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Mercury Sensors

Rational Design of "Turn-On" Allosteric DNAzyme Catalytic Beacons for Aqueous Mercury Ions with Ultrahigh Sensitivity and Selectivity**

*Juewen Liu and Yi Lu**

Mercury is a highly toxic heavy metal in the environment. Mercury exposure can cause a number of severe adverse health effects, such as damage in the brain, nervous system, immune system, kidney, and many other organs.[1] Mercury contamination comes from nature as well as from human activities, and an annual release of 4400 to 7500 metric tons of mercury into the environment was estimated by the United Nations Environment Programme (UNEP).[2] Therefore, highly sensitive and selective mercury sensors are very useful in understanding its distribution and pollution and in preventing mercury poisoning. Towards this goal, many fluorescent small-organic-molecule-based Hg^{2+} ion sensors have been reported, which change their emission properties upon binding to Hg^{2+} ions. Most of these sensors, however, require the involvement of organic solvent, show quenched emissions, and suffer from poor selectivity.[3,4] Only a few such sensors can detect Hg^{2+} ions in water with high sensitivity and selectivity.[5] Hg^{2+}-ion sensors based on foldamers,[6] oligonucleotides,[7] conjugated polymers,[8] genetically engineered cells,[9] enzymes,[10] antibodies,[11] transcriptional regulatory proteins,[12,13] DNAzymes,[14] and chemically modified optical fibers,[15,16] capillary optodes,[17] membranes,[18] electrodes,[19] mesoporous silica,[20] and nanoparticles[21] are also known. For environmental-monitoring applications, such as detection of Hg^{2+} ions in drinking water, a detection limit of lower than 10 nM (the toxic level defined by the U.S. Environmental Protection Agency (EPA)) is required. However, few reported mercury sensors can reach such sensitivity.[4,9,13] We are interested in using catalytic DNA or DNAzymes to design metal sensors that can achieve the goal.[22,23]

DNAzymes are DNA-based biocatalysts.[24] Similar to protein enzymes or ribozymes, DNAzymes can also catalyze many chemical and biological transformations, and some of the reactions require specific metal ions as cofactors. We have demonstrated highly effective fluorescent and colorimetric sensors for Pb^{2+} and UO_2^{2+} ions with DNAzymes.[22,23,25] These sensors showed picomolar to low nanomolar sensitivity and a thousand- to millionfold selectivity. In the presence of target metal ions, the fluorescence enhancement was generally greater than 10-fold, and signal generation took only 2 min or less. These sensors can be used at room temperature in aqueous solutions and no organic solvents are needed. Recently, DNAzyme-based electrochemical metal sensors have also been reported.[26] Compared with protein or RNA, DNA is relatively more cost effective to produce and more stable. DNAzymes can be denatured and renatured many times without losing their activities. Therefore, DNAzymes are useful in metal detection.

It was reported that Hg^{2+} ions can specifically bind in between two DNA thymine bases and promote these T–T mismatches to form stable base pairs (Figure 1 d).[7,27] This property was applied by Ono and Togashi to design a fluorescent sensor for detection of Hg^{2+} ions.[7] The sensor consisted of a single-stranded thymine-rich DNA strand with the 3′ and 5′ ends labeled with a fluorophore and a quencher, respectively. In the presence of Hg^{2+} ions, the two ends were brought close to each other, resulting in decreased fluorescence. A detection limit of 40 nM was reported.[7] Being sensitive and selective, this sensor was a "turn-off" sensor, and fluorescence intensity decreased in the presence of Hg^{2+} ions, which may give "false positive" results caused by external quenchers or other environmental factors that can also induce fluorescence decrease. The Hg^{2+}-ion stabilization effects on T–T mismatches have also been applied to design colorimetric sensors with DNA-functionalized gold nanoparticles to achieve a detection limit of 100 nM.[21] By using conjugated polymers for signal transduction, detection limits of 2.5 μM and 42 nM for colorimetric and fluorescent sensors, respectively, were reported.[8] In our previous DNAzyme work, we have designed a signaling method called a catalytic beacon in which the metal binding site in DNAzymes and the fluorescence signaling part are spatially separated.[22,23,28] We herein report that the thymine–mercury–thymine interaction can be used to modulate DNAzyme activities through allosteric interactions, resulting in a catalytic beacon with a detection limit of 2.4 nM.

Recently, we reported a UO_2^{2+}-specific DNAzyme isolated by in vitro selection.[23] The secondary structure of the DNAzyme shown in Figure 1 a contains a substrate strand (39S) and an enzyme strand (39E). 39S has a single RNA linkage (rA) that serves as the cleavage site. 39E binds 39S through two substrate binding arms. The catalytic core in 39E contains a stem loop (shown in blue) and an eight-nucleotide bulge (shown in green). Further studies indicated that the exact nucleotide sequence in the stem loop was unimportant for activity as long as such a structure was maintained. For example, when the stem loop was replaced with that shown in

[*] Dr. J. Liu, Prof. Y. Lu
Department of Chemistry
Beckman Institute for Advanced Science and Technology
University of Illinois at Urbana-Champaign
Urbana, IL 61801 (USA)
Fax: (+1) 217-333-2685
E-mail: yi-lu@uiuc.edu

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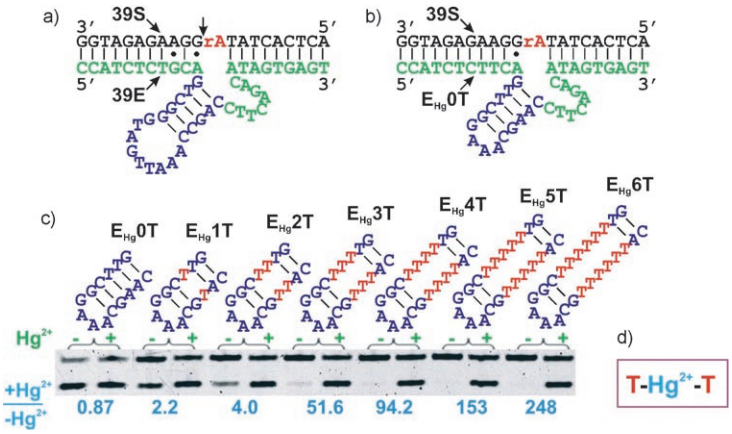

Figure 1. a) The secondary structure of the originally reported UO_2^{2+}-ion-specific DNAzyme. b) The new UO_2^{2+}-ion-specific DNAzyme with the replaced stem loop. One of the A·G mismatches was also replaced by an A–T base pair. c) The stem-loop part of DNAzymes with 0–6 T–T mismatches (top). Gel image showing the fraction of cleavage after 1-min reaction time in the absence or presence of 10 μM Hg^{2+} ions (middle). The ratio of the cleavage fraction after 1 min in the presence or absence of Hg^{2+} ions (bottom). d) Schematic diagram of a T–T mismatch stabilized by a Hg^{2+} ion.

Figure 1b, the DNAzyme was still active. In addition to the change made to the stem loop, one of the A·G mismatches in the substrate binding arm on the left side of cleavage site in Figure 1a was replaced by a A–T Watson–Crick base pair, whereas the other A·G mismatch closest to the cleavage site was maintained. The new enzyme strand was then named E_{Hg}0T (Figure 1b), which was used as a scaffold to engineer allosteric DNAzymes that can detect Hg^{2+} ions. In addition to having such a replaceable stem loop, we chose the uranium DNAzyme for Hg^{2+}-ion sensing for the following reasons: First, this DNAzyme is active only in the presence of UO_2^{2+} ions, and 1 μM UO_2^{2+} ions are sufficient to saturate its activity.[23] Unlike other common metal ions, UO_2^{2+} ions are not present in high concentrations in most environmental samples. Therefore, if the sensor system is saturated with UO_2^{2+} ions, external metals are unlikely to interfere with the detection. Even though uranium is a radionuclide, 1 μM UO_2^{2+} ions do not cause health or environmental concerns because uranium is ubiquitous in the environment, and even in drinking water, 130 nM uranium is allowed according to the U.S. EPA. As the sensing application requires only 500 μL or less of the sensor sample, the environmental impact is negligible. Second, the enzyme kinetics are fast. The E_{Hg}0T DNAzyme shown in Figure 1b has a rate constant of 2.0 min^{-1} in the presence of 1 μM UO_2^{2+} ions, which allows fast sensor response. Finally, the DNAzyme is relatively small in size and can be chemically synthesized and modified with high yields.

To incorporate Hg^{2+}-ion recognition elements into the DNAzyme, we used rational design methods and introduced between one and six T–T mismatches in the stem region of E_{Hg}0T (Figure 1c). All other nucleotides were kept the same. The sequence of E_{Hg}0T was designed in such a way that no stable secondary structures in the catalytic cores of all the DNAzymes (from E_{Hg}1T to E_{Hg}6T) involving the thymine insertions were predicted by the Mfold program.[29] As a result, the DNAzymes cannot fold into their active structures in the absence of Hg^{2+} ions. The addition of Hg^{2+} ions should quickly fold the DNAzymes into their active conformations without kinetic traps. Because the effect of Hg^{2+} ions is spatially located away from the UO_2^{2+}-ion binding site, such DNAzymes belongs to the group of allosteric DNAzymes.[30]

To test whether Hg^{2+} ions can enhance the activity of these thymine-rich DNAzymes, a 1 μM solution of the DNAzyme complexes was incubated with 10 μM Hg^{2+} ions for 10 min at room temperature. The substrate strand was labeled with a 6-carboxyfluorescein (FAM) fluorophore on the 5′ end. UO_2^{2+} ions were added to initiate the cleavage reaction. After 1 min, the reaction was stopped and the samples were loaded onto a 20% denaturing polyacrylamide gel to separate the cleaved and uncleaved substrate. As shown in Figure 1c, in all the DNAzymes with T–T mismatches, the fraction of cleavage was higher in the presence of Hg^{2+} ions, suggesting that Hg^{2+} ions indeed helped to stabilize the stem-loop structure and made the DNAzymes more active. For the E_{Hg}1T, E_{Hg}2T, and E_{Hg}3T DNAzymes, the cleavage bands in the absence of Hg^{2+} ions were also quite clear, suggesting that the DNAzymes may transiently fold into their active conformations even with several T–T mismatches. Such tolerability, however, dropped very quickly as the number of mismatches increases. For each DNAzyme, the ratio of the cleavage fraction in the presence and absence of Hg^{2+} ions was determined (bottom of Figure 1c), which approximately represents the magnitude of activity enhancement caused by Hg^{2+} ions. This value also positively correlates with the signal-to-background ratio for sensing applications.

In the above experiment, the DNAzymes were first allowed to equilibrate with Hg^{2+} ions, and then UO_2^{2+} ions were added to initiate the reaction. To detect Hg^{2+} ions, it is more desirable to add Hg^{2+} ions to the DNAzyme/UO_2^{2+}-ion mixture to initiate the cleavage reaction. Because E_{Hg}5T and E_{Hg}6T showed the highest activity enhancement by Hg^{2+} ions, the rates of cleavage initiated by adding 10 μM Hg^{2+} ions to the mixture of 1 μM DNAzyme and 1 μM UO_2^{2+} ions was calculated. Compared with the original DNAzyme E_{Hg}0T, which had a rate constant of 2.0 min^{-1}, the values for E_{Hg}5T and E_{Hg}6T were 0.61 and 0.45 min^{-1}, respectively. Therefore, DNAzymes with more T–T mismatches had lower rates, which could be explained by the fact that it took more time for longer DNA to find the right conformation. As a compromise between the rate of the reaction and the magnitude of activity enhancement, E_{Hg}5T was chosen for further studies.

The Hg^{2+}-ion catalytic beacon is shown in Figure 2a. The original E_{Hg}5T enzyme strand was extended on the 5′ end by five nucleotides, and the substrate was also extended accordingly to form base pairs with the extended enzyme. Such extensions were made to increase the hybridization efficiency between the two strands. To generate a signal, a fluorophore (FAM) was labeled on the 5′ end of the substrate, a quencher was labeled on the 3′ end of the enzyme, and an additional quencher was attached on the 3′ end of the substrate. Both

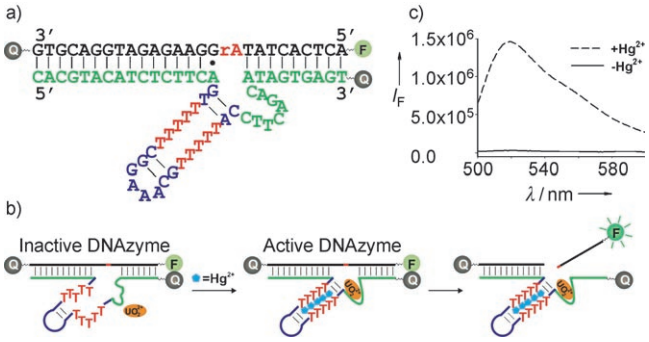

Figure 2. a) The secondary structure and modification of the Hg^{2+}-ion sensor DNAzyme. b) Schematic presentation of the sensor design. c) Fluorescence spectra of the sensor (with the DNAzyme and 1 μM UO_2^{2+} ions) in the absence of and 8 min after the addition of 0.5 μM Hg^{2+} ions. I_f = fluorescence intensity.

quenchers were black-hole quenchers. Such a dual-quencher labeling method gave very low background fluorescence and therefore allowed high signal enhancement.[31] The DNAzyme was mixed with UO_2^{2+} ions to become a mercury sensor (Figure 2b). In the absence of Hg^{2+} ions, the DNAzyme was incapable of binding UO_2^{2+} ions because the active secondary structure cannot form. The addition of Hg^{2+} ions quickly restored the stem-loop structure and activated the DNAzyme to cleave the substrate, releasing the fluorophore-labeled piece and resulting in increased fluorescence. The fluorescence spectra of the sensor before and 8 min after the addition of 500 nM Hg^{2+} ions is shown in Figure 2c, and an approximate 50-fold increase in the 520-nm peak was observed. Such a level of fluorescence increase is among the highest in functional nucleic-acid-based sensors.[32]

Given the very high fluorescence enhancement, the DNAzyme was titrated with varying concentrations of Hg^{2+} ions, and the kinetics of fluorescence enhancement at 520 nm was monitored. As shown in Figure 3a, higher concentrations of Hg^{2+} ions produced higher rates of emission enhancement. All the kinetic traces showed a roughly linear increase in the 1–2-min time window after the addition of Hg^{2+} ions, and therefore the rate of fluorescence increase in this window was calculated to quantify Hg^{2+}-ion concentration (Figure 3b). The Hg^{2+}-ion-dependent response had a sigmoid shape and was fit to a Hill plot with a Hill coefficient of 2.1. This result suggests that Hg^{2+}-ion binding to the DNAzyme is a cooperative process. Although the DNAzyme has five Hg^{2+}-ion binding sites, the DNAzyme is stable enough to cleave its substrate after binding approximately two Hg^{2+} ions. The detection limit was determined to be 2.4 nM based on 3σ/slope (inset of Figure 3b), which was an approximate 16-fold improvement over the previous oligonucleotide folding-based sensor.[7] Based on the best of our knowledge, among all the reported Hg^{2+}-ion sensors made from small and macromolecules, this catalytic beacon has the best detection limit. The U.S. EPA defined the toxic level of Hg^{2+} ions in drinking water to be two parts per billion or 10 nM, which can be covered by the beacon.

To test selectivity, the catalytic-beacon responses in the presence of 13 competing metal ions were assayed (Figure 4).

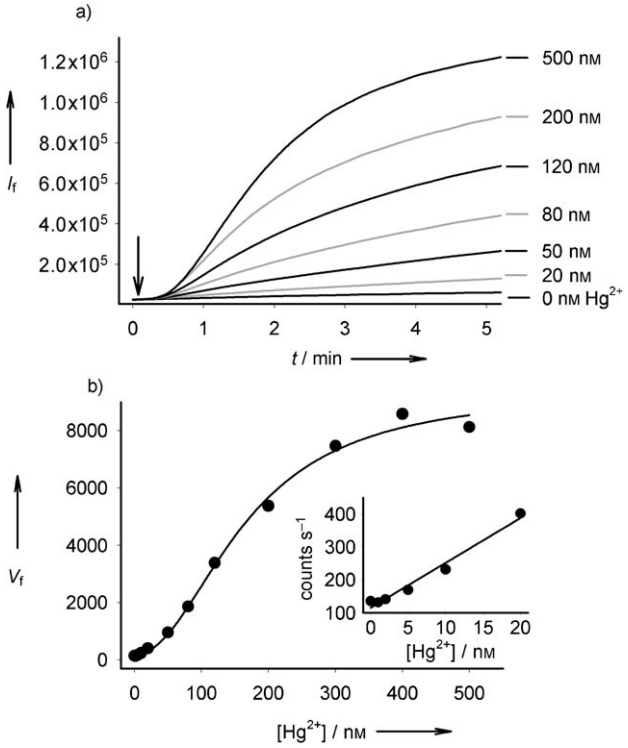

Figure 3. Sensitivity of the Hg^{2+} ion sensor. a) Kinetics of the fluorescence increase in the presence of varying concentrations of Hg^{2+} ions. b) Hg^{2+}-ion-dependent fluorescence increase rate. Rates were calculated in the time window of 1–2 min from (a). Inset: sensor responses at low Hg^{2+} ion concentrations. The *y* axis is the fluorescence counts increase per second. The DNAzyme and UO_2^{2+} ion concentrations were 100 nM and 1 μM, respectively.

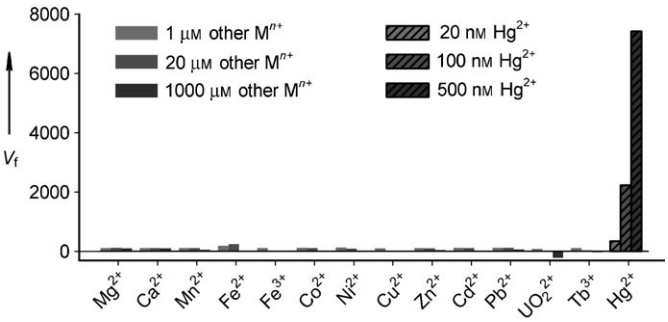

Figure 4. Selectivity of the Hg^{2+}-ion sensor. All competing metal ions were tested at 1, 20, and 1000 μM. For comparison, sensor responses to 20, 100, and 500 nM of Hg^{2+} ions were also presented. The DNAzyme and UO_2^{2+} ion concentrations were 100 nM and 1 μM, respectively.

Each metal was tested at three concentrations (1, 20, and 1000 μM). None of the metal ions gave responses higher than half of that produced by 20 nM Hg^{2+} ions, and the selectivity was determined to be at least 100000-fold higher for Hg^{2+} ions over any other metal ions (10 nM Hg^{2+} versus 1 mM competing metal ions).

In summary, we rationally designed a highly sensitive and selective catalytic beacon for mercury based on a uranium-

specific DNAzyme. Hg^{2+} ions enhanced the DNAzyme activity through allosteric interactions, and a series of allosteric DNAzymes with a varying number of thymine–thymine mismatches were tested. The optimal DNAzyme was labeled with fluorophores and quenchers to construct a catalytic beacon. The sensor has a detection limit of 2.4 nM, which is lower than the EPA limit of Hg^{2+} ions in drinking water. It is also highly selective and is silent to any other metal ions with up to millimolar concentration levels. The catalytic-beacon performance may be further improved by the incorporation of in vitro selections to optimize the allosteric interactions.[33] This work further demonstrated that DNAzymes are a great platform for metal sensing.

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