In Vitro Selection of Chromium-Dependent DNAzymes for Sensing Cr(III) and Cr(VI)

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Abstract

Chromium is a very important analyte for environmental monitoring, and developing biosensors for chromium is a long-standing analytical challenge. In this work, *in vitro* selection of RNA-cleaving DNAzymes was carried out in the presence of Cr^{3+}. The most active DNAzyme turned out to be the previously reported lanthanide-dependent Ce13d DNAzyme. While the Ce13d activity was ~150-fold lower with Cr^{3+} compared to that with lanthanides, the activity of lanthanides and other competing metals was masked by using a phosphate buffer, leaving Cr^{3+} the only metal that can activate Ce13d. With 100 μM Cr^{3+}, the cleavage rate is 1.6 h^{-1} at pH 6. Using a molecular beacon design, Cr^{3+} was measured with a detection limit of 70 nM, significantly lower than the U.S. Environmental Protection Agency (EPA) limit (11 μM). Cr(VI) was measured after its reduction by $NaBH_4$ to Cr^{3+}, and it can be sensed with a similar detection limit of 140 nM Cr(VI), lower than the EPA limit of 300 nM. This sensor was tested for chromium speciation analysis in a real sample, supporting its application for environmental monitoring. At the same time, it has enhanced our understanding on the interaction between chromium and DNA.

Introduction

Chromium (Cr) is an extremely useful metal for hardening steel, coating materials, pigments, and catalysis, yet it is mutagenic and carcinogenic.[1] Its toxicity and applications have raised serious environmental concerns. Cr has nine different oxidation states, with the trivalent Cr^{3+} and hexavalent Cr(VI) (present as $Cr_2O_7^{2-}$ in acid and CrO_4^{2-} in alkaline solutions) being the most common in the environment. The toxicity of chromium is strongly related to its oxidation state.[2] For example, a trace amount of Cr^{3+} is needed for glucose and fat metabolism, and thus Cr^{3+} is considered to be essential for life.[3] However, Cr^{3+} is still toxic at high concentrations, and Cr^{3+} is currently classified as a group 3 carcinogen.[2] Cr(VI) is much more toxic as a potent human carcinogen, which is attributed to its ability to cross the cell membrane and high oxidation potential.[4] To manage chromium contamination, it is important to measure its concentration and speciation information.

While many instrumentation methods were developed for chromium analysis, such as inductively coupled plasma atomic emission spectrometry,[5] few can offer on-site and real-time detection. In this regard, developing sensors is a very valuable. Many biopolymers have been tested for chromium detection, such as peptides,[6] antibodies,[7,8] protein enzymes,[9] and even whole bacterial cells.[10] Sensors based on nanomaterials were also reported.[11-15] Most of these sensors however suffer from low selectivity and poor stability.

Over the past two decades, DNA has emerged as a unique platform for metal sensing.[16-18] DNA is very stable, easy to modify, programmable and very versatile in metal binding.[19] DNAzymes are catalytic DNA isolated using *in vitro* selection.[20-23] By using specific metal ions during selection, it is possible to obtain metal-sensing DNAzymes.[24] Many divalent metals have been detected by DNAzymes, such as Pb^{2+},[25] Zn^{2+},[26] Cu^{2+},[27,28] UO_2^{2+},[29] Cd^{2+},[30] and Hg^{2+}.[31]

Recently, progresses were made also for monovalent Na^+,[32,33] and Ag^+,[34] and trivalent lanthanide ions.[35-38]

The interaction between chromium and DNA was studied mainly in biological systems. For example, Cr(VI) crosslinks DNA in rats and in cultured cells, although it has little interaction with isolated DNA. Cr^{3+} binds to the N7 position of guanine and also be chelated by the nearby phosphate backbone.[39] The binding constant of DNA to Cr^{3+} (3150 M^{-1}) is ~6-fold higher than that to Cr(VI). Since DNA interacts more strongly with Cr^{3+}, we chose it as the target metal, and the detection of Cr(VI) relied on its reduction to Cr^{3+}. In this work, we started with *in vitro* DNAzyme selection, followed by studying DNAzyme cleavage by Cr^{3+}, and ended with a biosensor for Cr^{3+} and Cr(VI) detection and speciation.

Materials and Methods

Chemicals. The DNA samples were obtained from Integrated DNA Technologies (IDT, Coralville, IA). See Table S1 for DNA sequences and modifications. Reagents for PCR were from New England Biolabs (Ipswich, MA). Chromium (III) chloride hexahydrate, other metal salts, and $NaBH_4$ were from Sigma-Aldrich. Note that chromium (III) chloride anhydrous salt should not be used, since it is very difficult to dissolve in water and gave inconsistent assay results. The contaminated water was supplied by GeoSyntec Consultants (Guelph, ON, Canada).

***In vitro* selection**. The in vitro selection method including PCR was reported previously.[36] For each round of selection, 200 μM Cr^{3+} was added to initiate cleavage (see Figure 1C for incubation time). The round 10 cleavage products were cloned and sequenced. In the second selection effort, to block the Ce13d sequence, the library was annealed with 150 pmol of the

blocker DNA in buffer A (50 mM MES, pH 6.0, 25 mM NaCl). The library was then incubated with Cr^{3+} for 1 h to induce cleavage. The round 6 cleaved product was sequenced.

Gel-based activity assays. To test the DNAzyme activity, the DNAzyme complex was formed by annealing the FAM-labeled substrate (1 μM) and enzyme (2 μM) in buffer A. The reaction was initiated by adding metal ions and quenched by transferring an aliquot of the sample into 8 M urea. The samples were then separated on a 15% dPAGE gel and analyzed using a ChemiDoc MP imaging system (Bio-Rad). To reduce $Cr_2O_7^{2-}$, a final of 5 mM $NaBH_4$ was added for 5 min prior to mixing it with the DNAzyme solution.

Cr(III) and Cr(VI) sensing. The DNAzyme-based sensor was prepared by annealing the FAM-labeled substrate (2.5 μM) and the quencher-labeled enzyme (5 μM) in buffer B (50 mM MES, pH 6.0, 25 mM NaCl, 0.8 mM phosphate). Then, 2 μL of the sensor solution was diluted with 7 μL buffer B, and 1 μL metal ion was added to initiate the reaction. After 1 h, 1 μL EDTA (25 mM) was added to quench the reaction, followed by adding 10 μL ethanol to facilitate releasing the cleavage product. Afterwards, the reaction solution was transferred to 80 μL HEPES buffer (50 mM, pH 7.5) and the fluorescence were monitored with a SpectraMax M3 microplate reader for 30 min. To detect Cr(VI), $Cr_2O_7^{2-}$ (7 μL) was first treated with 50 mM $NaBH_4$ (1 μL) in buffer B for 5 min. Then, 2 μL sensor was then added to initiate the reaction.

Results and Discussion

In vitro selection. To isolate Cr^{3+}-dependent DNAzymes, *in vitro* selection was carried out using the library shown in Figure 1A. It has a 5′-FAM label to track the selection progress. The rAG junction indicated by the arrowhead is the embedded cleavage site, where rA denotes for

ribo-adenine. This single RNA linkage has much lower stability compared to the rest DNA linkages.[40] The library diversity is from the 35 random nucleotides (N35) that are positioned near the cleavage site by the two base paired regions. The initial library contained ~10^{13} random DNA sequences. In each round of selection, 0.2 mM Cr^{3+} was added to induce cleavage (Figure 1B). The cleaved product was shorter, allowing its separation by denaturing polyacrylamide gel electrophoresis (dPAGE). After two rounds of PCR, the full-length library was re-generated to seed the next round of selection. The selection progress was tracked for each round (Figure 2A). The activity appeared to plateau at round 10 and this library was sequenced.

Sequence analysis. Out of the 38 sequences obtained (Table S2), 37 of them are very similar to the previously reported Ce13d DNAzyme that is active with trivalent lanthanides.[37] The structure of Ce13d is shown in Figure 1C, which differs from Clone 5 (a representative sequence from the current selection) mainly by the number of thymine bases in the box. Our previous studies on Ce13d already showed that these thymine bases are unimportant for activity.[37,41,42] To further confirm this, Clone 5 and Ce13d were tested in presence of 10 μM Ce^{3+} or 100 μM Cr^{3+} (Figure 1D), and the two DNAzymes indeed showed the same activity. Out of the two metals, Ce^{3+} is much more efficient. For example, Ce13d has a rate of 1.6 h^{-1} with 100 μM Cr^{3+}, which is 15-fold slower than that with 10 μM Ce^{3+}. Therefore, the Cr^{3+}-dependent activity is ~150-fold lower compared to that with Ce^{3+}.

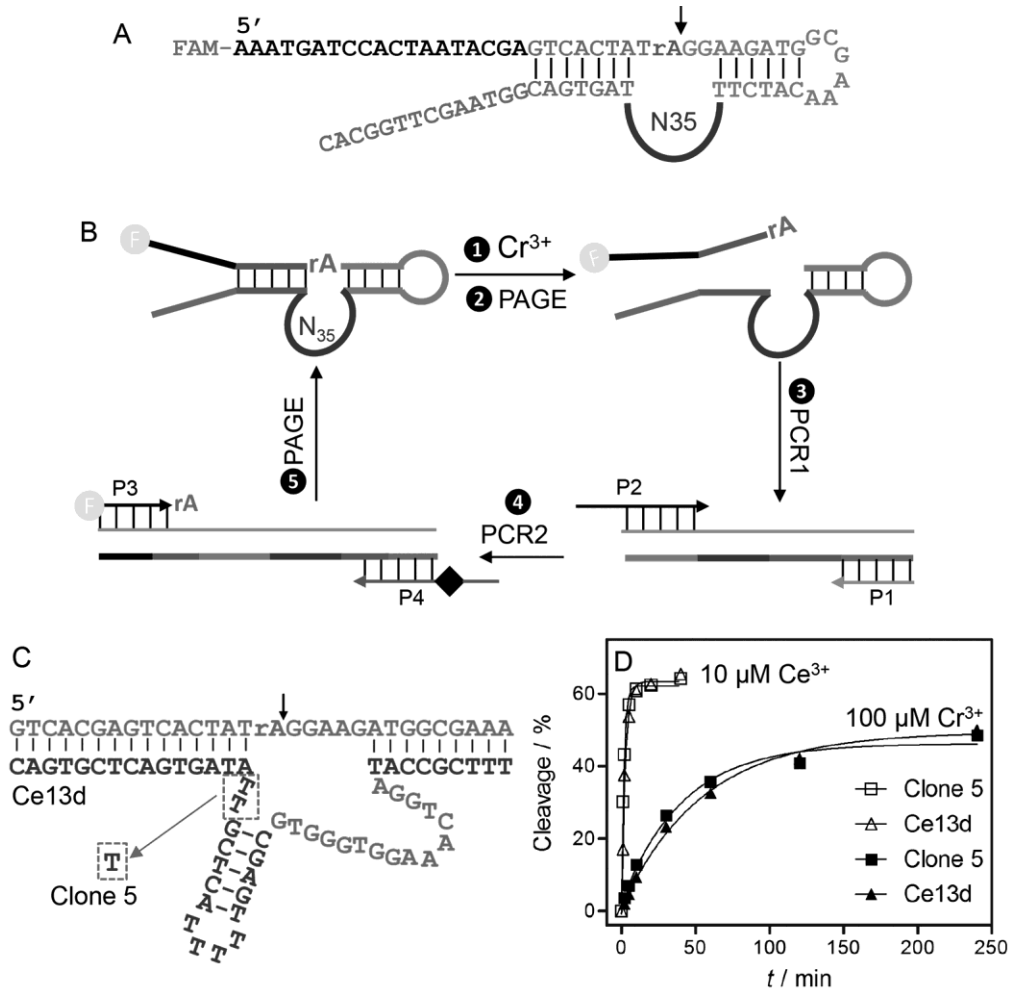

Figure 1. (A) The DNA library used for the Cr^{3+} selection containing a single scissile RNA linkage (rA), and 35 random nucleotides (N35). (B) The scheme of *in vitro* selection with five main steps. Two rounds of polymerase chain reactions (PCR) are used to amplify the selected sequences and re-construct the full-length library. (C) The secondary structures of the Ce13d DNAzyme. It differs from Clone 5, a representative sequence from the currently selection, mainly in the number of the T bases in the box. (D) Cleavage kinetics of Clone 5 and Ce13d in presence of 10 μM Ce^{3+} or 100 μM Cr^{3+} tested in buffer A (50 mM MES, pH 6.0, 25 mM NaCl).

New selection blocking Ce13d. Since Ce13d is only moderately active with Cr^{3+}, we decided to perform a new selection blocking the Ce13d related sequences in the library and thus fostering potential new sequences with higher Cr^{3+}-dependent activity. Ce13d is a unique DNAzyme with a stretch of 16 conserved nucleotides in its catalytic loop (Figure 1C in the big box). This allows us to inactivate the Ce13d-type sequences in the library by adding a blocking DNA complementary to these conserved nucleotides (top of Figure 2B). Using this method, we succeeded in Cd^{2+} and Cu^{2+}-dependent selections.[30,43] Using this strategy, the selection progress is shown in Figure 2B. While we indeed eliminated the Ce13d sequences, the obtained DNAzymes were however 90% the EtNa DNAzyme,[33] accompanied with less than 10% of the 17E (Figure S1). EtNa is Na^{+}-dependent DNAzyme we reported recently, and its cleavage activity is accelerated in organic solvents. EtNa is however inactive with Cr^{3+}.[33] The emergence of EtNa suggests that buffer-dependent activity dominated the selection and the intended Cr^{3+} failed to produce competitive cleavage activity after blocking Ce13d. 17E was previously isolated in many *in vitro* selections in the past two decades.[23,44] 17E is the most active with Pb^{2+} and is also active with many transition metals such as Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+} and Ca^{2+}. The activity of 17E with Cr^{3+} is also negligible (Figure S2), and therefore, it is unlikely to be useful for detecting Cr^{3+} either. After these selection efforts, only three previously reported DNAzymes were produced: 17E, EtNa, and Ce13d. Among them, Ce13d has the best activity with Cr^{3+}.

In the above selections, we did not include a negative selection step (e.g. removing DNAzyme sequences active with non-Cr^{3+} metals). Although the best DNAzyme Ce13d is much more active with many other metal ions than with Cr^{3+}, this cannot be solved by negatively selection. We completely eliminated Ce13d using the blocker DNA, and the resulting

DNAzymes were inactive with Cr^{3+}. Therefore, had a negative selection step been incorporated, it would be unlikely to produce better DNAzymes.

This selection outcome has important chemical implications. The role of polyvalent metal ions in Ce13d cleavage is known to be interacting with the scissile phosphate linking the rAG junction.[41] It is likely that Cr^{3+} is playing a similar role here. For interacting with the phosphate, the size and charge of the metal and its affinity with phosphate are important. Trivalent lanthanide ions are the most efficient in performing this task for Ce13d. The size of Cr^{3+} is only ~0.5 Å while the lanthanides are between 0.8 and 1.1 Å. This might explain the lower rate by Cr^{3+}. Using lanthanide ions for in vitro selection has resulted in a number of highly efficient DNAzymes in addition to the Ce13d,[35-37] while using Cr^{3+} only yielded Ce13d. This implies limited interaction modes between Cr^{3+} and DNA to allow favorable RNA cleavage. Cr^{3+} has a very slow ligand exchange rate.[45] After binding (e.g. to DNA bases), the complex may not dissociate easily, making the system very difficult to adjust dynamically to a favorable configuration for catalysis.

Recurrence of previously reported DNAzymes was reported a few times previously,[46,47] with the 8-17 DNAzyme being a prevalent example.[20] Such so called tyranny of the small motif exist due to its statistic advantages. For example, the 8-17 DNAzyme has only 13 nucleotides with a few quite tolerating mutations. However, Ce13d is not an obvious tyranny sequence since it is longer and highly conserved.[41] We believe its appearance is due to it is the optimal and probably the only solution for Cr^{3+}.

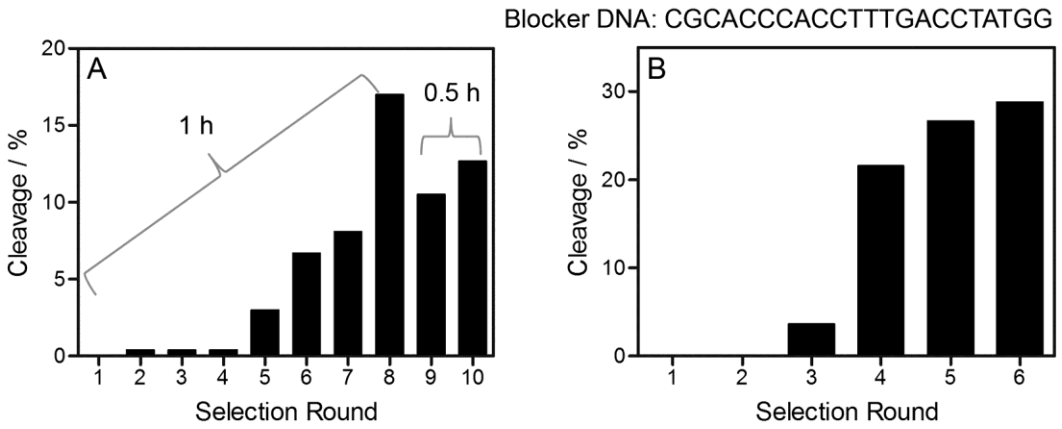

Figure 2. (A) Progress of the first selection experiment (with 0.2 mM Cr^{3+} in buffer A). (B) Progress of the blocked selection (25 µM Cr^{3+} in buffer A for 1 h incubation). The blocker DNA sequence complementary to the conserved nucleotides in Ce13d is also shown.

Masking interfering ions by phosphate. To fully understand the selectivity of Ce13d, we measured its cleavage with various divalent and trivalent metal ions (Figure 3A). Since Ce13d is similarly active with all the lanthanides,[37] we only tested Ce^{3+}. With 10 µM metals, only Ce^{3+}, Y^{3+} and Pb^{2+} produced significant cleavage, while Cr^{3+} induced just a moderate amount of cleavage after 1 h. The cleavage yield significantly increased with 100 µM Cr^{3+}, while 100 µM Zn^{2+} and Mn^{2+} also induced a trace amount of cleavage. To use Ce13d for Cr^{3+} detection, we need to mask the competing metal ions (namely lanthanides, Y^{3+} and Pb^{2+}). Lanthanides, Y^{3+} are hard Lewis acids and they should have very high affinity with inorganic phosphate. Pb^{2+}, Zn^{2+} and Mn^{2+} are borderline metal and may also be sequestered by phosphate.

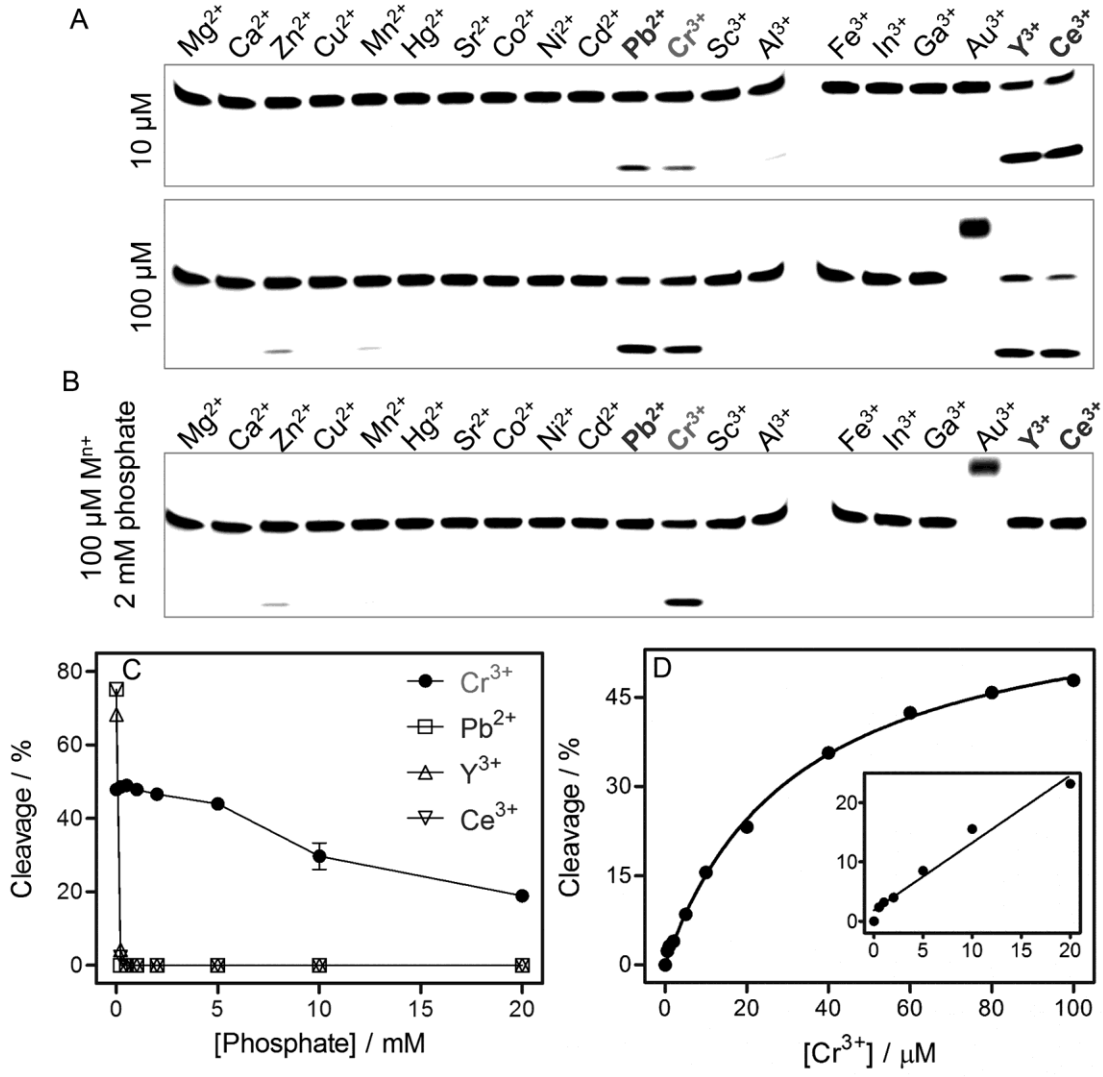

Figure 3. (A) Gel images showing cleavage of Ce13d with 10 or 100 µM of various metal ions. 100 µM Au^{3+} strongly bound to DNA and retarded DNA migration. (B) Masking competing metals 2 mM phosphate. (C) Phosphate concentration dependent Ce13d activity with 100 µM Ce^{3+}, Y^{3+}, Pb^{2+} or Cr^{3+}. (D) The activity of Ce13d with different Cr^{3+} concentrations. Inset: linearly increase of cleavage at low Cr^{3+} concentrations. All the reactions were with 1 µM DNAzyme in buffer A for 1 h.

To test this idea, we carried out the reaction in the presence of 2 mM phosphate. Indeed, only Cr^{3+} yielded a high cleavage and nearly all the rest metals were silent (Figure 3B). To have a quantitative understanding, we measured cleavage of these four metals as a function of phosphate concentration (Figure 3C). As low as 0.2 mM phosphate fully inhibited 0.1 mM competing metals. Cr^{3+} retained a similar cleavage even with 2 mM phosphate and ~50% inhibition was achieved with 20 mM phosphate. With 1 mM phosphate, the Ce^{3+}-dependent activity decreased from 0.4 min^{-1} to 1.8×10^{-4} min^{-1} (Figure S3). We estimated the inhibition effect was more than 2200-fold stronger for Ce^{3+}. Phosphate only exists in trace amount in most natural waters (e.g. US EPA criterion of maximally 0.03 mg/L or ~300 nM for uncontaminated lakes), and such background phosphate should not affect our DNAzyme performance.

After finding a simple masking agent, we next studied Cr^{3+}-dependent activity of Ce13d. The cleavage yield gradually increased with increasing Cr^{3+} concentration (Figure 3D), from which we obtained an apparent K_d of 32 μM Cr^{3+}, ~9-fold larger than that with Ce^{3+}.[37,41] Therefore, Cr^{3+} has a lower affinity to Ce13d compared to the lanthanides. The low Cr^{3+} concentration region has a linear response (inset), which is useful for analytical applications.

Detection of Cr^{3+}. After confirming the selectivity and activity of Ce13d for Cr^{3+} in phosphate buffer, we next tested Cr^{3+} detection. Our biosensor design is shown in the inset of Figure 4A. A FAM fluorophore was labeled at the 3′-end of the substrate strand, and a dark quencher was labeled at 5′-end of the enzyme.[37,48,49] After hybridization, the fluorescence was quenched because of close proximity between the FAM and quencher. After incubating with Cr^{3+}, the substrate is cleaved and the fluorescence is increased. Since Ce13d is quite slow with Cr^{3+}, we did not monitor the signalling kinetics but only measured the final fluorescence after 1 h. A Cr^{3+}-concentration dependent fluorescence enhancement was observed (Figure 4B). The change

in fluorescence at the 526 nm peak is plotted against Cr^{3+} concentration (Figure 4C). We obtained a detection limit of 70 nM Cr^{3+} (inset of Figure 4C, based on 3σ/slope calculation), which is significantly lower than maximal limit of Cr^{3+} in drinking water defined by the U.S. Environmental Protection Agency (EPA) (570 $\mu g \cdot L^{-1}$ or 11 μM). Using the same DNAzyme in a phosphate-free buffer, we reported a detection limit of 1.7 nM Ce^{3+}, and this 41-fold difference in detection limit is attributable to the slower rate of Ce13d in the presence of Cr^{3+}. We also tested the sensor specificity under two metal concentrations (Figure 4A). With the aid of phosphate, we indeed suppressed the sensor response to most other metals. We noticed that Zn^{2+} also has a weak response, which is attributable to its cleavage activity (Figure 4B).

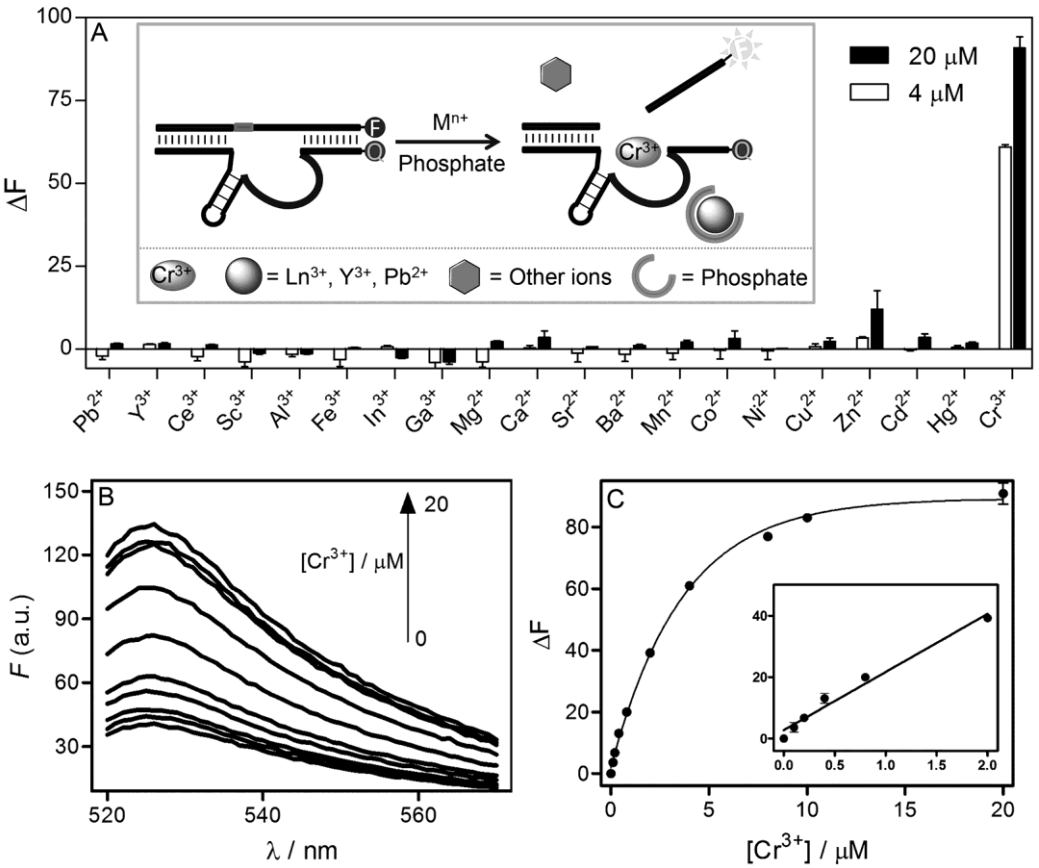

Figure 4. (A) Sensor specificity test with 4 μM and 20 μM different metal ions. The assays were performed in 50 mM MES, pH 6.0, 25 mM NaCl and 0.8 mM phosphate (1 h incubation). Inset: a scheme showing the sensor design for Cr^{3+} detection. (B) Fluorescence spectra of the sensor after incubating with different concentrations of Cr^{3+}. (C) Fluorescence enhancement at 526 nm with different Cr^{3+} concentrations. Inset: sensor with a linear response at low Cr^{3+} concentrations.

Detection of Cr(VI). After demonstrating Cr^{3+} detection, we next explored the possibility of detecting Cr(VI). For this purpose, $Cr_2O_7^{2-}$ was used as the metal source. No cleavage was observed with up to 20 μM $Cr_2O_7^{2-}$ (Figure 5A, open dots). Interestingly, after treating $Cr_2O_7^{2-}$ with $NaBH_4$, concentration-dependent cleavage occurred likely due to its reduction to Cr^{3+} (Figure 5A, solid dots). The successful reduction of $Cr_2O_7^{2-}$ to Cr^{3+} was also supported by its color changed from yellow to green (Figure S4A). We also tested ascorbate as a reducing agent, but the product failed to cleave the DNAzyme (Figure S4B). This is likely due to the chelation of Cr^{3+} by ascorbate. This result suggests that Ce13d can also be used to detect $Cr_2O_7^{2-}$. Using this method, we measured $Cr_2O_7^{2-}$ concentration-dependent response of the sensor. Again, $Cr_2O_7^{2-}$ alone failed to show any fluorescence change (open squares, Figure 5B), while after treatment with $NaBH_4$, the expected sensor response was observed (open circles). The detection limit for $Cr_2O_7^{2-}$ was determined to be 140 nM (Figure 5B, inset), also lower than the EPA maximal contamination level of hexavalent chromium in drinking water (16 $\mu g \cdot L^{-1}$ or 300 nM).

To test this sensor for real water samples, a Cr(VI) contaminated water was used, which has a strong yellow color (Figure S5). After dilution, the sample was first measured directly to determine the Cr^{3+} concentration. Then the sample was reduced by $NaBH_4$ followed by

determination of the total Cr^{3+}. The difference was then the Cr(VI) concentration. The concentrations of Cr^{3+} and Cr(VI) were measured to be 18 μM and 970 μM, respectively, which is consistent with to the ICP and colorimetric measurement within the error range (Table S3). This study supports the use of our sensor in real water samples and for chromium speciation analysis.

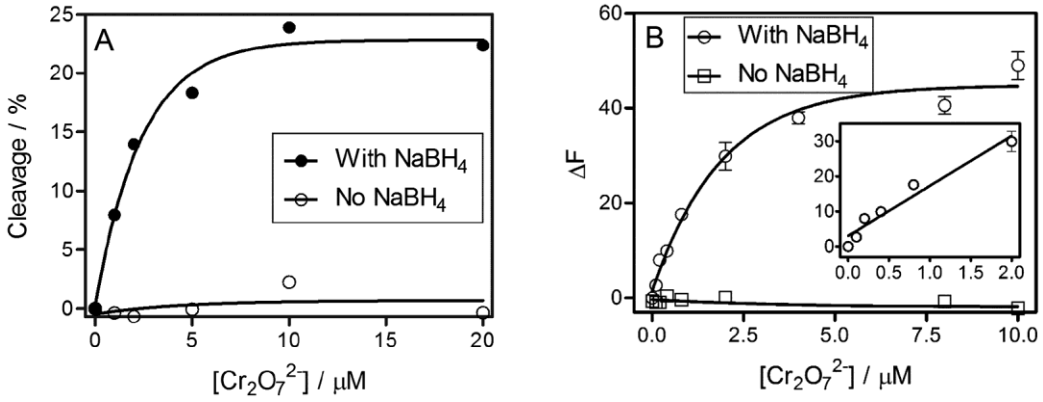

Figure 5. (A) The fraction of Ce13d substrate cleavage as a function of $Cr_2O_7^{2-}$ concentration with and without pre-treatment with $NaBH_4$. (B) The fluorescence enhancement of the sensor at different $Cr_2O_7^{2-}$ concentrations with or without pre-treatment with $NaBH_4$. Inset: the linear response at low $Cr_2O_7^{2-}$ concentrations.

Conclusions

In summary, we made extensive *in vitro* selection efforts to isolate RNA-cleaving DNAzymes that can work in the presence of Cr^{3+}. It is very likely that Ce13d is the optimal DNAzyme that can be activated by Cr^{3+}, although Cr^{3+} is ~150-fold less active compared to trivalent lanthanide ions such as Ce^{3+}. After blocking the Ce13d sequences in the library, no DNAzyme with Cr^{3+}-

dependent activity was obtained. As such, we used Ce13d as a sensor for Cr^{3+}. The distinct chemical property difference between Cr^{3+} and its competing metal ions allowed us mask the competing metals, leaving the Cr^{3+} activity intact. After achieving selectivity, this Ce13d DNAzyme was converted to a catalytic beacon for Cr^{3+} detection with a detection limit of 70 nM. Detection of hexavalent chromium was achieved by reducing the sample using $NaBH_4$ to convert it to Cr^{3+}, and a similar detection limit was obtained. Finally, a preliminary example of chromium speciation analysis of a real water sample was demonstrated.

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