A Cleavable Molecular Beacon for Hg^{2+} Detection based on Phosphorothioate RNA Modifications

Po-Jung Jimmy Huang, Feng Wang and Juewen Liu*

Department of Chemistry, Waterloo Institute for Nanotechnology

Waterloo, Ontario, Canada, N2L 3G1.

Email: liujw@uwaterloo.ca

Abstract

Mercury is a highly toxic heavy metal and detection of Hg^{2+} by biosensors has attracted extensive research interest in the last decade. In particular, a number of DNA-based sensing strategies have been developed. Well-known examples include thymine-Hg^{2+} interactions and Hg^{2+}-activated DNAzymes. However, these mechanisms are highly dependent on buffer conditions or require hybridization with another DNA strand. Herein, we report a new mechanism based on Hg^{2+}-induced cleavage of phosphorothioate (PS) modified RNA. Among the various metal ions tested, Hg^{2+} induced the most significant cleavage (~16%), while other metals cleaved less than 2% of the same substrate. The uncleaved substrate undergoes desulfurization in the presence of Hg^{2+}. This cleavage reaction yields a similar amount of product from pH 3.5 to 7 and in the temperature range between 20 °C and 90 °C. Various PS RNA junctions can be cleaved with a similar efficiency, but PS DNA junctions cannot be cleaved. A molecular beacon containing three PS RNA modifications is designed, detecting Hg^{2+} down to 1.7 nM with excellent selectivity. This sensor can also detect Hg^{2+} in the Lake Ontario water sample, although its response is significantly masked by fish tissues.

Introduction

Mercury is a highly toxic heavy metal.[1] Due to its bioaccumulative property, long-term exposure to even low concentrations of mercury causes serious organ damages. To achieve on-site and fast detection, many small molecules, peptides, lipids, and nucleic acids have been develop into biosensors for Hg^{2+}.[2, 3] In the past decade, DNA-based mercury sensors have attracted extensive interest, which might be related to the programmability of DNA and their ease of modification. First, the thymine-Hg^{2+}-thymine interaction has been extensively utilized.[4-10] In this case, Hg^{2+} bridges two thymine bases with no other metals being able to perform the same reaction.[11] However, thymine coordination is not strong enough, and sensors based on this mechanism suffer from variations in buffer composition. For example, Hg^{2+} can be masked by many common chemicals (e.g. even by a moderate concentration of NaCl).[12] A second strategy for sensing Hg^{2+} is to use it as a cofactor to assist DNAzyme catalysis.[6, 13, 14] However, DNAzymes with modified bases are often needed to achieve high affinity and such DNAzymes are not commercially available.[13] We reason that new strategies with a simple chemical reaction based on the DNA platform might further advance the mercury sensing field.

Strong thiophilicity is an important feature of Hg^{2+} that may separate it from other common metal ions. This property has not been widely explored in its sensing, especially in the DNA sensor field. One way to introduce a sulfur atom to a DNAzyme is via phosphorothioate (PS) modification, where one of the non-bridging oxygen atoms in the DNA phosphate backbone is replaced by a sulfur.[15, 16] Unlike a terminal thiol, PS can be placed at any position within a DNA and even made in tandem. It is also cost-effective to synthesize (~$3 per modification from most commercial sources). PS modification is a common tool in chemical biology to increase DNA stability against nuclease degradation,[17] and to probe ribozyme and DNAzyme mechanisms.[14, 18-22] In addition,

because of its affinity for thiophilic metals, PS-modified DNA can functionalize and assemble both metal and semiconductor nanoparticles.[23-27]

We recently selected a lanthanide-dependent DNAzyme, named Ce13d.[28] This DNAzyme can cleave an RNA/DNA chimeric substrate in the presence of trivalent lanthanide ions. By introducing a single PS modification at the cleavage junction, Ce13d is then active with a number of toxic thiophilic metals including Hg^{2+}.[14] A PS modification was also introduced in a DNA library for selecting a Cd^{2+}-specific DNAzyme.[29] In this study, we noticed that even the substrate alone (without DNAzyme) can be cleaved by Hg^{2+}. This non-specific cleavage is unique to Hg^{2+} among the various tested divalent metal ions. We reason this might be a new mechanism for Hg^{2+} detection. Since this is a chemical reaction (instead of simple binding), it may produce a very distinct signal and is less affected by the buffer conditions. At the same time, it does not require an enzyme strand, and thus allows more versatility in sensor design. We herein study this Hg^{2+}-dependent cleavage reaction in detail and the resulting knowledge is applied to design a highly sensitive and selective Hg^{2+} sensor.

Materials and Methods

Chemicals. The fluorophore/quencher-modified DNAs were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The unmodified and PS-modified DNAs were from Eurofins (Huntsville, AL). The exact DNA sequences used in this study are listed in Table 1. Cerium chloride heptahydrate, ammonium cerium nitrate, magnesium chloride tetrahydrate, manganese chloride tetrahydrate, cobalt chloride hexahydrate, nickel chloride, copper chloride dihydrate, zinc chloride, cadmium chloride hydrate, mercury perchlorate, lead acetate, lithium chloride,

sodium chloride, rubidium chloride, potassium chloride, calcium chloride dihydrate, cesium chloride, strontium chloride hexahydrate, barium chloride, silver nitrate, yttrium chloride hexahydrate, scandium chloride hydrate, chromium chloride hexahydrate, indium chloride, gallium chloride, aluminum chloride hydrate, and nickel chloride were purchased from Sigma-Aldrich and iron chloride tetrahydrate, and iron chloride hexahydrate, were from Alfa Aesar. The solutions were made by directly dissolving the salts in Milli-Q water. 2-(N-morpholino)ethanesulfonic acid (MES), 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), EDTA disodium salt dihydrate, sodium acetate trihydrate, and sodium chloride were from Mandel Scientific Inc. (Guelph, ON). Sodium phosphate monobasic dihydrate and sodium phosphate dibasic heptahydrate were from Fisher Scientific.

Gel-based assays. The stock DNAzyme complex was first prepared by annealing the FAM-labeled substrate strand (5 μM) and the Ce13d enzyme strand (7.5 μM) in buffer A (25 mM NaCl, 50 mM MES, pH 6). Gel-based activity assays were performed with a final concentration of 0.7 μM of the complex or substrate strand alone in the presence of 10 μM metal ion. The reactions were quenched with 8 M urea at designated time points. The products were separated on 15% dPAGE gels and analyzed using a Bio-Rad ChemiDoc MP imaging system.

Mass spectrometry. The PS-modified substrate (1 μM, 2 mL) was incubated with 10 μM metal ions in buffer A for 2 h. The sample was then desalted using a Sep-Pak C18 column (Waters) and then lyophilized overnight. The dried samples were dissolved in Milli-Q water without additional buffer to a final concentration of 40 μM. The sample was mixed with water-methanol (1:1) containing 0.1% formic acid to facilitate ionization and analyzed on a Micromass Q-TOF Ultima Global mass spectrometer using electrospray ionization (ESI).

Hg^{2+} sensing. The stock molecular beacon was first annealed in 500 mM HEPES, pH 7.5 and stored in a -20 °C freezer overnight. For each assay, 100 µL of sensor (10 nM) was used. The sensing kinetics studies were carried out in 96 well plates (100 µL for each well) and were monitored with a M3 microplate reader (SpectraMax).

Table 1. The DNA samples used in this work. The asterisk denotes for PS modification.

DNA names	**Sequences and modifications (from the 5′-end)**
PO-substrate	GTCACGAGTCACTATrAGGAAGATGGCGAAA-FAM
PS-substrate	GTCACGAGTCACTATrA*GGAAGATGGCGAAA-FAM
Ce13d	TTTCGCCATAGGTCAAAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Mass sub	TATrA*GGAAGA
rC*G	GTCACGAGTCACTATrC*GGAAGATGGCGAAA-FAM
rC*T	GTCACGAGTCACTATrC*TGAAGATGGCGAAA-FAM
*rAG	GTCACGAGTCACTAT*rAGGAAGATGGCGAAA-FAM
Beacon	Iowa Black FQ-GrA*GCGCTArA*GAAATrA*GCGCTC-FAM

Results and Discussion

DNAzyme activity. We noticed cleavage of PS-modified RNA by Hg^{2+} during our study of a lanthanide-dependent DNAzyme.[14] Figure 1A shows the structure of the Ce13d DNAzyme, which was selected in the presence of a cerium salt.[28] It contains a substrate strand with a single RNA linkage (rA, ribo-adenosine) and a DNA enzyme strand. Figure 1B shows the structure of the cleavage junction (rA·G) with a normal phosphate linkage. This is an interesting DNAzyme since it is active only with trivalent lanthanide ions (moderate cleavage in the presence of Pb^{2+}, Figure 1D). After a single PS modification at the cleavage junction (Figure 1C), the lanthanide activity

was significantly suppressed, and the DNAzyme becomes active with thiophilic metals such as Cd^{2+} and Pb^{2+} (Figure 1E). This makes it a useful sensor for detecting these toxic heavy metals as a group.[14]

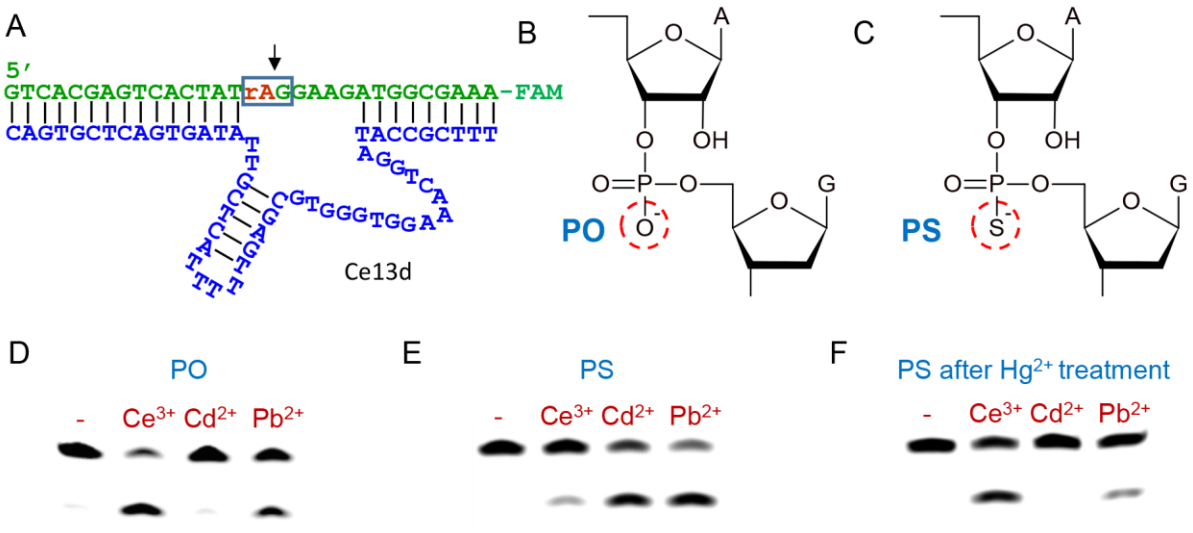

Figure 1. (A) The secondary structure of the Ce13d DNAzyme, containing a substrate strand (in green) and an enzyme strand (in blue). The cleavage junction is highlighted by the box and its structure is shown in (B). (C) The cleavage junction with a single PS modification. Gel images of Ce13d cleavage of (D) the normal PO substrate, (E) the PS substrate, and (F) the PS substrate after Hg^{2+} treatment and purification in the presence of different metal ions.

As a control experiment, the substrate strand alone (no enzyme strand) was treated with the metals. We noticed ~16% cleavage with Hg^{2+}, while the remaining 84% were still at the original length (Figure 2A). This reaction is highly specific to Hg^{2+} and no other metal shows observable cleavage. Therefore, we aim to explore the feasibility of developing this reaction into a biosensor for Hg^{2+}. To understand the mechanism, we isolated the uncleaved substrate after Hg^{2+} treatment using gel electrophoresis. Then this Hg^{2+} treated substrate was hybridized with the Ce13d DNAzyme and

incubated with different metal ions. Interestingly, it is cleaved more by Ce^{3+}, followed by Pb^{2+}, while Cd^{2+} is completely inactive (Figure 1F). This pattern is similar to that in Figure 1A. This strongly suggests that at least two reactions occurred for the Hg^{2+} treatment. First, ~16% of the substrate underwent cleavage. Second, the remaining uncleaved fraction became the PO substrate (i.e. the sulfur atom removed by Hg^{2+}), which is called desulfurization.[30]

Desulfurization and cleavage. The Hg^{2+}-induced cleavage is related to its strong thiophilicity to bind the sulfur atom. This neutralizes the charge of the sulfur, making it a better leaving group in the nucleophilic attack by the 2′-OH group of the ribo-adenosine. At the same time, Hg^{2+} may extract the sulfur from the substrate. The simple gel-based assay only signals cleavage but the amount of desulfurization is unclear. To further understand it, we developed the following assay. The PS substrate was mixed with 10 μM of various metal ions and the samples were split into two. One was analyzed directly by gel electrophoresis to measure cleavage (Figure 2A). From this group, we only observed significant cleavage with Hg^{2+}, while Cu^{2+} and Pb^{2+} showed very moderate cleavage (<2%, Figure 2C, black bars). The other group was further treated with Hg^{2+} and then analyzed by gel (Figure 2B). If the first metal treatment induced desulfurization, Hg^{2+} would not be able to induce further cleavage. The quantification is in the red bars of Figure 2C. It can be observed that Hg^{2+} still induced a similar level of cleavage for all the metals, suggesting that only Hg^{2+} induced desulfurization under our experiment conditions. This result further supports the use of this mechanism for Hg^{2+} detection.

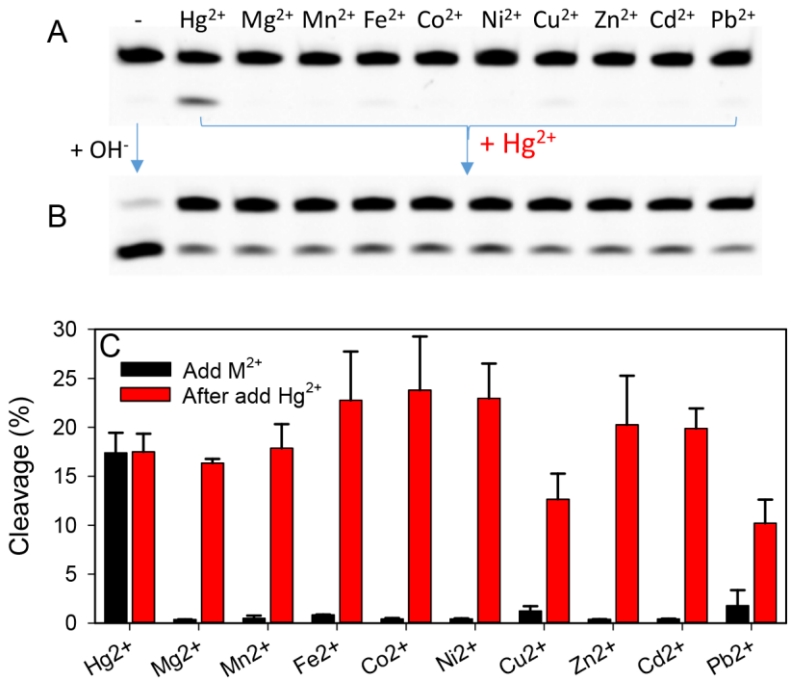

Figure 2. (A) Gel image showing the cleavage of the PS substrate with 10 μM of various divalent metal ions after 1 h incubation. The first lane is a negative control without any metal. (B) The samples in (A) after further treatment with Hg^{2+} for additional 1 h. The first lane was treated by NaOH to fully cleave the substrate. (C) Quantification of cleavage in (A) (black bars) and (B) (red bars).

To further confirm our hypothesis, we treated a shortened substrate (10-mer) containing a PS RNA modification with Hg^{2+} and analyzed the sample by mass spectrometry. The original mass is 3132.2 and after the treatment, we observed a reduction of mass by 16 and peaks from cleavage products, while the original peak completely disappeared (Figure 3A). On the other hand, if the substrate is treated with the same concentration of Co^{2+}, the major peaks are from the original PS

substrate (Figure 3B). This experiment also supports the desulfurization and cleavage mechanism induced by mercury.

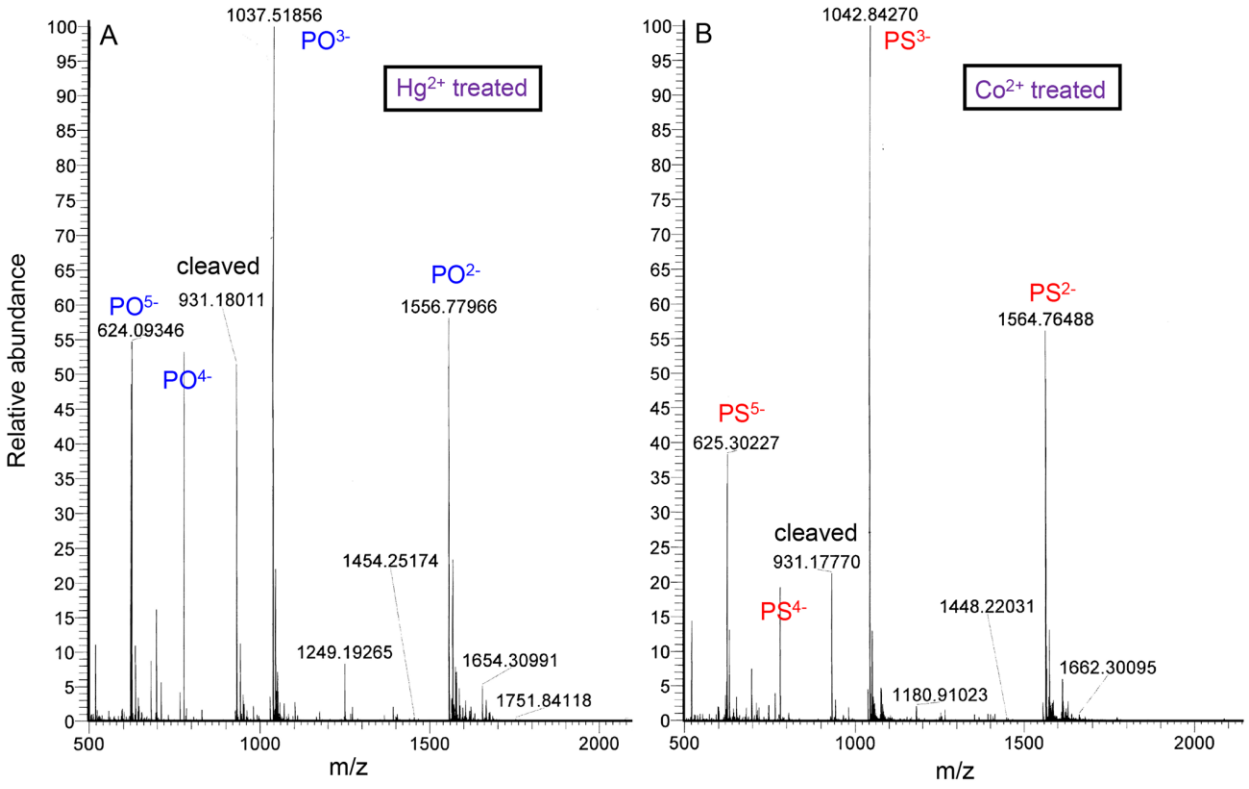

Figure 3. ESI mass spectrometry analysis of a PS-containing RNA cleavage after treatment with (A) Hg^{2+}, or (B) Co^{2+}. The sequence is TATrA*GGAAGA (Mass sub in Table 1). The PO and PS marked on the peaks represent different charged species of the full length molecule (PO means desulfurized). The cleavage product is marked by 'cleaved'.

Hg^{2+} cleavage conditions. Given the excellent specificity, we are interested in comparing this new Hg^{2+} sensing mechanism with the popular T-Hg^{2+}-T method.[31, 32] Thymine binding requires its

deprotonation and thus is strongly pH-dependent. In general, binding reactions are strongly dependent on temperature and high temperature disrupts Hg^{2+} binding due to DNA denaturation. Since the affinity is weak between thymine and Hg^{2+}, even weak ligands such as chloride can inhibit the binding reaction at physiological conditions.[12, 33] Therefore, it is difficult to measure the true Hg^{2+} concentration in a water sample without knowing the exact anion composition. In the work below, we test the buffer conditions for this new Hg^{2+} recognition mechanism based on RNA cleavage.

First, we studied the effect of the junction base composition. The current junction is denoted by rA*G, where the asterisk represents the PS modification. When it is replaced by rC*G or rC*T, a similar amount of cleavage was observed in the presence of Hg^{2+} (Figure 4A). However, if the position of the PS bond is shifted by one nucleotide (e.g. *rAG), no cleavage is observed (the first bar in Figure 4A). Therefore, this cleavage is independent of junction base composition and is only related to the presence of 2′-OH (i.e. needs to be PS RNA). The generality in junction allows more freedom in subsequent biosensor design.

Since water samples might exist in a diverse range of pH values, next the effect of pH was studied (Figure 4B). The amount of Hg^{2+}-induced cleavage remained at ~16-20% from pH 3.5 to 7.0. At even higher pH, the fraction of cleavage decreased gradually and reached ~5% at pH 8.5. The reason for the decreased cleavage might be related to Hg^{2+} hydrolysis. Therefore, this sensing mechanism can be used in many buffer conditions.

The effect of temperature was studied next (Figure 4C). The free substrate in the absence of Hg^{2+} was stable and only ~1% cleavage occurred after incubating at 90 °C for 5 min. With Hg^{2+}, the cleavage was low at low temperatures and increased linearly until 20 °C. After that, the fraction

of cleavage barely changed. Finally, we studied the effect of reaction time (Figure 4D). The cleavage proceeds with the first order rate constant at 1.8 min^{-1}. The reaction is largely finished in 2 min. Overall, this is a very stable reaction that is quite insensitive to DNA sequence, pH, temperature and reaction time over a large range, which makes it ideal for biosensor design.

Finally, we measured the effect of Hg^{2+} concentration. A gel image is shown in Figure 4F, where an increased fraction of cleavage is observed with more Hg^{2+}, making quantitative detection possible. The quantification is shown in Figure 4E and a linear relationship is observed until 1 μM Hg^{2+} was added. Since our DNA concentration was 1 μM, the reaction between these two is quantitative. Such a linear relationship also suggests that Hg^{2+} is not catalytic. Once a Hg^{2+} ion is associated with the substrate, it is consumed by either assisting cleavage or desulfurization.

The above measurements indicate that Hg^{2+}-induced cleavage of PS RNA is an analytically useful reaction. Unlike the thymine-Hg^{2+} interaction, the reaction between PS RNA and Hg^{2+} is less affected by buffer conditions. Differing from DNAzyme reactions, the cleavage here does not require an enzyme strand, bringing more simplicity and versatility for sensor design. A downside however, is the relatively low fraction of DNA cleaved compared to that which underwent desulfurization. Since only the cleaved DNA can produce signal, this might compromise sensitivity.

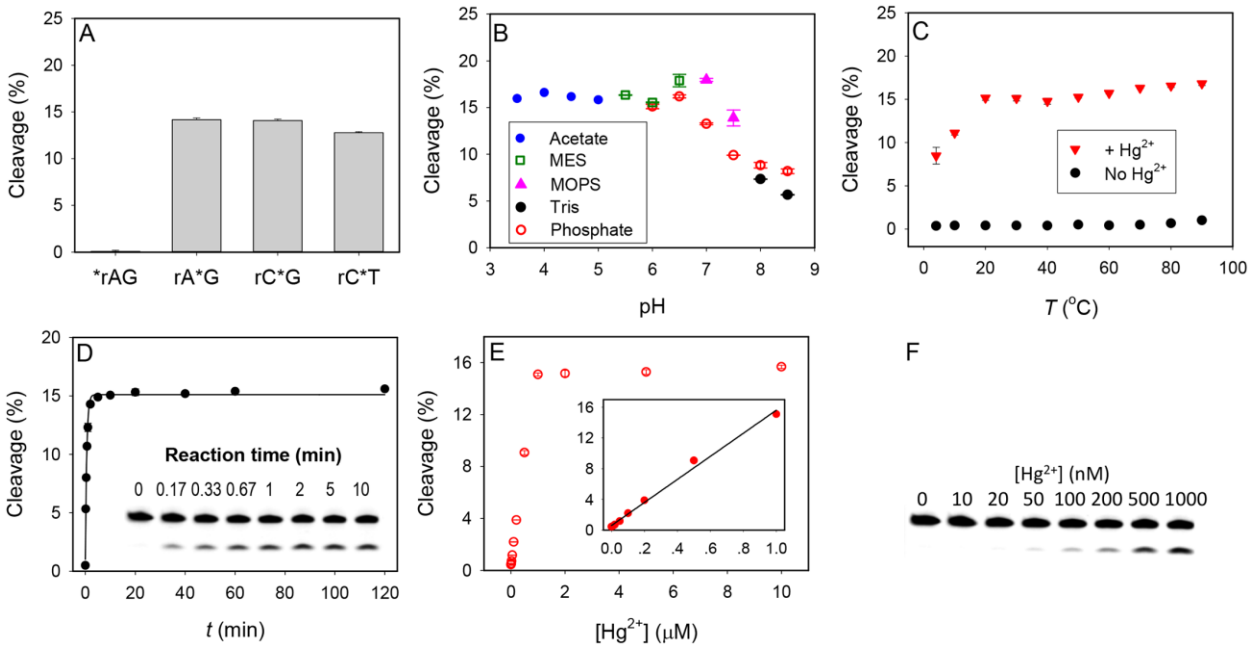

Figure 4. Optimization of the Hg^{2+} cleavage reaction. (A) Effect of the base composition of the PS cleavage junction. (B) Effect of pH. (C) Effect of temperature. (D) Effect of reaction time. Inset is a gel image showing the cleavage kinetics. (E) Effect of Hg^{2+} concentration. (F) A gel picture showing cleavage as a function of Hg^{2+} concentration.

A Hg^{2+} sensing beacon. This simple cleavage reaction allows various sensor design strategies. For this initial proof-of-concept work, a molecular beacon with three PS RNA linkages was used. A molecular beacon involves a fluorophore and a quencher respectively labeled on either end of a DNA hairpin, producing suppressed signal.[34] Cleavage separates the fluorophore from quencher to enhance fluorescence.[35] To increase cleavage yield, three PS RNA linkages were included in our beacon (inset of Figure 5C). Using more PS cleavage sites may not increase sensitivity at low Hg^{2+} concentrations. However, at high Hg^{2+} concentrations (e.g. Hg^{2+} is in excess), the cleavage yield should be higher. If cleavage of each PS site is independent, with three sites, the yield should

increase from 16% for a single site to 41% to three sites (e.g. the uncleaved fraction is $(0.84)^3$). We first measured the sensor signaling kinetics as a function of Hg^{2+} concentration (Figure 5A). A higher Hg^{2+} concentration produces stronger signal enhancement, and the signal is stabilized in ~1 min. The fluorescence intensity at 3 min is then plotted as a function of Hg^{2+} concentration (Figure 5B), where a linear response is observed until 50 nM Hg^{2+}. Further increase of Hg^{2+} concentration does not produce more signal. The detection limit is calculated to be 1.7 nM based on signal stronger than three times the background variation. Finally, we measured sensor selectivity. Other than Hg^{2+}, only Ag^{+} produced a moderate signal, which can be explained by its strong thiophilicity. We also measured the sensor response of Hg^{2+} with 100-fold excess of competing metal ions (Figure S1). Most divalent metal ions have no effect on Hg^{2+} sensing, while all the trivalent metal ions suppressed the sensor signal. This is attributed to the fluorescence quenching and DNA condensation effect of high valent metals.

We also measured the sensor response in Lake Ontario water samples (Figure S2). The signal was masked when Hg^{2+} was below 10 nM, and Hg^{2+} can be detected at 20 nM or higher. This response indicates that certain chemicals in the lake water can tightly bind to ~10 nM Hg^{2+}. After that, Hg^{2+} can be quantitatively detected. Finally, we challenged the reaction with fish tissue and Hg^{2+} signal was observed only with more than 100 μM Hg^{2+} (Figure S3). Therefore, this reaction cannot compete with the proteins and other molecules that can bind Hg^{2+} even more strongly in biological samples. We reason that this sensor is more useful for detecting Hg^{2+} in environmental water samples.

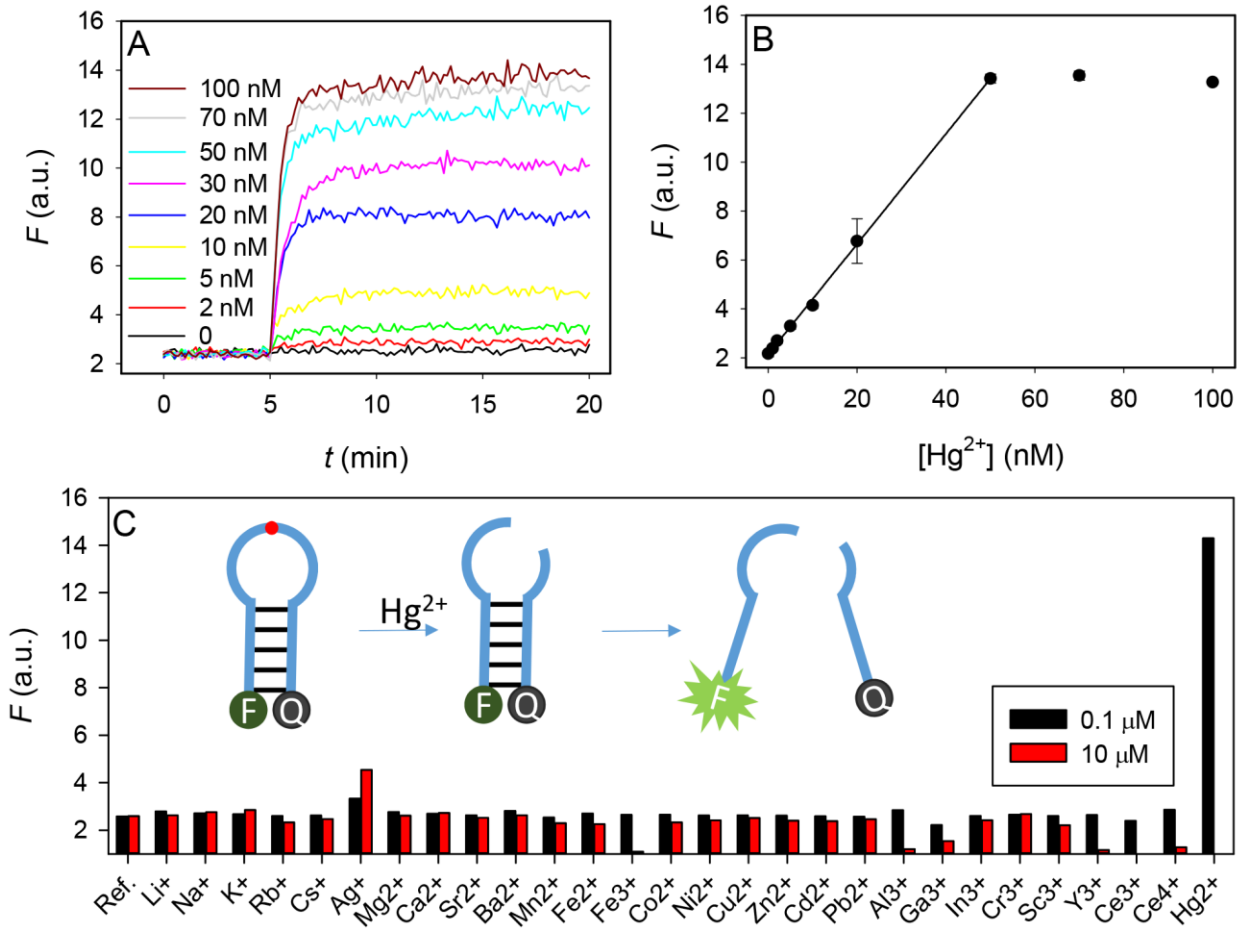

Figure 5. (A) Sensor signaling kinetics at various Hg^{2+} concentrations. (B) Sensor signal at 3 min after adding Hg^{2+} as a function of Hg^{2+} concentration. (C) Sensor selectivity test. Inset: scheme of sensor design. Reaction buffer: 50 mM HEPES, pH 7.5.

Conclusions. In summary, we studied metal-induced cleavage of PS-modified RNA, and Hg^{2+} is the most efficient metal for this reaction. The cleavage reaction yield is ~16%, while the remaining sample underwent desulfurization as indicated by mass spectrometry. Unlike other DNA-based Hg^{2+} sensing mechanisms, this cleavage reaction is quite independent of pH, temperature, and

reaction time. The reaction requires only a single nucleic acid strand and no enzyme needs to be hybridized, allowing more versatility and simplicity in sensor design. We designed a molecular beacon with three embedded PS-RNA modifications that can detect Hg^{2+} down to 1.7 nM with excellent selectivity.

Acknowledgments

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Supporting Information Available: additional metal interference data, cleavage in fish tissue and detection in Lake Ontario water. This information is available free of charge via the Internet at http://pubs.acs.org/.

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