavage band in the gel (Fig. 3C), suggesting that these guanines were readily methylated by DMS. For S3, however, we observed reduced or disappeared bands corresponding to the abovementioned guanines, indicating Na^+ binding. This result was consistent with the aforementioned Tb^{3+} luminescence experiment, further confirming that the S3 splitting retained its ability to bind Na^+.

The fact that the S3 site (*i.e.*, the $A_{1.1}G$ junction) could be split was quite unexpected because the two flanking nucleotides are highly conserved. We previously demonstrated that the mutation of $A_{1.1}G$ to other nucleotide combinations (*e.g.*, $A_{1.1}A$, $A_{1.1}T$, or $T_{1.1}T$) completely inhibited Na^+ binding,[5b] and changing the $rA_{1.1}G$ substrate to other junctions (*e.g.*, $rA_{1.1}A$, $rA_{1.1}T$, and $rU_{1.1}T$) inhibited the DNAzyme activity to almost background level (Fig. S4†), yet it retained Na^+ binding. Notably, the S2 split site could not tolerate splitting, even though it is right next to S3. Therefore, the interaction between $A_{1.1}$ and the next G might be important for linking two fragments together to form the Na^+ binding structure.

In addition, it is quite surprising that splitting at the S1 and S4 sites disrupted Na^+ binding. The S1 and S4 splits are both in the double-stranded regions, and the flanking nucleotides are unlikely to be directly involved in Na^+ binding. Indeed, splitting at the substrate binding arm has been reported for several DNAzymes and ribozymes, for which the enzyme activity can be modulated by altering the substrate/enzyme hybridization.[12d,16] We reason that the loss of Na^+ binding by S1 and S4 may also be due to the disrupted duplex regions (*e.g.* the 2 to 4 base pairs cannot stably form after splitting) that cannot be rescued by Na^+ binding.

Split Ce13d retained Na^+ binding affinity and specificity

So far, we have systematically split Ce13d at both the substrate and enzyme strands, with S3 being the only viable split site. It is quite surprising that splitting can be done at such a critical position. We then quantitatively measured its Na^+ binding. To do this, a 2AP probe was used again, but this time it was incorporated into the enzyme strand. We previously found that by replacing the A_8 position with a 2AP, Na^+ binding caused a specific 2AP fluorescence decrease (Fig. 4A).[7a]

Using this A_8-to-2AP modified enzyme strand, we performed a Na^+ titration experiment, and a gradually decreasing fluorescence for intact Ce13d was observed, as was expected (Fig. 4B, red trace). For split Ce13d (S3), a similar progressive fluorescence drop was seen with higher Na^+ concentrations (Fig. 4B, blue trace). As a control, Li^+ did not produce any significant fluorescence change for the split structure (Fig. 4B, black trace). Therefore, the fluorescence decrease was specific for Na^+. The K_d values for intact Ce13d and split Ce13d were calculated to be 44 mM and 57 mM Na^+, respectively (differ only by ~30%). Therefore, splitting has little effect on the affinity of Na^+ binding. Using this S3 split structure, the same experiment was also carried out with other monovalent cations. The addition of 100 mM Na^+ induced more than a 40% drop in fluorescence, while none of the other monovalent ions triggered an obvious decrease (Fig. 4C), suggesting that the Na^+ specificity of Ce13d was maintained after splitting.

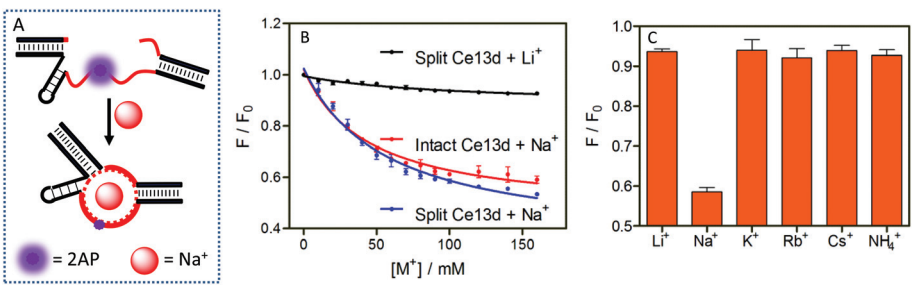

Fig. 4 (A) A scheme showing the Na^+-induced fluorescence decrease of split Ce13d with the A_8-2AP modified enzyme strand. (B) Relative fluorescence intensity at 370 nm as a function of salt concentration for the intact and split Ce13d. (C) Normalized fluorescence intensity at 370 nm before and after adding 100 mM of various monovalent metal ions.

Na^+-dependent folding of split Ce13d probed by FRET

After identifying a viable splitting site, we went back to our initial goal of using FRET to probe Na^+ binding. We hypothesized that the two splitting points might be separated by a relatively large distance in the absence of Na^+, due to the flexible nature of the enzyme loop. When a FRET pair is labeled around this split site, these two dyes might experience a large distance drop upon the addition of Na^+, thus resulting in a strong FRET enhancement (Fig. 5A). If true, this FRET signal could provide further evidence to support the split model, and the results may offer a new mechanism for Na^+ sensing.

We inserted the donor (FAM) and acceptor (TAMRA) labels next to the split sites (Fig. 5A), and observed a weak acceptor fluorescence at 580 nm after exciting the donor, which was due to the initial long distance between them (Fig. 5B, black trace). With the addition of 100 mM Na^+, we observed an obvious increase in the acceptor fluorescence (Fig. 5B, red trace), attributable to the energy transfer from the donor. We further studied the fluorescence response with different Na^+ concentrations, and the fluorescence emission ratio of acceptor to donor (A/D) was used for signal quantification. The results showed an increased A/D ratio with higher Na^+ concentrations (Fig. 5C), suggesting a Na^+-dependent folding. However, the maximal enhancement of the A/D ratio was only ~1.5-fold. Therefore, further optimization is required to produce a more sensitive FRET signal for Na^+ detection, and this study has only pointed out the feasibility. The folding was saturated at 80 mM Na^+, and the K_d was determined to be ~26 mM, comparable to the K_d value obtained with other methods.

To test metal specificity, FRET was measured with other monovalent ions. Among the tested ions, Na^+ yielded the highest A/D ratio change, and a moderate FRET signal was observed for K^+ (Fig. 5D). Other ions produced less than 10% of change. Compared with this FRET signal, the above 2AP fluorescence was more specific without any obvious K^+ response, which is attributable to 2AP only affected by its local stacking environment. On the other hand, FRET relies on the distance change between the fluorophores. We suspect that the response from K^+ might be due to the guanine-rich aptamer core that could interact more favorably with K^+ in this split setting. To confirm the metal selectivity of Ce13d, its enzymatic activity was measured using the cleavable substrate. Indeed, it showed exceptional specificity towards Na^+, more than 100-fold faster than those of other ions, and K^+ did not show noticeable activation (Fig. S5†).

As a control, we also tested an inactive mutant of Ce13d-G5C (inset of Fig. 5E). With this single point mutation, the Ce13d cleavage activity was fully abolished, and Na^+ binding was also inhibited for the intact DNAzyme.[7b] For the split DNAzyme, none of the tested metals showed a strong FRET signal, including Na^+ (Fig. 5E). We further measured the FRET of the split substrate using the 17E DNAzyme. Again, all the monovalent ions failed to change the fluorescence significantly (Fig. 5F). These results strongly suggest that the FRET signal, which occurred in the presence of Na^+, is a result from the binding of Na^+ to the split aptamer instead of non-specific interactions. In addition, the ability to split at the cleavage site might be quite unique for the Ce13d DNAzyme.

Most metal-specific RNA-cleaving DNAzymes do not have a well-defined aptamer motif, since metal specificity is mainly from metals interacting with the scissile phosphate group.[17] The NaA43 and Ce13d DNAzymes are probably the first examples of directly isolating metal-dependent aptamers *via* DNAzyme selections.[4a,b,5b,18] We recently reported another example of a silver aptamer from a DNAzyme selection.[18,19] Li and co-workers have isolated a series of aptazymes in the presence of bacterial and cancer cells, and those enzymes are likely dependent on proteins instead of metal ions.[20]

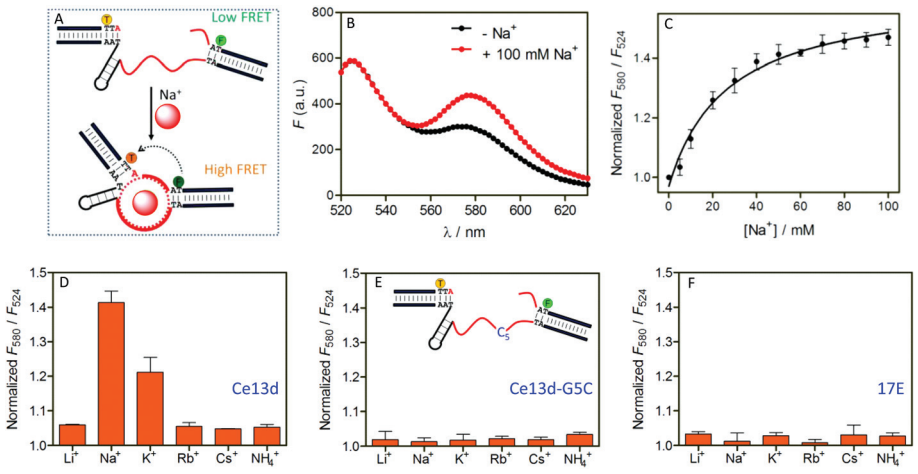

Fig. 5 (A) A scheme illustrating Na^+-induced FRET signal change of a dual-labeled split Ce13d. (B) Fluorescence spectra of the dual-labeled split Ce13d in the absence or presence of 100 mM Na^+. (C) Normalized FRET ratio (F_{580}/F_{524}) as a function of Na^+ concentration. (D) FRET response to 50 mM monovalent ions using (D) Ce13d, (E) Ce13d-G5C and (F) 17E as the enzyme strand with the split substrate. Inset of (E): A cartoon showing the structure of Ce13d-G5C. It differs from Ce13d by a single point mutation.

Conclusions

In summary, we studied the folding of a Na^+ aptamer embedded in an RNA-cleaving DNAzyme in its intact and split constructs using FRET. The intact aptamer showed minimal FRET signal change, which was attributed to its rigid global structure. With the help of various previously developed methods, we screened a series of split structures in terms of their Na^+ binding ability, and identified a viable construct by splitting at the cleavage site in the substrate strand. This work provides a robust system to characterize split aptamers, which might be useful in future studies given the versatile applications of split aptamers.[11d,21] The Na^+-dependent folding of this split DNAzyme provided interesting insights of its conformational change upon Na^+ binding. Based on the previous literature on ribozymes, many interesting experiments can be performed to further understand the folding and dynamics of this DNAzyme, such as using spermine to probe folding,[22] and ^{23}Na NMR to probe metal binding dynamics.[23] While it seems difficult to use such a FRET signal for Na^+ detection, this split aptamer may serve as a promising tool for the design of novel Na^+ sensors by using other signaling mechanisms such as electrochemistry,[24] color,[25] and fluorescence.[26]

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References

1 (*a*) W. Zhou, R. Saran and J. Liu, *Chem. Rev.*, 2017, **117**, 8272; (*b*) X. B. Zhang, R. M. Kong and Y. Lu, in *Annual Review Of Analytical Chemistry*, ed. R. G. Cooks and E. S. Yeung, Annual Reviews, Palo Alto, 2011, vol. 4, p. 105; (*c*) R. K. Sigel and A. M. Pyle, *Chem. Rev.*, 2007, **107**, 97; (*d*) W. L. Ward, K. Plakos and V. J. DeRose, *Chem. Rev.*, 2014, **114**, 4318; (*e*) K. Hwang, P. Hosseinzadeh and Y. Lu, *Inorg. Chim. Acta*, 2016, **452**, 12.

2 A. N. Lane, J. B. Chaires, R. D. Gray and J. O. Trent, *Nucleic Acids Res.*, 2008, **36**, 5482.

3 H. Ueyama, M. Takagi and S. Takenaka, *J. Am. Chem. Soc.*, 2002, **124**, 14286.

4 (*a*) S.-F. Torabi, P. Wu, C. E. McGhee, L. Chen, K. Hwang, N. Zheng, J. Cheng and Y. Lu, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 5903; (*b*) P.-J. J. Huang, J. Lin, J. Cao, M. Vazin and J. Liu, *Anal. Chem.*, 2014, **86**, 1816; (*c*) W. Zhou, R. Saran, Q. Chen, J. Ding and J. Liu, *ChemBioChem*, 2016, **17**, 159; (*d*) W. Zhou, R. Saran, J. Ding and J. Liu, *ChemBioChem*, DOI: 10.1002/cbic.201700184.

5 (*a*) S.-F. Torabi and Y. Lu, *J. Mol. Evol.*, 2015, **81**, 225; (*b*) W. Zhou, Y. Zhang, P. J. Huang, J. Ding and J. Liu, *Nucleic Acids Res.*, 2016, **44**, 354.

6 M. Vazin, P.-J. J. Huang, Ż. Matuszek and J. Liu, *Biochemistry*, 2015, **54**, 6132.

7 (*a*) W. Zhou, J. Ding and J. Liu, *Nucleic Acids Res.*, 2016, **44**, 10377; (*b*) W. Zhou, J. Ding and J. Liu, *ChemBioChem*, 2016, **17**, 1563.

8 (*a*) J. C. F. Lam and Y. Li, *ChemBioChem*, 2010, **11**, 1710; (*b*) Y. He and Y. Lu, *Chem. – Eur. J.*, 2011, **17**, 13732; (*c*) D. Mazumdar, N. Nagraj, H.-K. Kim, X. Meng, A. K. Brown, Q. Sun, W. Li and Y. Lu, *J. Am. Chem. Soc.*, 2009, **131**, 5506; (*d*) H. K. Kim, I. Rasnik, J. W. Liu, T. J. Ha and Y. Lu, *Nat. Chem. Biol.*, 2007, **3**, 762; (*e*) H. K. Kim, J. Liu, J. Li, N. Nagraj, M. Li, C. M. Pavot and Y. Lu, *J. Am. Chem. Soc.*, 2007, **129**, 6896; (*f*) D. A. Lafontaine, D. G. Norman and D. M. J. Lilley, *EMBO J.*, 2001, **20**, 1415; (*g*) J. W. Liu and Y. Lu, *J. Am. Chem. Soc.*, 2002, **124**, 15208.

9 G. Lee, J. Yoo, B. J. Leslie and T. Ha, *Nat. Chem. Biol.*, 2011, **7**, 367.

10 Y. Liu and D. Sen, *J. Mol. Biol.*, 2010, **395**, 234.

11 (*a*) Z. Zhang and J. Liu, *ACS Appl. Mater. Interfaces*, 2016, **8**, 6371; (*b*) M. N. Stojanovic, P. de Prada and D. W. Landry, *J. Am. Chem. Soc.*, 2000, **122**, 11547; (*c*) F. Li, J. Zhang, X. Cao, L. Wang, D. Li, S. Song, B. Ye and C. Fan, *Analyst*, 2009, **134**, 1355; (*d*) A. K. Sharma, A. D. Kent and J. M. Heemstra, *Anal. Chem.*, 2012, **84**, 6104; (*e*) Y. Wen, H. Pei, Y. Wan, Y. Su, Q. Huang, S. Song and C. Fan, *Anal. Chem.*, 2011, **83**, 7418.

12 (*a*) J. L. Richards, G. K. Seward, Y. H. Wang and I. J. Dmochowski, *ChemBioChem*, 2010, **11**, 320; (*b*) B. K. Ruble, J. L. Richards, J. C. Cheung-Lau and I. J. Dmochowski, *Inorg. Chim. Acta*, 2012, **380**, 386; (*c*) D. Y. Wang and D. Sen, *J. Mol. Biol.*, 2001, **310**, 723; (*d*) J. Liu and Y. Lu, *Anal. Chem.*, 2004, **76**, 1627; (*e*) P.-J. J. Huang and J. Liu, *Chem. Commun.*, 2014, **50**, 4442; (*f*) T. Kuwabara, M. Warashina, T. Tanabe, K. Tani, S. Asano and K. Taira, *Mol. Cell*, 1998, **2**, 617; (*g*) E. Mokany, S. M. Bone, P. E. Young, T. B. Doan and A. V. Todd, *J. Am. Chem. Soc.*, 2009, **132**, 1051.

13 E. L. Rachofsky, E. Seibert, J. T. Stivers, R. Osman and J. B. Ross, *Biochemistry*, 2001, **40**, 957.

14 S. Shimron, J. Elbaz, A. Henning and I. Willner, *Chem. Commun.*, 2010, **46**, 3250.

15 (*a*) W. T. D. Lin, P.-J. J. Huang, R. Pautler and J. Liu, *Chem. Commun.*, 2014, **50**, 11859; (*b*) M. Zhang, H.-N. Le, X.-Q. Jiang, B.-C. Yin and B.-C. Ye, *Anal. Chem.*, 2013, **85**, 11665.

16 D. Y. Wang, B. H. Lai, A. R. Feldman and D. Sen, *Nucleic Acids Res.*, 2002, **30**, 1735.

17 W. Zhou, R. Saran, P.-J. J. Huang, J. Ding and J. Liu, *ChemBioChem*, 2017, **18**, 518.

18 R. Saran, K. Kleinke, W. Zhou, T. Yu and J. Liu, *Biochemistry*, 2017, **56**, 1955.

19 (*a*) R. Saran and J. Liu, *Anal. Chem.*, 2016, **88**, 4014; (*b*) R. Saran, L. Yao, P. Hoang and J. Liu, *Biochimie*, 2017, DOI: 10.1016/j.biochi.2017.07.001.

20 (*a*) M. M. Ali, S. D. Aguirre, H. Lazim and Y. Li, *Angew. Chem., Int. Ed.*, 2011, **50**, 3751; (*b*) Z. Shen, Z. Wu, D. Chang, W. Zhang, K. Tram, C. Lee, P. Kim, B. J. Salena

and Y. Li, *Angew. Chem., Int. Ed.*, 2016, **55**, 2431; (*c*) S. He, L. Qu, Z. Shen, Y. Tan, M. Zeng, F. Liu, Y. Jiang and Y. Li, *Anal. Chem.*, 2014, **87**, 569.

21 Z. Y. Liu, W. Zhang, L. Z. Hu, H. J. Li, S. Y. Zhu and G. B. Xu, *Chem. – Eur. J.*, 2010, **16**, 13356.

22 (*a*) B. M. Chowrira, A. Berzal-Herranz and J. M. Burke, *Biochemistry*, 1993, **32**, 1088; (*b*) H. L. Lightfoot and J. Hall, *Nucleic Acids Res.*, 2014, **42**, 11275.

23 H. Deng and W. H. Braunlin, *J. Mol. Biol.*, 1996, **255**, 476.

24 L. Y. Feng, Z. J. Zhang, J. S. Ren and X. G. Qu, *Biosens. Bioelectron.*, 2014, **62**, 52.

25 S. Cheng, B. Zheng, M. Z. Wang, M. H. W. Lam and X. W. Ge, *Talanta*, 2013, **115**, 5061.

26 Y. Bai, F. Feng, L. Zhao, Z. Chen, H. Wang and Y. Duan, *Analyst*, 2014, **139**, 1843.