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An *In Vitro* Selected DNAzyme Mutant Highly Specific for Na^+ in Slightly Acidic Conditions

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Abstract

Sodium is one of the most ubiquitous metal ions in biology, while its DNA-based probes were reported only recently. A Na^+-specific RNA-cleaving DNAzyme named NaA43 is active with Na^+ alone. In this work, we used $Co(NH_3)_6^{3+}$ as the intended metal cofactor for *in vitro* selection, but obtained a mutant of the NaA43 DNAzyme. The mutant was named NaH1, which differs from NaA43 by only two nucleotides. NaA43 has an optimal of pH 7.0, while the optimal pH for NaH1 is 6.0. This difference might be due to our selection being performed at pH 6.0. Compared to other competing monovalent ions, NaH1 also displays an excellent selectivity for sodium and a fast catalytic rate of 0.11 ± 0.01 min^{-1} with 50 mM Na^+. At low Na^+ concentrations, the selected DNAzyme exhibited a higher cleavage rate than NaA43 and thus a tighter apparent K_d of 12.0 ± 1.6 mM Na^+. Furthermore, the NaH1 DNAzyme was engineered into a fluorescent Na^+ biosensor by labeling a fluorophore/quencher pair that the sensor had a detection limit of 223 μM Na^+. Preliminary work on detection Na^+ in serum was demonstrated as well. This study provides a useful mutant that works in a slightly acidic environment, which might be useful for sensing Na^+ in acidic *in vivo* environment.

Keywords: Biosensors, sodium, SELEX, aptamers, deoxyribozymes

Introduction

Sodium is one of the most common and important metal ions in the environment and in biology. A high sodium level is a reflection of physiological disorders such as high blood pressure and water retention. Over the years, various methods have been developed to detect Na^+ and most efforts were focused on crown ether-based fluorescent chelators.[1-2] Using DNA as a platform for developing metal sensors has been practiced for nearly two decades and many DNA sequences have been identified to bind to a wide range of metals.[3-8] These mainly include two classes of functional DNA molecules: aptamers and DNAzymes.[3, 9-10] Aptamers perform a simple binding function, while DNAzymes are catalysts and they usually require a divalent metal ion for activity,[11-13] such as Pb^{2+},[14] Zn^{2+},[15] Cu^{2+},[16-17] UO_2^{2+},[18] Cd^{2+},[19] Ca^{2+},[20] and Hg^{2+}.[21-22] Recently, trivalent lanthanides were also used in DNA-based catalysis.[23-27] Aside from Ag^+,[28-29] binding monovalent cations (e.g. K^+,[30] and Tl^+) mainly relied on G-quadruplex DNA.[31]

Using DNA for Na^+ recognition has not caught much attention until 2015.[32] Before that, Na^+ was mainly used as a buffer to control ionic strength without considering its specific binding by DNA.[33] Recently, a few highly Na^+-specific RNA-cleaving DNAzymes have been reported. The NaA43 DNAzyme was selected by Lu and coworkers,[32] and it has a high sequence analogy to a lanthanide-dependent DNAzyme named Ce13d discovered in our lab.[25, 34] Further assays indicated that Ce13d also requires Na^+,[35-36] suggesting a common Na^+ aptamer in these two DNAzymes.[37-39] We also discovered the EtNa DNAzyme that uses a different mechanism for specific Na^+ recognition.[38, 40-41]

So far, most of the work has been focused on the characterization of previously selected DNAzymes. Rational mutations were performed to understand individual nucleotides important for catalysis and metal binding,[34, 39] while efforts on new selections were limited. New selections under different conditions can explore a larger sequence space and may offer new insights. In this work, we report a mutant of NaA43, which was discovered from a selection effort using a cobalt complex as the intended metal cofactor. We call it a mutant since it is very similar to NaA43 in DNA sequence and it also works with Na^+ alone with excellent specificity to Na^+. However, this mutant has a different pH optima. This study broadens our understanding of

Na^+-dependent DNAzymes and reports the performance of a sodium sensor derived from this mutant.

Materials and Methods

Chemicals. All the DNA samples used in the *in vitro* selection and sensing experiments were from Integrated DNA Technologies (Coralville, IA). The rest of the DNAs were from Eurofins Genomics (Louisville, KY). Hexammine cobalt (III) chloride and other metal salts were from Sigma-Aldrich. Sodium chloride, acetate acid, tris(hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)ethanesulfonic acid (MES), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA) were from Mandel Scientific Inc. (Guelph, Ontario, Canada). Lithium hydroxide was from Alfa Aesar. Sso Fast EvaGreen supermix was from Bio-Rad. T4-DNA ligase, deoxynucleotide (dNTP) solution mix, Taq DNA polymerase with ThermoPol buffer, 10× gel loading dye, and low molecular weight DNA ladder were from New England Biolabs. All solutions used in this work were prepared with Milli-Q water.

***In Vitro* Selection.** The experiment of *in vitro* selection followed an established protocol in our lab with a few minor modifications. For each round of selection, 10 μM of $Co(NH_3)_6Cl_3$ was introduced to the library in selection buffer (50 mM MES buffer, pH 6.0, 25 mM NaCl, 1 mM EDTA). After 1 h of incubation, 8 M urea was added to quench the reaction. The active sequences with 28 nucleotides cleaved were then separated from the inactive library using 10% dPAGE (denaturing polyacrylamide gel electrophoresis) and amplified by two rounds of polymerase chain reactions (PCR) following previously reported thermocycling protocols. In PCR1, the cleaved sequences were extended and amplified to generate full-length templates for PCR2. In PCR2, two modified primers were used to regenerate the library. The P3 primer contained a 6-carboxyfluorescein (FAM) fluorophore on its 5′-end terminus and a rA base on its 3′-end terminus. The P4 primer contained a polymer spacer that can stop the polymerase reaction. All the DNA sequences related to the selection are listed in Table S1.

Deep Sequencing. To prepare the sample for deep sequencing, PCR1 was performed on the round 6 library. Then, PCR2 was performed to introduce specific index sequences into the library for the Illumina sequencing. Instead of P3 and P4, the forward primer (P701) and the

reverse primer (P501) were used with their sequences listed in Table S1. The PCR product was then purified with 2% agarose gel (120 V, 50 min). A gel/PCR DNA fragment extraction kit (IBI Scientific) was used to extract the library from the gel. Finally, the purified DNA sample was eluted in 20 μL of Milli-Q water. The DNA concentration measured with a NanoDrop spectrophotometer was ~10 ng/μL. The sample was shipped to McMaster University for sequencing.

Activity Assays. For cleavage activity assays, the DNAzyme complex was prepared by annealing the FAM-labeled substrate and enzyme (ratio 1:1.5) in sodium-free buffer A (50 mM HEPES buffer, pH 7.6, 25 mM LiCl) to minimize background cleavage. Assays were performed with a final concentration of 0.3 μM of DNAzyme complex in buffer B (50 mM MES buffer, pH 6.0, 25 mM NaCl). A small volume (1 μL) of NaCl was added to initiate the cleavage reaction. After different incubation time periods, the reaction was quenched with 8 M urea. The cleavage products were quantified by 15% dPAGE gel and analyzed by a Bio-Rad Chemi-Doc MP imaging system. For pH-dependent assays, acetate buffer was used for pH 4.5 and pH 5.0, and HEPES buffer was used for pH 7.0 and pH 7.5. All the buffer pH was adjusted using 1 M LiOH to avoid Na^+.

Fluorescence-Based Na^+ Sensing. Sensor signaling kinetics were measured in 96-well plates using a microplate reader (Infinite F200Pro, Tecan). The excitation wavelength was 485 nm and the emission wavelength was 535 nm. The sensor complex was prepared by annealing the FAM-labeled substrate (5 μM) and the quencher-labeled enzyme (7.5 μM) in buffer B. Next, 2 μL of the sensor complex was diluted in 93 μL buffer (50 mM MES buffer, pH 6.0). The background signal was first monitored for at least 5 min, followed by a quick addition of 5 μL of NaCl to induce the cleavage reaction. The fluorescence was continuously monitored for 1 h with 20 s intervals. For detection in serum, FBS was filtered through a nitrocellulose membrane to remove aggregated proteins. The filtered FBS (1 μL) was added to the sensor solution and the fluorescence was monitored for at least 5 min. Different concentrations of NaCl were then added in the plate followed by monitoring fluorescence for 1 h.

Results and Discussion

***In vitro* selection.** The original goal of this work was to obtain a DNAzyme that works in the presence of $Co(NH_3)_6^{3+}$. Due to the exchange inertness of Co^{3+}, $Co(NH_3)_6^{3+}$ can only interact with nucleic acids via outer-sphere coordination.[42] DNAzymes working with this complex might offer new insights into the cleavage mechanism. Our *in vitro* selection followed an established protocol in the lab with an initial library containing approximately 10^{14} random DNA sequences (Figure 1A),[25] and the detailed procedures will not be repeated here. The design of the library is shown in Figure 1B with 50 random nucleotides and a single RNA linkage (rA, ribo-adenine) serving as the cleavage site. After incubating at pH 6.0 with $Co(NH_3)_6Cl_3$, the cleaved sequences were harvested using gel electrophoresis and then amplified by two PCR reactions to seed the next round of selection. With a FAM label, the cleavage yield of each round was monitored (Figure 1C). A gradual increase in the cleavage yield was observed (blue bars), and ~24% of the library was cleaved at round 6. At this point, we checked the activity with the reaction buffer alone without $Co(NH_3)_6^{3+}$. However, cleavage was still observed (black bar), suggesting that $Co(NH_3)_6^{3+}$ might not be required for the reaction. At this point, we stopped the selection and sent the round 6 library for deep sequencing.

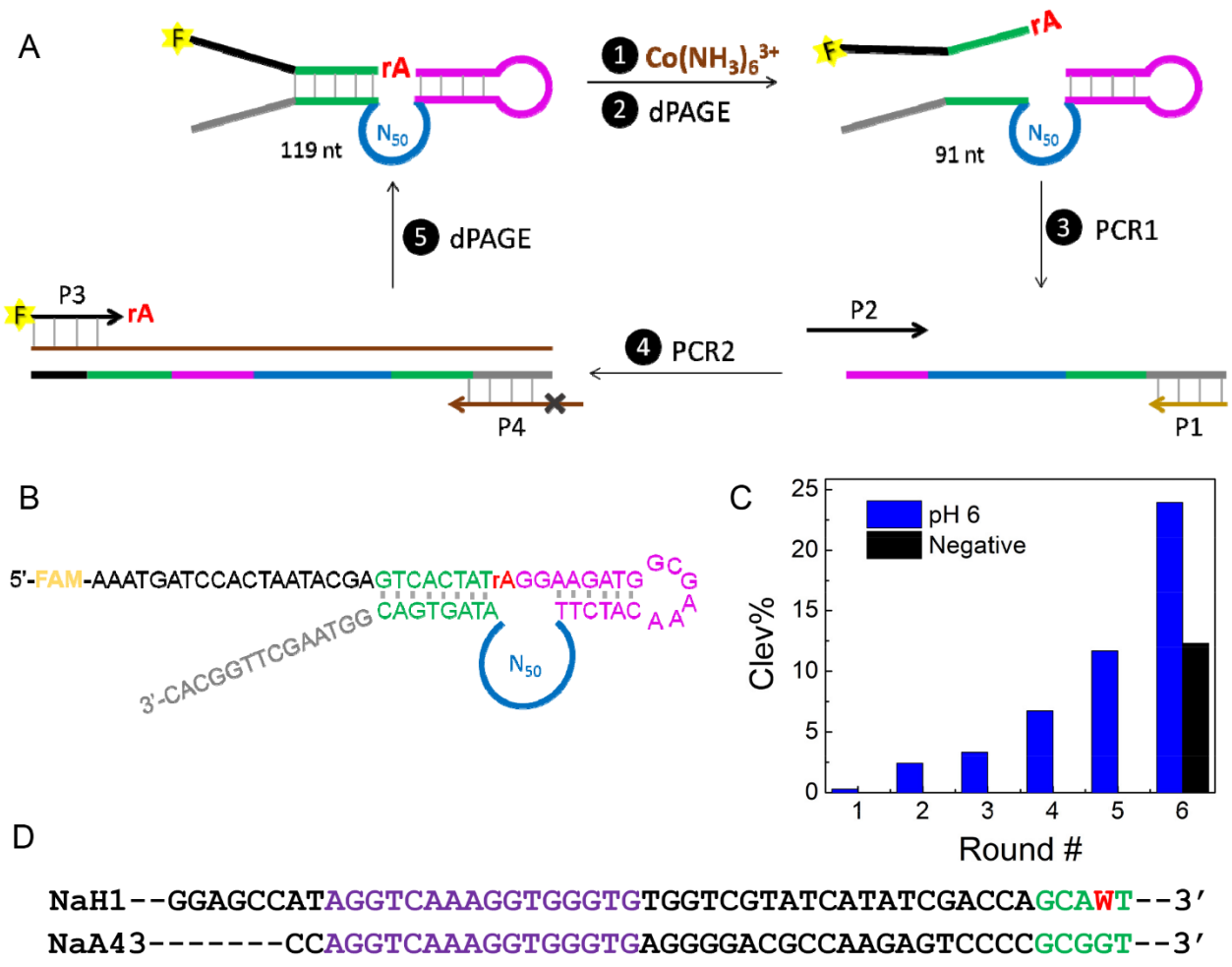

Figure 1. *In vitro* selection. (A) Illustration of five main steps of our $Co(NH_3)_6^{3+}$-dependent DNAzyme selection. Two PCR steps were used to amplify the cleaved sequence and regenerate the full-length library. The P3 primer had a FAM label and the rA, while P4 had a polymer spacer to stop PCR extension (denoted by a cross) beyond this point. Two denaturing polyacrylamide gel electrophoresis (dPAGE) steps were carried out to separate the cleavage product and to recover the positive strand from the duplex after PCR, respectively. (B) The sequence of the initial library containing an N_{50} randomized region with a FAM labeled. (C) Progress of the selection showing the cleavage percentage of each round at pH 6. Throughout the selection, $Co(NH_3)_6Cl_3$ was 10 μM with 1 h incubation. The black bar was the cleavage yield at round 6 with buffer alone (no $Co(NH_3)_6^{3+}$). (D) Sequence alignment of NaH1 with NaA43. W means A or T.

Sequence analysis. The deep sequencing yielded a total of 97,533 sequences. After sequence alignment, 21,919 reads were assembled into more than one thousand families. The most populated 200 families accounted for around 50% of the analyzed sequences. Table S2 lists the sequences from the top 20 families. Among them, 19 families contain a 16-nt motif of AGGTCAAAGGTGGGTG (purple colored) in the N_{50} region. This 16-nt motif was also found in the Ce13d and NaA43 DNAzymes.[35-36] Compared to the NaA43 DNAzyme (Figure 1D), the major difference lies in two nucleotides at the end of the N_{50} region. Statistically, this position has roughly an equal chance of being T and A in our library, while NaA43 is a G at this position.

Due to sequence similarity, the secondary structures of Ce13d (Figure 2A) and NaA43T DNAzymes (Figure 2B) are presented for comparison. NaA43T was designed based on the NaA43 DNAzyme but with a shortened hairpin loop whose activity remained.[35] They had the same substrate strands with a FAM labeled at the 3′-end. The enzyme strand binds to the substrate via two duplex regions. For the newly selected sequences, we used Mfold to predict their secondary structures (Figure S1).[43] For example, the family #1 was named NaH1 and the cis-cleaving NaH1 sequence was folded in Figure 2C. Using NaA43 as a reference, we designed its trans-cleaving structure in Figure 2D, which was used for subsequent activity analysis. By examining the nucleotides in blue, NaH1 is more similar to NaA43 than to Ce13d. These three DNAzymes all have the conserved 16-nt loop highlighted in dark red, which is responsible for Na^+ binding.[35]

The main difference in these three DNAzymes lies in the few nucleotides in blue. The Ce13d (Figure 2A) requires both Ce^{3+} and Na^+ for activity. The small motif of $TG_{23}GCG$ in NaA43 was believed to replace the role of Ce^{3+} in the cleavage reaction. The guanine at the 23 position (G_{23}) cannot be mutated and it was thought to be critical for catalysis.[35] Interestingly, NaH1 has a TACG motif on this region, and the critical guanine in NaA43 was removed. A closer examination of our selection library did not yield any sequence with the exact TGGCG motif and likely we obtained a mutant of NaA43. On the other hand, in the original NaA43 selection paper,[32] sequences containing a TACG motif were found in their Class A-I but with incomplete Na^+-binding loops. Therefore, our NaH1 is a new sequence that was not reported before either from *in vitro* selection or from rational mutations. Since this sequence appears to be

interesting, we focused on it in this work. Future efforts will be made with $Co(NH_3)_6^{3+}$ in a Na^+-free buffer, which may eliminate such Na^+-dependent sequences.

In vitro selection sometimes yielded similar sequences, and a representative example is the 17E DNAzyme.[44] The recurrence or tyranny of 17E has been a common problem in divalent metal-dependent selections. Li and coworkers attributed it to the small size, tolerance to mutation and high activity of the 17E motif.[44-45] In the case of NaA43 and NaH1, the sequence requirement is more stringent since the 16-nt motif is highly conserved. When a typical divalent metal ions are absent and when Na^+ is present, this motif appears to be a good solution for achieving activity.[25, 32, 46]

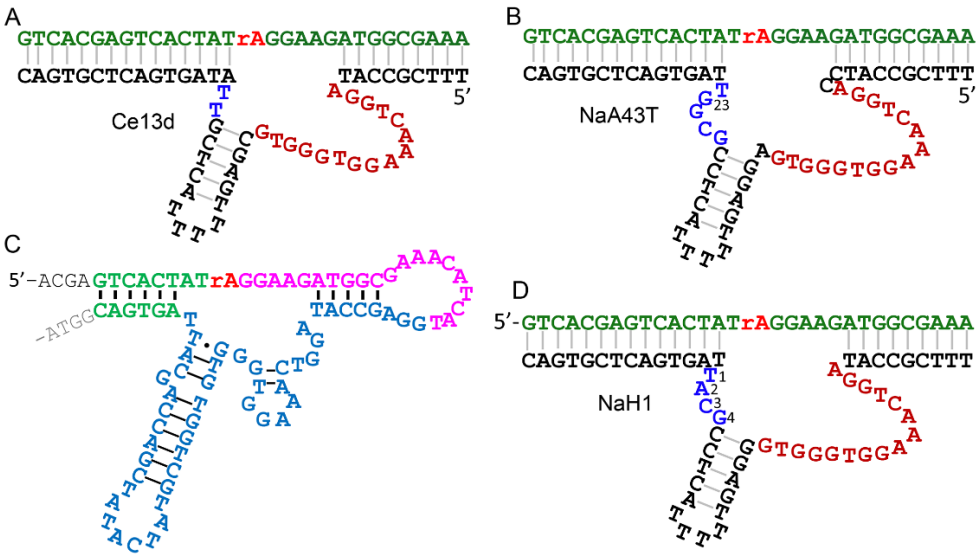

Figure 2. The secondary structures of (A) the Ce13d and (B) the NaA43T DNAzymes (NaA43 with a shortened hairpin). (C) The cis-cleaving structure of a selected Na^+-dependent DNAzyme. (D) The trans-cleaving form of the selected DNAzyme with a shortened hairpin structure.

Biochemical assays. Since $Co(NH_3)_6^{3+}$ was used as the intended cofactor during the selection, we tested its effect on NaH1. We first measured the trans-cleaving NaH1 with $Co(NH_3)_6^{3+}$ but in a buffer without Na^+. In this case, no cleavage activity was observed (Figure S2). Addition of $Co(NH_3)_6^{3+}$ in the presence of Na^+ slightly inhibited the activity of NaH1 (cleavage yield decreased from 49% to 32%, Figure S2). Its insufficient inhibition still allowed NaH1 to survive

the selection process. Overall, $Co(NH_3)_6^{3+}$ is not a cofactor for NaH1, and this DNAzyme works with Na^+ alone.

To characterize our newly selected NaH1 DNAzyme, we measured its cleavage kinetics in the presence of various concentrations of Na^+ at pH 6.0, the pH of our selection (Figure 3A). The kinetic traces were fitted into a first-order equation, $\%P_{cleavage,t} = \%P_{max}(1 - e^{-kt})$, where $\%P_{max}$ is the maximum cleavage yield at the end of the reaction and k is the cleavage rate constant. In the presence of 50 mM NaCl, NaH1 displayed a cleavage rate of 0.11 ± 0.01 min^{-1}, which was around 3-fold faster compared to that of NaA43T at the same condition. The cleavage rate increased with a rising concentration of Na^+ and approached saturation beyond 50 mM Na^+ (Figure 3B). This data was then fitted into a one-site binding curve with an apparent dissociation constant (K_d) of 12.0 ± 1.6 mM Na^+ at pH 6.0. The K_d of NaA43 using gel-based assays was estimated to be ~70 mM Na^+ at pH 7.0.[32] Therefore, our NaH1 had a tighter Na^+ binding affinity.

To confirm the cleavage activity of NaH1 and NaA43T, assays were carried out in the presence of 10 mM Na^+ for both DNAzymes side-by-side (Figure 3C). The cleavage rate of NaA43T was ~0.02 min^{-1} (pH 7.0), which agreed with the literature. With 10 mM Na^+, NaH1 (pH 6.0) had a cleavage rate that was about 3-fold higher. NaH1 was tested in pH 6.0 buffer while NaA43T was in pH 7.0 buffer, since these are their respective optimal pH for activity (*vide infra*).

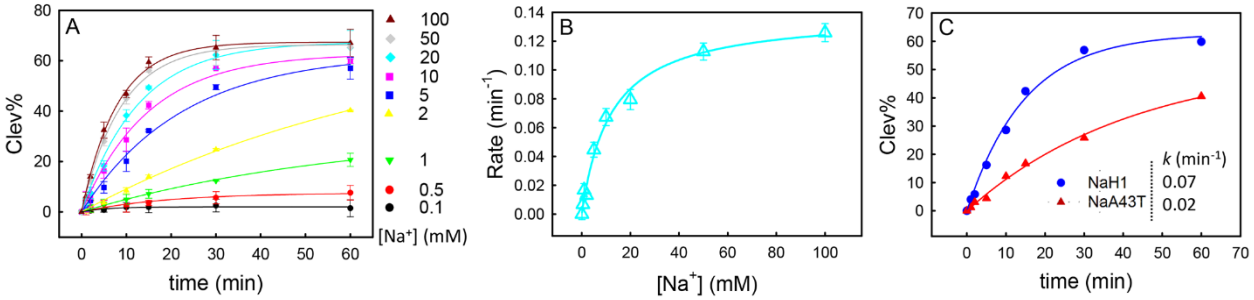

Figure 3. (A) Kinetic profile of the NaH1 DNAzyme with various concentrations of NaCl at pH 6.0. (B) The cleavage rate of NaH1 as a function of Na^+ concentration. (C) A comparison of the cleavage kinetics of the DNAzymes with 10 mM NaCl. NaH1 was tested at pH 6.0, while NaA43T at pH 7.0.

pH-dependent activity and Na^+ specificity. NaH1 differs from NaA43T mainly on 2 nucleotides. Since NaH1 exhibited a higher cleavage rate at low Na^+ concentrations, a systematic comparison was made for a better understanding of these two Na^+-dependent DNAzymes. The reaction condition of NaA43 as reported was 50 mM Bis-Tris, pH 7.0, 90 mM LiCl, and it was also selected at this pH.[69] Our NaH1 was selected using 50 mM MES buffer, pH 6.0. A difference in pH during selection could result in different DNAzymes.[5] To have a full understanding, we compared the kinetics of NaH1 and NaA43T in the presence of 10 mM NaCl at both pH's. At pH 6.0, NaH1 displayed a higher cleavage rate than NaA43T (Figure 4A), while at pH 7.0, NaH1 was slower (Figure 4B).

After noticing this difference, pH-dependent assays were performed with 10 mM Na^+ in various pH buffers (Figure 4C). The activity of NaH1 increased from pH 4.5 to pH 6.0 and then decreased at higher pH. The optimal reaction condition of NaH1 turned out to be pH 6.0, our selection condition. In contrast, the highest activity of NaA43 was observed at pH 7.0. The fact that we obtained NaH1 instead of NaA43 might be related to the lower pH of our selection. This is quite interesting since the selection seems to be able to sense such a small pH difference and the pH selectivity was achieved by just changing a few nucleotides.

After understanding the effect of pH, we wondered whether the Na^+ specificity of NaH1 was retained. A total of 20 metal ions including monovalent, divalent, and trivalent ions were tested at pH 6.0 (Figure 4D). NaH1 showed a significant cleavage with Na^+ but not with other monovalent ions tested. The quantification is shown in Figure 4E. The only exception was Pb^{2+}, which also had a significant cleavage. RNA cleavage by Pb^{2+} has been commonly observed in many DNAzymes.[25, 40, 47] Importantly, other monovalent metals and all physiologically relevant metals were all silent.

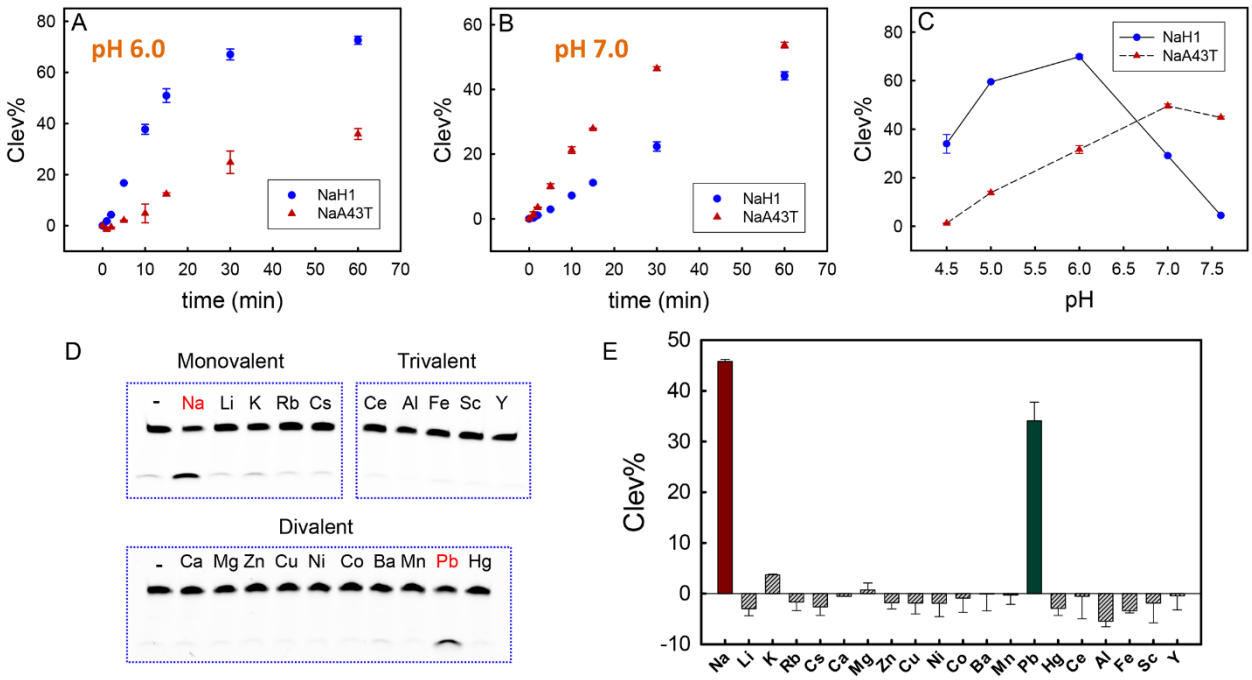

Figure 4. The kinetics studies of the NaA43T and NaH1 DNAzymes with 10 mM NaCl at (A) pH 6.0, and (B) pH 7.0. (C) Cleavage yield of NaH1 and NaA43T with 10 mM NaCl at different pH values after 30 min incubation. (D) Gel micrographs showing the cleavage in the presence of monovalent (10 mM), divalent (1 mM) and trivalent (100 μM) ions. (E) Quantification of the NaH1 cleavage yield with 20 different metal ions at pH 6.0 for 1 h. The background cleavage (without metal) was subtracted. The concentrations of 10 mM, 1 mM, and 100 μM were used for monovalent, divalent, and trivalent ions, respectively.

A Na^+ biosensor. Due to the superior activity of NaH1 at low pH, a fluorescence-based biosensor was further designed for Na^+ detection (Figure 5A). A FAM label was placed on the 3′-end of the substrate strand. A quencher was labeled at the 5′-end of the enzyme strand. Upon hybridization, the fluorescence was quenched in a duplex structure. In the presence of Na^+, the cleavage reaction can release the FAM-labeled fragment and induce a fluorescence signal. The 5′-end of the enzyme strand was shortened by two nucleotides to ensure a fast release of the fluorophore after cleavage.

For the detection, the background fluorescence of 100 nM sensor complex was first monitored for 10 min. In the absence of Na^+, the background fluorescence was quite stable over

1 h (Figure 5B). A higher Na^+ concentration resulted in faster fluorescence increase, and saturation was observed with up to ~20 mM Na^+. The initial rates of the fluorescence traces were quantified from 10 to 15 min after Na^+ addition (Figure 5C). After fitting the curves with a one-site binding equation, an apparent K_d of 4.2 ± 0.5 mM was obtained. The calibration curve was linear when Na^+ was below 2 mM. The detection limit was calculated to be 223 µM Na^+ based on 3σ/slope (σ is the standard deviation of the background signal). The lower pH optima of this DNAzyme might be useful for detection of Na^+ in acidic cellular environment such as the endosome and in cancer cells.

Using NaA43 for detecting Na^+ in live cells has already been demonstrated.[32] In this work, we studied a different biological sample matrix, serum.[48] In the presence of 1% FBS, the background signal was low (Figure 5D), indicating that our sensor was stable in the serum. Fluorescence increase was observed when Na^+ was added. A linear response was obtained with Na^+ concentration below 20 mM and the detection limit was 676 µM Na^+ (Figure 5E).

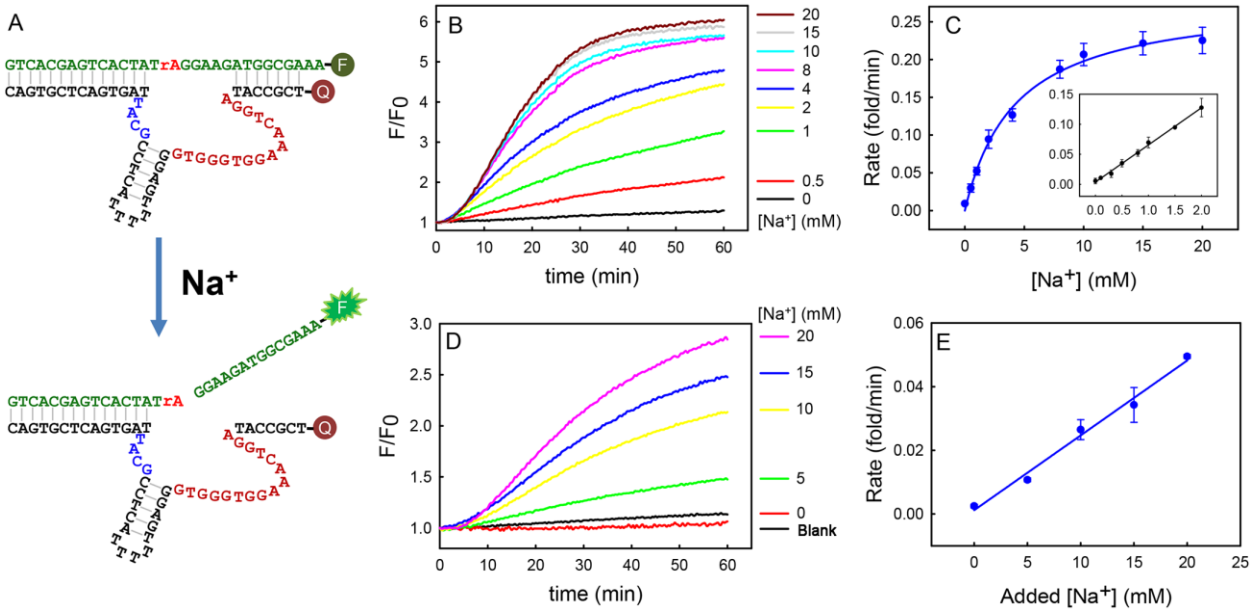

Figure 5. (A) The Na^+ DNAzyme sensor design. (B) Sensor signaling kinetics with various concentrations of Na^+. (C) Quantification of the rate of fluorescence increase as a function of Na^+ concentration, and the data were fitted using $v = v_{max} \cdot [M^+]/(K_d + [M^+])$, where v is the reaction rate, and $[M^+]$ is the Na^+ concentration. Inset: the linear response at low [Na^+] concentrations. (D) Sensor response in the presence of 1% serum. (E) A linear response between the fluorescence signal and [Na^+] in 1% serum.

Conclusions

In conclusion, a new Na^+-dependent DNAzyme mutant was discovered by a selection effort using a cobalt complex as the intended metal cofactor. A selected DNAzyme, named NaH1, possesses high selectivity for sodium over other monovalent ions and a fast catalytic rate of 0.11 ± 0.01 min^{-1} with 50 mM Na^+ at pH 6. With a few nucleotides mutated in the small loop, NaH1 is more active than the previously reported NaA43 at low Na^+ concentrations, especially at pH 6.0. pH-dependent assays revealed that NaH1 had a higher cleavage activity at pH ~6.0, while NaA43 was more active at neutral pH. A careful comparison showed that these two DNAzymes differ only by one or two nucleotides. A fluorescent Na^+ sensor was also demonstrated with an apparent K_d value of 4.2 ± 0.5 mM and a limit of detection of 223 μM Na^+.

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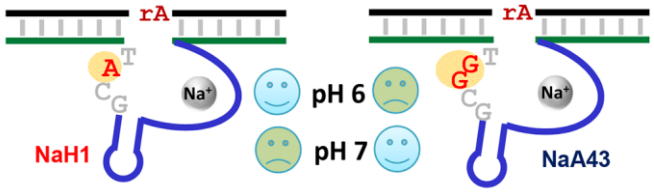
For graphical abstract



In vitro selection yielded a mutant of a previously reported sodium-specific DNAzyme with a slightly acidic pH optima and the same excellent specificity for Na^+.