Chapter 5

DNAzyme-Based Sensing for Metal Ions in Ocean Platform

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Abstract

The ocean contains a number of metal ions that are either beneficial or detrimental to marine lives or ecology. Developing sensors for on-site and real-time detection of these metal ions plays an important role in our understanding the ocean as well as its protection. DNAzymes, DNA molecules with enzymatic functions, have emerged as a new class of sensing molecules for metal ions, because DNAzymes with high affinity and specificity for almost any metal ions at a specific oxidation state can be obtained through in vitro selection. By integrating the DNAzyme with different signal transduction molecules, such as fluorophores or nanoparticles, DNAzyme-based sensors for a broad range of metal ions with high sensitivity (with limit of detection down to ppt) and selectivity (with over a million fold) have been reported. In this chapter, we summarize recent progress in DNAzyme-based sensors for metal ions and describe detailed protocols in designing fluorescent and colorimetric sensors for uranium and mercury. The diverse range of metal ions it can detect as well as its excellent sensing properties makes DNAzyme an excellent choice for ocean sensing.

Key words: DNAzyme, Sensing, Seawater, Uranium, Mercury, Colorimetric sensor, Fluorescent sensor, Fluorophores, Nanoparticles, Ocean

1. Introduction

The ocean contains many metal ions, such as copper, molybdenum, and vanadium, that are important for marine organisms and plants (1). At the same time, heavy metal ion contaminations from sewage run-offs and air pollution (such as mercury from power plants) are becoming an increasingly serious problem in ocean. Monitoring these metal ions in seawater is very important to protect the ocean from environmental contamination which can threaten the safety of the ecosystem including human being. The recent damage of Fukushima Daiichi Nuclear Power Plant due to tsunami that hit Japan on March 11, 2011 and the resulting release of several toxic radioactive metals into the ocean highlights the importance of

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developing effective metal sensors for on-site and real-time detection. However, accurate and fast detection of metals in seawater is quite challenging because of their low concentration (nM to pM range) and high interferences from the competing species (2). Thus, seawater generally has to go through several processes, including preconcentration and separation, before complicated instrumental analysis can be performed (3, 4). Furthermore, most detection methods rely on expensive and sophisticated instruments, such as inductively coupled plasma mass spectrometry and atomic absorption spectrometry, which are not suitable for on-site real-time monitoring (2, 5, 6).

DNA has been regarded as a passive molecule, containing genetic information of a living organism. However, in 1994, the existence of short DNA strands with active catalytic functions has been reported and they were called catalytic DNA, deoxyribozyme, or DNAzyme (7). These DNAzymes perform catalytic function, such as cleavage of the complementary substrate strand, in the presence of metal ion cofactors. Since this reaction occurs in response to certain metal ion cofactors, DNAzymes can be used as a universal platform for their detection. Furthermore, because these DNAzymes can be obtained through in vitro selection from a large DNA library in the presence of metal cofactors (7), DNAzymes specific for a variety of metal ions, including Pb^{2+} (7, 8), Cu^{2+} (9–11), Zn^{2+} (12), Co^{2+} (13, 14), Mn^{2+} (15), Hg^{2+} (16), and UO_2^{2+} (17), have been selected. Recognizing this new capability, we and others have converted these DNAzymes as a new class of metal ion sensors (18–32). In this chapter, we summarize recent advances in and provide detailed protocols for DNAzyme-based fluorescent and colorimetric sensors for detection of metal ions, using uranium and mercury sensing as examples.

2. DNAzyme-Based Metal Detection

2.1. Uranium-Specific DNAzyme

Uranium is one of the radioactive metals which exist ubiquitously in our environment, and it is also a core component of nuclear energy generation and nuclear weapon. Therefore, precise monitoring of uranium in our environment is highly demanded to protect human health and our environment.

Recently, our group has used in vitro selection to obtain a DNAzyme with high specificity for uranyl (UO_2^{2+}), which is the most stable form of uranium in water (see Fig. 1) (17). UO_2^{2+}-specific DNAzyme is composed of two strands of DNA: a substrate strand (39S) and an enzyme strand (39E). The DNAzyme remains hybridized in the absence of UO_2^{2+}. However, the substrate strand which contains hydrolytically susceptible ribo-adenosine (rA) is

Fig. 1. The secondary structure of an UO_2^{2+}-specific DNAzyme. Reproduced by permission of National Academy of Sciences of the United States of America (17).

cleaved by the enzyme strand in the presence of UO_2^{2+} and breaks into two fragments. Uranyl-specific cleavage is a highly efficient multiple turnover reaction, with dissociation constant of 469 nM for uranyl, making it an ideal choice as UO_2^{2+} sensor (33).

2.2. Fluorescence-Based Sensors

In order to design a sensor, at least two components are needed: target recognition and signal transduction. The high sensitivity and selectivity of DNAzyme in response to specific analytes allow them to serve as an ideal target recognition element for sensing. However, we also need a part which transforms the recognition event into detectable signal.

Since DNA can be chemically modified with a variety of organic fluorescent dyes of choice, DNAzymes have been converted into fluorescent sensors (8, 24). Recently, our group developed uranyl-specific fluorescent sensor by introducing a fluorophore on the substrate strand of the DNAzyme and a quencher on its enzyme strand (see Fig. 2a) (17). With this simple design, the catalytic activity of the DNAzyme remains intact and the fluorescent uranyl sensor with a detection limit of 11 parts per trillion (45 pM) and selectivity of more than one million fold over other metal ions has been observed. This sensor rivals one of most sensitive analytical instruments for uranium detection.

This strategy can also be applied to detection of other metal ions, such as mercury. Hg^{2+} can bind specifically to two DNA thymine (T) mismatches and form stable thymine–Hg^{2+}–thymine complex. We introduced several thymine mismatches to the catalytic site of the uranyl DNAzyme, which is only activated in the presence of Hg^{2+} (See Fig. 2b). By attaching fluorophore/quencher pairs to the DNAzyme complex, the DNAzyme could function as highly sensitive and selective Hg^{2+} sensor in aqueous solution with detection limit of 2.4 nM and no interference from competing metal ions (34).

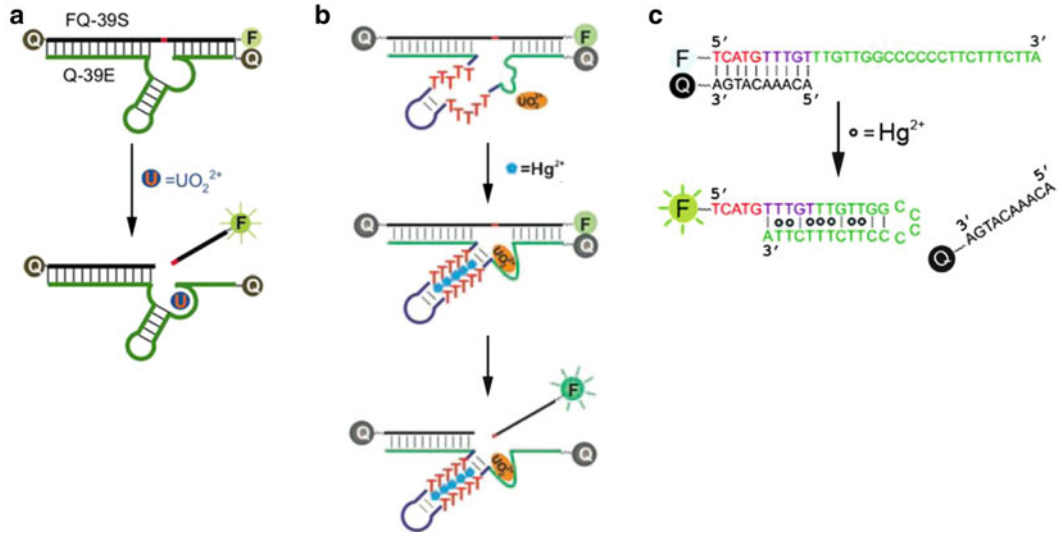

Fig. 2. DNAzyme-based fluorescent metal ion sensors: (**a**) uranyl sensor, (**b**, **c**) mercury sensors. Sensor (**b**) needs uranyl for detection of mercury while sensor (**c**) does not. Reproduced by permission of National Academy of Sciences of the United States of America (17), Wiley-VCH Verlag GmbH & Co. KGaA (34) and the Royal Society of Chemistry (35).

While this modified uranyl DNAzyme sensor shows excellent performance for detection of Hg^{2+}, it functions only in the presence of micromolar concentration of UO_2^{2+}. A fluorescence sensor which is also based on the thymine (T) mismatches but does not need UO_2^{2+} has also been developed from our group (see Fig. 2c) (35). This "turn-on" sensor shows excellent performance in water with 3.2 nM (0.6 ppb) detection limit and no response from other metal ions. The practical application of this sensor has also been demonstrated for detection of mercury in pond water. These few examples suggest that DNA can be an ideal tool for detection of broad range of metal ions in seawater.

2.3. Interaction Between Gold Nanoparticles and DNA: The Labeled and Label-Free Systems

Even though fluorophores are commonly used as signal reporter molecules for the development of high performance sensors, the requirement of analytical instruments such as fluorimeters makes them less convenient for on-site and remote monitoring. On the other hand, noble metal nanoparticles such as gold nanoparticles (AuNPs) have several unique optical properties (36). For instance, AuNPs have extremely high extinction coefficients; so the color of a few nanomolar AuNP can be distinguished with naked eye without the need of any instrument. In addition, the extinction wavelength of AuNPs can be significantly altered by changing the distances between the nanoparticles. For example, 13 nm AuNPs have reddish color in dispersed phase while their color changes bluish after aggregation. These interesting optical properties of AuNPs make them an ideal reporter for colorimetric sensing.

While DNA has the capability to identify a broad range of target molecules, they can also interact with AuNPs in a certain way and control their aggregation and disassembly status. This essential role of DNA will construct direct correlation between the presence of analyte and the color change of the sensor. The DNA-based AuNP colorimetric sensors can be designed in either labeled or label-free methods based on the way DNA interacts with AuNPs (26, 27, 37).

2.3.1. Labeled U Colorimetric Sensing

In the labeled method, AuNPs are chemically functionalized with DNA and their aggregation and disassembly status is controlled by the hybridization of the DNA (37, 38). Two batches of AuNP–DNA are first prepared by conjugating AuNPs with two different DNA arm strands (Arm 5′ and 3′) through Au-thiol chemistry. When both batches of AuNP–DNA are mixed together, they stay dispersed with reddish color due to the strong negative charge of the DNA. In the presence of the bridging DNA, however, which is complementary to both arm strands, the AuNPs aggregate and the color of the AuNP becomes blue because the distance between the AuNPs becomes close (see Note 1).

Uranyl colorimetric sensor uses uranyl-specific DNAzyme complex (39S and 39E) as the bridging DNA to crosslink AuNPs (see Fig. 3) (26). While the AuNPs of the sensor stay assembled in the absence of uranyl, they start to disassemble in its presence due to the cleavage of the substrate strand (39S). The disassembly of the AuNPs can be significantly accelerated by introducing two invasive DNA strands which are complementary to both ends of the substrate strands (39). The addition of this invasive DNA can help to shorten the detection time.

Figure 4 summarizes various properties of the optimal-labeled uranyl sensor. As shown in Fig. 4a, the blue shift of the plasmonic resonance of the AuNPs could be observed by UV-vis in the presence of uranyl within 30 min and the detection limit was found to be 50 nM. This is lower than the maximum contamination level of uranium (130 nM) defined by the US Environmental Protection Agency (EPA). Furthermore, no color change of the sensor occurred on competing metal ions, which indicates that the sensor only responses to uranyl and not to other metal ions (See Fig. 4b).

2.3.2. Label-Free U Colorimetric Sensing

The label-free method is based on the different adsorption properties of single-stranded (ss) DNA and double-stranded (ds) DNA on the surface of citrate modified AuNP (27, 40). Although citrate-capped AuNPs are negatively charged, they are naturally unstable in the presence of NaCl and can be easily aggregated. Because ssDNA is flexible and can uncoil its structure, positively charged bases of the ssDNA can be easily exposed and adsorbed on negatively charged AuNP surface, resulting in enhanced stability of AuNPs even in the presence of NaCl. On the other hand, because dsDNA is relatively rigid and surrounded by negatively charged phosphate backbone, its binding with AuNP is negligible.

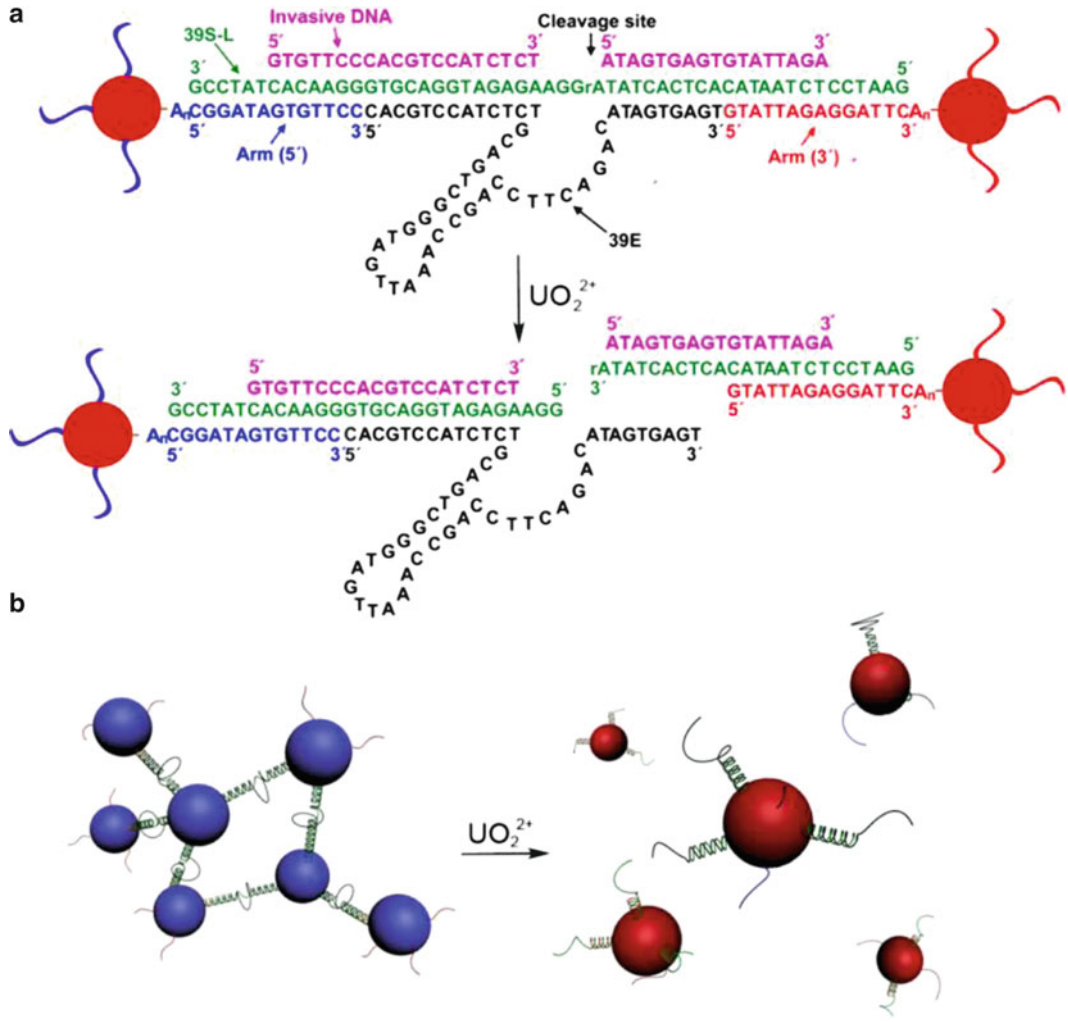

Fig. 3. Scheme of labeled colorimetric UO_2^{2+} sensor: (**a**) Labeled sensor need chemical modification between AuNP and DNA. (**b**) The blue-colored AuNP aggregates disassemble in the presence of UO_2^{2+}, resulting in color change of the sensor. Reproduced with permission of ref. 26, copyright of 2008 American Chemical Society.

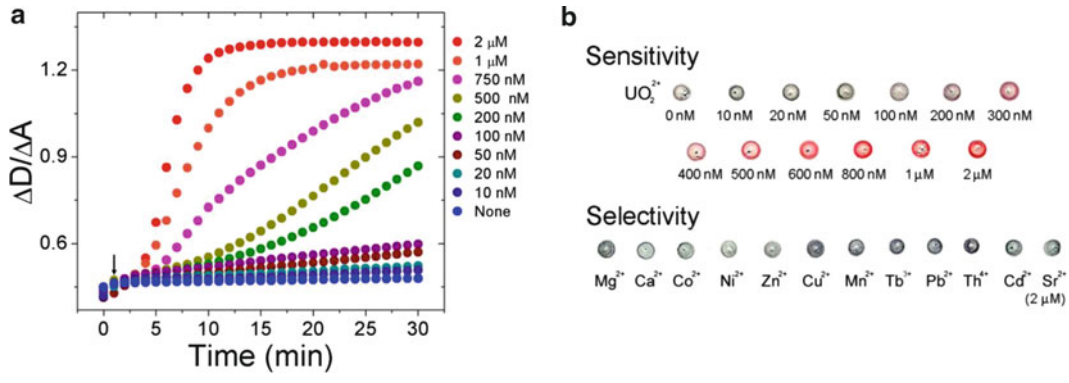

Fig. 4. Properties of labeled uranyl colorimetric sensor: (**a**) Variation of AuNP disassembly kinetics as a function of uranyl concentration (**b**) Image of the color of labeled sensor in response to different concentrations of UO_2^{2+} and other metal ions. Reproduced with permission of ref. 26, copyright of 2008 American Chemical Society.

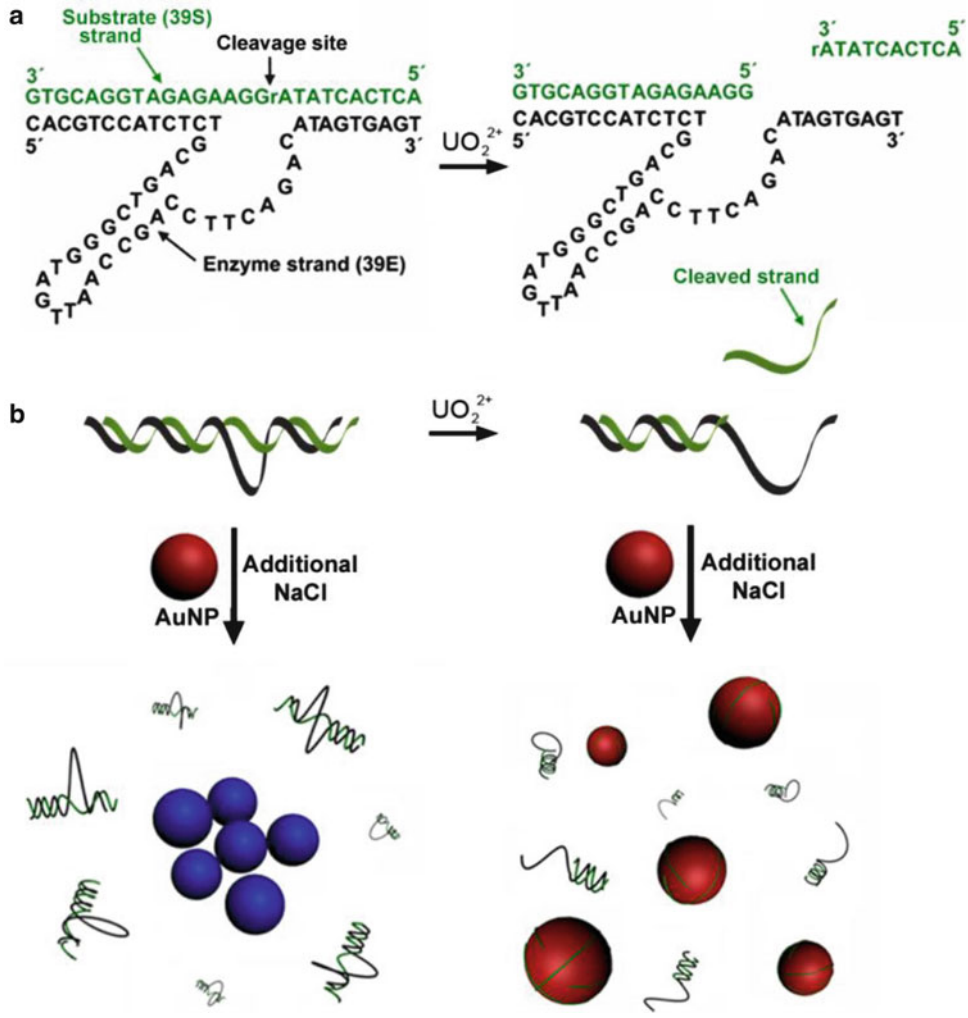

Fig. 5. Scheme of the label-free colorimetric uranyl sensor: (**a**) Label-free sensor does not need chemical modification between DNA and AuNPs. (**b**) The sensor is based on different adsorption properties of ssDNA and dsDNA on citrate modified AuNP. The amount of ssDNA is determined by the concentration of uranyl. Reproduced with permission of ref. 26, copyright of 2008 American Chemical Society.

Therefore, NaCl induced aggregation of AuNPs will occur in the existence of dsDNA.

The label-free uranyl sensor can be prepared by, first hybridizing unmodified 39E and 39S strands to form DNAzyme complex (see Fig. 5). The DNAzyme complex is then treated with uranyl for 6 min, resulting in the release of the short 10-mer DNA strand from the substrate that can interact with AuNPs. The uranyl-dependent cleavage reaction can be quenched by quickly mixing concentrated TRIS base solution with the DNAzyme solution, which results in a shift of the pH from 5.5 to 8. Since UO_2^{2+} DNAzyme is most active at pH 5.5 but nonactive at pH 8 (33), the

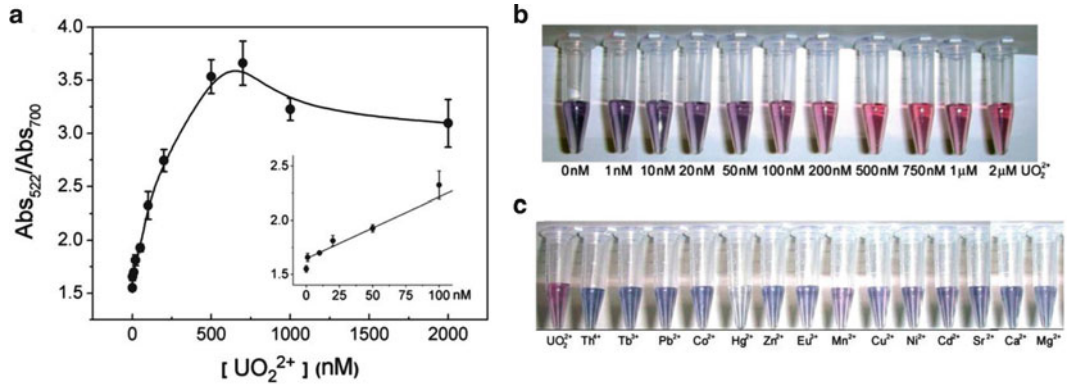

Fig. 6. Properties of label-free uranyl colorimetric sensor: (**a**) Variation of the aggregation status of the label-free sensor at different concentration of uranyl. (**b**) Color change of label-free sensor in response to different amount of uranyl. (**c**) Image of the color of label-free sensor treated with competing metal ions. Reproduced with permission of ref. 26, copyright of 2008 American Chemical Society.

cleavage reaction is quenched after the addition of TRIS. Once 13 nm AuNPs are added to the solution containing DNA, the color of the AuNP will change in correlation to the amount of uranyl in the solution.

The color change of the AuNPs treated with various uranyl concentrations has been monitored by comparing the extinction ratios between 522 and 700 nm by UV-vis (Fig. 6). The sensitivity of sensor was found to be as low as 1 nM, which is even lower than that of the labeled sensor. The color change can also be easily observed by naked eye. No color change of the sensor was observed when it was treated with other metal ions, indicating that the label-free sensor has excellent selectivity over competing metal ions.

2.3.3. Experimental Protocols of U Sensors

Fluorescence Sensor

In order to form DNAzyme complex, 60 nM 39S with both fluorescein and quencher labeled on each end (FQ-39S) and 60 nM quencher modified 39E (Q-39E) were used. The two strands were annealed in a buffer containing 300 mM $NaNO_3$ and 50 mM MES (pH 5.5) by heating the solution up to 70°C and cooling down to room temperature in 1 h (see Fig. 2a).

The detection can be accomplished using a fluorimeter. Five hundred microliters of the prepared sensor solution was transferred to a quartz cuvette with a 0.5 cm path length. The cuvette was placed in a fluorimeter (FluoroMax-P; Horiba Jobin Yvon, Edison, NJ). The excitation and emission wavelengths were set at 490 and 520 nm, respectively. After the initial measurement, a small volume of concentrated metal solution was added to initiate the cleavage reaction.

Labeled Colorimetric Sensor

Thirteen nanometer AuNPs were synthesized by reducing $HAuCl_4$ with sodium citrate based on previous literature (see Note 2). AuNP–

DNA conjugates were formed by reacting thiol modified DNA with AuNPs. First, thiol modification placed on Arm 5′ strand was activated by mixing 9 μL of 1 mM Arm 5′ strand, 1 μL of 500 mM pH 5.5 MES buffer, and 1.5 μL of 10 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP) solution in a microcentrifuge tube and keeping them for an hour. A parallel procedure was performed with Arm (3′) strand to activate thiol modification on Arm (3′) strand.

In order to react Arm (5′) strand with AuNPs, 3 mL of 13 nm AuNP solution was mixed with 9 μL Arm (5′) strand in a scintillation vial and left overnight. The process was repeated with Arm (3′) strand to react the Arm (3′) strand with AuNPs. The next day, 300 μL of 1 M NaCl and 15 μL of 500 mM TRIS acetate buffer (pH 7.6) were added to each scintillation vial and were stored for another day.

To prepare the labeled sensor, AuNPs functionalized with Arm strands were purified to remove excess DNA. Both 500 μL of Arm (5′) functionalized AuNPs and Arm (3′) functionalized AuNPs were, respectively, placed in 1.5 mL microcentrifuge tube and were centrifuged at 16,110 × *g* for 15 min. The supernatant was replaced with 500 μL of 100 mM NaCl, 50 mM MES (pH 5.5) solution. After additional purification process, the supernatant was replaced with 250 μL of 300 mM NaCl, 50 mM MES (pH 5.5) buffer. In order to form AuNP aggregates, both AuNP–DNA solutions were mixed with 10 μL of 10 μM elongated substrate strand (39S-L), and 20 μL of 10 μM enzyme strand (39E) and the combined solution was heated up to 55 °C and cooled down to room temperature for about 1 h. DNA directed AuNP aggregation should happen, which can be notified by the color change of AuNP solution. The microcentrifuge tube containing the AuNP aggregates should be centrifuged for about 1 min followed by supernatant replacement with 120 μL of 300 mM NaCl, 50 mM MES (pH 5.5) solution to remove excess DNA (39S-L and 39E) in the solution (see Note 3).

Before detection of uranyl, a pair of invasive DNA strands were added to the sensor solution to expedite sensing process. Three hundred and eighty-one microliters of 50 mM MES buffer (pH 5.5), 4.5 μL of 0.2 mM Inva (5), and 4.5 μL of 0.2 mM Inva (3) were mixed with 60 μL sensor solution. Uranyl was added 1 min after the addition of invasive DNA strands and the detection typically took 30 min. Color change can be monitored by either UV-vis or naked eye.

Label-Free Colorimetric Sensor

In order to form DNAzyme complex, 4 μL of 100 μM 39S strand were mixed with identical amount of 39E strand in 70 μL 300 mM NaCl, 10 mM MES buffer (pH 5.5) in a microcentrifuge tube and mixture was annealed by heating it up to 80 °C and cooling it down to room temperature (see Note 4). For the reaction with uranyl, 77 mL of the DNAzyme complex were transferred to a new

tube and treated with uranyl for 6 min. To quench UO_2^{2+}-induced cleavage reaction, 1.19 μL of 500 mM TRIS base solution and 56 μL Millipore water were added to the tube containing DNAzyme and uranyl with quick vortexing. In order to observe color change of the sensor, 76 μL of 10 nM AuNPs were added to the tube containing DNAzyme and uranyl. The color change of the solution, which can be monitored by either UV-vis or naked eye, will be determined by the amount of the uranyl in the solution.

2.3.4. Comparison of the Labeled and Label-Free Colorimetric Sensors

DNA–AuNP-based colorimetric sensors have recently received significant attention because the detection can be made for a broad range of analytes at a low cost without the need of any complicated instrument. But it is noteworthy to emphasize that in most cases, DNA–AuNP-based sensors are operated by either labeled or label-free method.

Although both methods take advantage of the altered optical property of the AuNP induced by the introduction of the analytes, the difference in the basic principle of both sensors make their general properties significantly different. Therefore, it is worthwhile to compare the two sensing systems.

In terms of sensor performance, the label-free sensor is much more sensitive than the labeled sensor. Because the labeled sensor relies on uranyl dependent disassembly of AuNPs from aggregated states, certain amount of uranyl concentration and reaction time is necessary for the detection to be recognized. These factors result in relatively lower detection limit (50 nM) and slower kinetics (30 min) of the labeled sensor. On the other hand, because the label-free sensor allows UO_2^{2+}-induced cleavage reaction to happen in the absence of AuNPs and AuNPs are added to the DNA solution afterwards, just to monitor the amount of the released short DNA strands, the reaction can be completed much quickly (6 min) with high sensitivity (with 1 nM detection limit).

In terms of sensor preparation and handling, both sensors have distinct properties as well. The labeled sensor requires more time and effort to be prepared as DNA–AuNP conjugation and AuNP aggregation processes are needed. However, once the labeled sensors are prepared, it could be more convenient to be used because UO_2^{2+} detection can be completed in a single step, by adding it to the sensor solution. On the other hand, although the label-free sensor can be relatively easily prepared, multiple handling steps such as UO_2^{2+} treatment, quenching, and AuNP addition, are required, for detection.

Finally, for practical applications, the labeled sensor has higher flexibility, due to its high stability of AuNP–DNA conjugation and simpler handling process. In contrast, the label-free sensor is normally restricted by the narrow operational condition, such as ionic strength, and multiple handling steps needed for detection.

3. Application of DNAzyme for Ocean Sensing

Because the DNAzyme has been demonstrated to target a broad range of metal ions with excellent sensitivity and selectivity in buffer solution, it holds great promise to serve as an ideal platform for detection of metals in ocean. In comparison with buffer solutions, the ocean water contains much higher level of various metal ions, including Na^+, Mg^{2+}, Ca^{2+}, and K^+. Although these metal ions might not affect the selectivity of a DNAzyme, they can still have considerable effect on its property. Therefore, the DNAzyme-based sensor will have to be optimized so that it can have maximal performance in the ocean. To obtain a DNAzyme with the best performance in the seawater, it would also be possible to carry out in vitro selection to obtain new DNAzymes directly in seawater.

While it is likely that fluorescence-based sensors could be adapted for ocean sensing without much alteration on the sensor designs, colorimetric sensors may have to be reengineered to adapt the high salt environment in seawater due to the vulnerability of AuNPs to salt. Among the two colorimetric sensors, the labeled sensor has advantages on ocean sensing application as DNA conjugated AuNPs are more resistant to salt-induced aggregation. On the other hand, since the label-free sensor uses naturally unstable citrate-capped AuNPs, the samples have to be diluted to appropriate concentration before optimal sensing performance could be obtained. In both cases, the sensors need to be recalibrated in ocean water with a standard addition method.

Although the DNAzyme sensors can be used for detection of metal ions in solution phase, it can also be integrated into various devices, such as nanocapillary array membranes, microfluidic devices, and dip sticks (41–44). The possibility of adapting DNAzymes onto diverse devices gives us the opportunity to expand the capability of DNAzyme for ocean sensing in other detection platforms.

4. Notes

1. Because the AuNPs are linked by DNA base-pairing interactions, the general property of the labeled sensor is highly dependent on the NaCl concentration.
2. In order to prevent AuNPs from sticking on the glass surface, the scintillation vials were first incubated in 10 M NaOH solution for an hour, and then rinsed with distilled water and subsequently with Millipore water copious times.

3. Even though DNA–AuNP conjugates stored at room temperatures for months can still form aggregates, the degradation of DNA can cause change of the sensing property and performance. To obtain consistent results, it is desirable to use freshly prepared conjugates for sensor preparation.
4. It should be emphasized that exact same concentration of 39E and 39S strands should be used for the hybridization of DNAzyme complex to minimize the background signal.

5. Future Directions

In this study, we have discussed the possibility of using DNAzyme based sensors for ocean sensing, using UO_2^{2+} and Hg^{2+} as examples. Although detection of both UO_2^{2+} and Hg^{2+} is meaningful, there are a number of other metal ions that can be important targets. For example, as a result of the tragic nuclear plant meltdown in Fukushima, Japan, several radioactive metals, such as cesium, plutonium, and strontium, have been released to the ocean that are highly harmful to not only human beings, but also the whole ecosystem (45, 46). Therefore, the development of facile method for detecting these metals becomes crucial. Furthermore, the ocean contains numerous metals that can be important resources. For example, seawater contains 250 billion tons of lithium which exceeds the amount of lithium stored on earth (47). Considering the highly increasing demand of these metals, it will be very useful to detect these metals with high sensitivity in the ocean. The DNAzyme can serve as one of the best candidates for these purposes as DNAzymes specific for almost any target metal ion can be obtained via the in vitro selection process. Furthermore, as the selectivity of the DNAzyme originates from a specific sequence, and each DNAzyme can be labeled with a different reporter group, it is possible use different DNAzymes in one system for multiplex sensing of various metal ions in the ocean.

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