



Reusable evanescent wave DNA biosensor for rapid, highly sensitive, and selective detection of mercury ions

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ABSTRACT

Mercury ions (Hg^{2+}) are a highly toxic and ubiquitous pollutants requiring rapid and sensitive on-site detection methods in the environment and foods. Herein, we report an evanescent wave DNA-based biosensor for rapid and very sensitive Hg^{2+} detection based on a direct structure-competitive detection mode. In this system, a DNA probe covalently immobilized onto a fiber optic sensor contains a short common oligonucleotide sequences that can hybridize with a fluorescently labeled complementary DNA. The DNA probe also comprises a sequence of T–T mismatch pairs that binds with Hg^{2+} to form a T– Hg^{2+} –T complex by folding of the DNA segments into a hairpin structure. With a structure-competitive mode, a higher concentration of Hg^{2+} leads to less fluorescence-labeled cDNA bound to the sensor surface and thus to lower fluorescence signal. The total analysis time for a single sample, including the measurement and surface regeneration, was under 6 min with a Hg^{2+} detection limit of 2.1 nM. The high specificity of the sensor was demonstrated by evaluating its response to a number of potentially interfering metal ions. The sensor's surface can be regenerated with a 0.5% SDS solution (pH 1.9) over 100 times with no significant deterioration of performance. This platform is potentially applicable to detect other heavy metal ions or small-molecule analytes for which DNA/aptamers can be used as specific sensing probes.

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1. Introduction

Mercury ions (Hg^{2+}), a highly toxic and ubiquitous pollutant, can cause a number of severe health problems, such as brain damage, kidney failure, mitosis impairment, and nervous system defects (Sekowski et al., 1997; WHO, 1991; Clarkson and Magos, 2006; Nolan and Lippard, 2008). Human exposure to Hg^{2+} stems from natural waters contaminated by industrial sources, including coal and gold mining, solid waste incineration, wood pulping, fossil fuel combustion, and chemical manufacturing (Chu and Porcella, 1995; US EPA, 2001; Rytuba, 2003; Nolan and Lippard, 2008). U.S. Environmental Protection Agency (EPA) has mandated a drinking water upper limit of 2 ppb (10 nM) for Hg^{2+} to protect the public (US EPA, 2001).

Traditional analytical methods for Hg^{2+} monitoring in water include inductively coupled plasma mass spectrometry (ICP-MS) (Fong et al., 2007), atomic absorption spectroscopy (Guo et al., 1996), and cold vapor atomic fluorescence spectrometry (Bloom and Fitzgerald, 1988; US EPA, 1999). These methods, although accurate and with low detection limits, are labor-intensive and require

expensive and sophisticated instrumentation, as well as complicated and multistep sample preparation, which prohibit on-line and real-time monitoring. Therefore, much effort has been devoted to cost-effective, rapid, and on-site detection of Hg^{2+} , including colorimetric, electrochemical, and optical sensors employing small fluorescent organic molecules (Caballero et al., 2005; Ha-Thi et al., 2007; Nolan and Lippard, 2007, 2008), functional polymers (Liu et al., 2007), proteins (Matsushita et al., 2005; Wegner et al., 2007), oligonucleotides (Ono and Togashi, 2004; Chiang et al., 2008), foldamers (Zhao and Zhong, 2006), and metal nanoparticles (Lee et al., 2007; Xue et al., 2008; Wang et al., 2008) as sensing elements.

Recently, oligodeoxyribonucleotide-based sensors have been designed based on the ability of Hg^{2+} (but not of other metal ions) to bind to thymine–thymine (T–T) mispairs in DNA to form T– Hg^{2+} –T complexes. (Ono and Togashi, 2004; Chiang et al., 2008; Wang et al., 2008; Xue et al., 2008; Han et al., 2009; Xu et al., 2009; Zhu et al., 2009; Wang et al., 2010). However, challenges still remain in obtaining high sensitivity and selectivity, as well as in how to simplify complicated design and operation for a rapid and on-site detection of Hg^{2+} (Wang et al., 2008). Herein, we report a novel and highly sensitive evanescent wave optical biosensor for the detection of Hg^{2+} in aqueous solution based on this cation's specific interactions with T bases of DNA to form T– Hg^{2+} –T complexes.

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The biosensor's sensing time, sensitivity, specificity, and reusability have been validated.

2. Experimental methods

2.1. Chemicals and reagents

All HPLC-grade oligonucleotides (DNA) were purchased from Eurofins MWG Operon (Huntsville, AL). The aminated DNA probe sequence was 5'-NH₂-(CH₂)₆-ATGTTTGTGTTGGCCCCCTTCTTTCTTA-3', that of the fluorescently labeled complementary DNA sequence 5'-Cy5.5-ACAAACAT-3', and that of the non-complementary DNA sequence (used for control) 5'-Cy5.5-TCCCGAGA-3'.

Acetone, 2-(*N*-morpholino)ethanesulfonic acid (MES), bovine serum albumin (BSA), 3-aminopropyltriethoxysilane (APTS), and glutaraldehyde (GA) were purchased from Sigma–Aldrich (St. Louis, MO). All other reagents, unless otherwise specified, were purchased from the Fisher Scientific (Pittsburgh, PA). A DNA probe stock solution was prepared with a 10 mM phosphate buffered solution (PBS, pH 7.4). Complementary and non-complementary DNA oligonucleotides were dissolved in a 10 mM MES buffer (pH 7.0) and kept frozen at -20 °C for storage. A 1 mM Hg²⁺ stock solution was prepared in ultrapure water and stored at 4 °C; its standard concentrations were prepared from the stock solution by serial dilutions in 10 mM MES buffer (pH 7.0). All other solutions, including 200 μM bivalent metal salts, were prepared with a Millipore Milli-Q system ultrapure water.

2.2. Instrumentation: evanescent wave all-fiber bisensing platform

The portable evanescent wave all-fiber biosensing platform was as previously described (Long et al., 2009, 2011). Briefly, the laser beam from a 635-nm pulse diode laser with pigtail was directly launched into a single-mode fiber of a single multi-mode fiber coupler. The laser light then entered the multi-mode fiber with a diameter of 600 μm and numerical aperture of 0.22 from the single-mode fiber. The excitation light from the laser, through the fiber connector, was coupled to a fiber probe. The incident light propagated along the length of the probe via total internal reflection. The evanescent wave generated at the surface of the probe then interacted with the surface-bound fluorescently labeled analyte complexes and caused excitation of the fluorophores. The collected fluorescence was filtered by means of a bandpass filter and detected by photodiodes through a lock-in detection. The probe was embedded in a glass flow cell with a flow channel having a nominal dimension of 60 mm in length and 2 mm in diameter. All reagents were delivered by a flow delivery system operated with a peristaltic pump. The controls of fluid delivery system and data acquisition and processing were automatically performed by the built-in computer.

2.3. Immobilization of DNA probes onto fiber optic sensor surface

Details of the fabrication and preparation of the combination tapered fiber optical sensor were described previously (Long et al., 2008). The immobilization of DNA probes onto its surface was performed as follows. The sensor was first cleaned with a piranha solution (H₂SO₄/H₂O₂, 3:1 (v/v)), then aminated by immersion in a 2% (v/v) APTS acetone solution for 60 min, followed by an acetone wash (three times), an ultrapure water wash, and drying in an oven for 30 min at 110 °C. To immobilize a DNA probe, the aminated sensor was immersed in a 5.0% (v/v) GA solution for 1 h at 37 °C, washed with water, and then immersed in 1 mL of a 0.5 μM DNA probe in PBS (pH 7.4) solution overnight at 4 °C. The sensor

surface was then dipped in a 2 mg/mL BSA solution for 1 h to block the remaining aldehyde sites.

2.4. Mercury ion analysis

Water samples containing different concentrations of Hg²⁺ were mixed with Cy5.5-cDNA at a fixed concentration. The mixture was pumped into a flow cell at a rate of 300 μL/min for 30 s and allowed to bind to the DNA probe for 4 min. Meanwhile, the fluorescence signal was collected. The sensing surface was then regenerated with a 0.5% SDS solution (pH 1.9) for 60 s and washed with a PBS solution.

To evaluate potential environmental sample matrix effects on Hg²⁺ detection, spiked samples of tap water, of commercially available bottled water, and of tertiary effluent from a wastewater treatment plant (Pinery, Colorado, USA) were tested at concentrations of 20, 100, and 200 nM. To assess the specificity of the sensor, its responses to such potentially interfering metal ions as Ca²⁺, Zn²⁺, Fe²⁺, Cu²⁺, Fe³⁺, Sn²⁺, and Pb²⁺ at concentrations up to 20 μM were evaluated.

3. Results and discussion

The sensor scheme employed herein is based on a portable evanescent wave biosensing platform in which both the transmission of the excitation light and the collection and transmission of fluorescence are achieved through a single multi-mode fiber optic coupler (Long et al., 2008). When light propagates through a fiber optic on the basis of total internal reflection (TIR), a thin electromagnetic field (the "evanescent wave") is generated, which decays exponentially with the distance from the interface with a typical penetration depth of up to several hundred nanometers (Andrade et al., 1985; Shriver-Lake et al., 1995; Marazuela and Moreno-Bondi, 2002). This evanescent wave can excite fluorescence in the proximity of the sensing surface, e.g., in fluorescently labeled DNA bound to the optical sensor surface. The short range of the evanescent wave allows it to discriminate between bound and unbound fluorescent compounds, hence eliminating the normally required washing steps. In this newly developed Hg²⁺ sensing system, DNA probes that contain initial binding DNA sequences and a T–T mismatch structure for binding to Hg²⁺ was first immobilized onto fiber optical sensing surface (Fig. 1).

3.1. Immobilization of DNA onto sensor surface

Covalent immobilization of a DNA probe on an aminated fiber optic sensing surface was achieved using a glutaraldehyde coupling strategy (Fig. 1a). A six-carbon alkyl group was added as a spacer between the surface and the probe to reduce steric hindrances for DNA hybridization. To evaluate the immobilization results and to confirm that the observed fluorescence signal was from hybridization between cDNA and the complementary sequence of the immobilized DNA probe, we conducted two control experiments whose results are shown in Fig. 1b. First, 30 nM fluorescently labeled non-complementary DNA was delivered over the sensing surface; no fluorescent signal was observed, thereby indicating no non-specific adsorption of DNA. In addition, a 10 nM Cy5.5-cDNA solution containing no salt was delivered to the sensor's surface; no fluorescence signal was detected, suggesting no hybridization. These results showed that the fluorescent signal was not obtained by either non-specific adsorption of Cy5.5-cDNA on the sensor surface or excitation of free Cy5.5-cDNA in solution. When 0.5 nM complementary DNA was introduced, however, a fluorescent signal was detected. As the concentration of cDNA was increased to 10 nM, a signal-to-noise ratio (the ratio of the maximum fluorescent signal to the base line) of over 20 was obtained (Fig. 1b). These

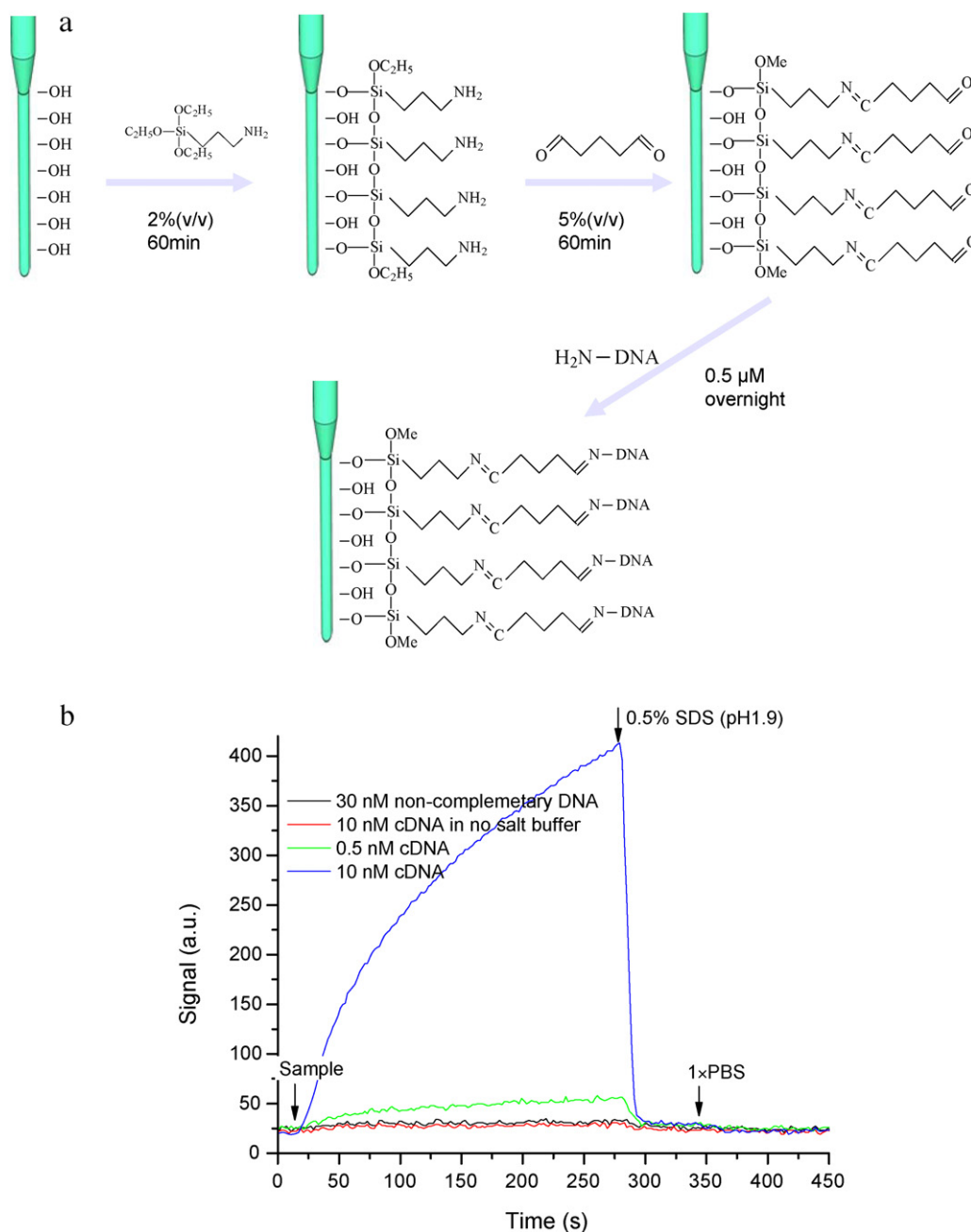


Fig. 1. Immobilization of DNA onto optical fiber sensor's surface. (a) Schematics of the procedures employed for DNA probe immobilization using glutaraldehyde covalent coupling; (b) Sensor's performance and responses to various controls.

results demonstrated a high sensitivity of the sensor for detecting specific DNA hybridization.

3.2. Structure-competitive sensing mechanism of Hg^{2+} detection

Fig. 2 illustrates the structure-competitive mode based sensing mechanism for Hg^{2+} detection based on the ability of T–T sequences in a DNA to form T– Hg^{2+} –T complexes in a specific manner. The DNA probe immobilized onto the fiber optical sensor surface contains a short sequence (8 bps, shown in purple and red in Fig. 2) at the 5' end of the DNA probe which is complementary to, and can therefore hybridize with, fluorescently (Cy5.5) labeled cDNA (shown in green in Fig. 2). The DNA probe also contains T–T mismatch pairs, which can bind with Hg^{2+} to form the T– Hg^{2+} –T complex by folding of the

DNA probe segments (shown in blue and red in Fig. 2) into a hairpin structure.

During one detection cycle, Hg^{2+} solutions of different concentrations and the fluorescently-labeled cDNA of a fixed concentration were mixed, and the mixture was added to the DNA probe on the sensor's surface. Because the fluorescence-labeled cDNA and Hg^{2+} competitively bind to the probe immobilized on the surface, the higher the concentration of Hg^{2+} , the less of cDNA binds with the DNA probe and, therefore, the lower the resultant fluorescence signal. Real-time monitoring of the fluorescence signal can be carried out as the binding reaction occurring between the Cy5.5-cDNA and the immobilized DNA probe, reflecting the reaction rates. The binding of the cDNA to the DNA probe led to a rise in fluorescence over time and eventually reached a plateau.

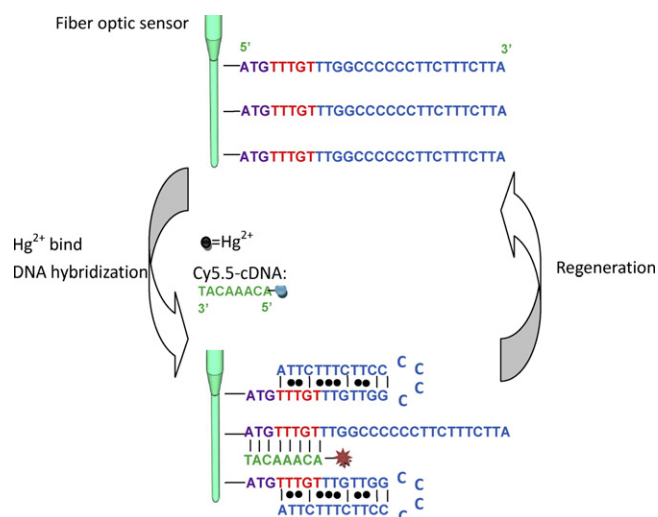


Fig. 2. Schematic representation of a structure-competitive sensing mechanism of Hg^{2+} detection.

A 4-min reaction time was chosen considering the signal intensity level required, as well as the goal of reducing the assay length.

Several “turn-off/on” fluorescent sensors based on T- Hg^{2+} -T structure have been developed for Hg^{2+} detection in aqueous solution (Ono and Togashi, 2004; Chiang et al., 2008; Wang et al., 2008). Because “turn-off” sensors are susceptible to interferences (e.g., decrease of fluorescence resulted from other quenchers or external environmental species), they can give “false positive” results; therefore, “turn-on” sensors are preferred (Wang et al., 2008). The latter, however, usually require longer reaction time (>1 h) for hybridization of fluorescence-labeled and quencher-labeled strand DNAs before detecting Hg^{2+} . Based on its direct structure-competitive detection mode, the portable optical biosensor presented here is simpler and can be extended to other analytes for which specific sensing probes are available. For example, numerous high-affinity and highly specific aptamers, which are the single-stranded DNA/RNA, have been selected against a number of target molecules including small organics, metal ions, proteins, and even cells (Rajendran and Ellington, 2008; Sefah et al., 2009). These aptamers can potentially be used as sensing probes for the detection of small molecule analytes and heavy metal ions through the detection mode presented herein.

3.3. Dose-response measurements of Hg^{2+}

Fig. 3a and b shows the temporal fluorescence signal during a typical test cycle for Hg^{2+} detection using the optical sensor scheme developed herein, including the introduction of a mixture of the fluorescence-labeled cDNA and Hg^{2+} , the competitive binding between cDNA/ Hg^{2+} and DNA probe, and sensor regeneration. One can see that the introduction of Hg^{2+} at different concentrations to the sensing interface induced proportional decreases in the fluorescence signal.

Fig. 3c shows the calibration curves (each being the average of three independent curves) for Hg^{2+} , which were normalized by expressing the signal of each standard point as the ratio to that of the blank sample containing no Hg^{2+} . The signal intensities were fitted to a 4-parameter logistic equation as follows:

$$y = \frac{A_1 - A_2}{1 + ([Ac]/[Ac_0])^p} + A_2 \quad (1)$$

where $[Ac]$ is the analyte concentration; A_1 , A_2 are the upper and lower asymptote (background signal) to the titration curve; $[Ac_0]$ is the analyte concentration at inflection; and p is the slope at the

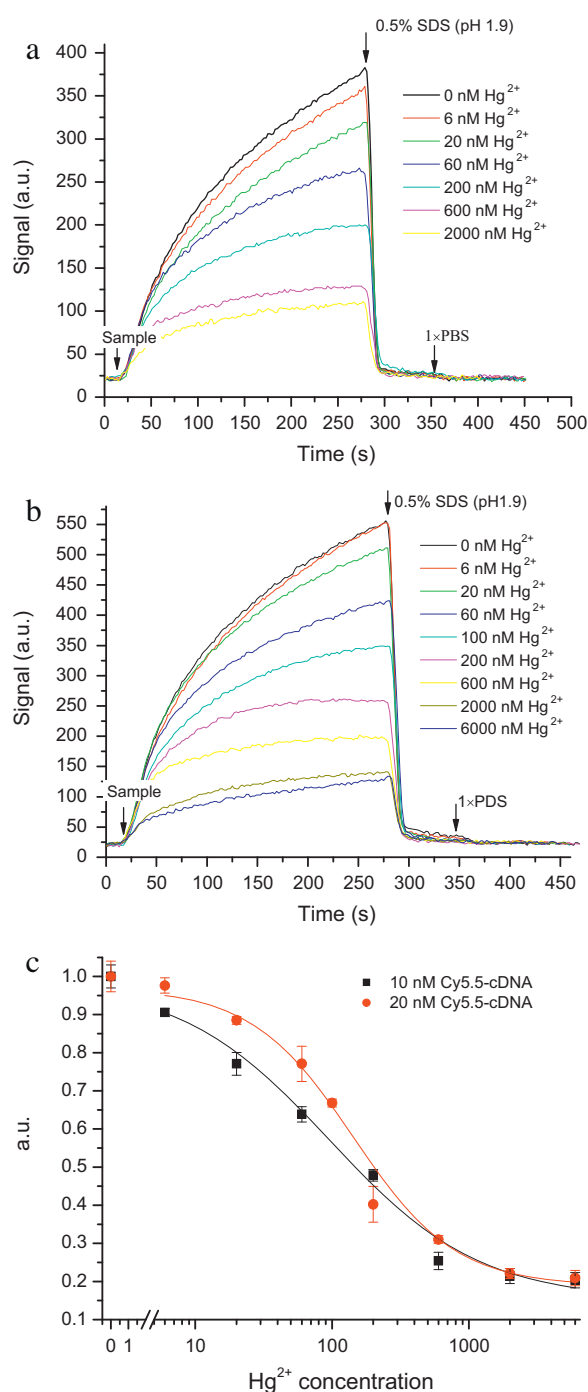


Fig. 3. Signal traces observed as the mixture of 10 nM (a) or 20 nM (b) cDNA and Hg^{2+} solutions of various concentrations flow over the sensor's surface containing immobilized DNA probe. (c) Dose-response curves as the normalized fluorescence signals with respect to the concentration of Hg^{2+} in structure-competitive assays at 10 and 20 nM cDNA.

inflection point. The error bars in the figure correspond to the standard deviations of the data points in triplicate experiments, with the standard deviations of all the data points being within 5%.

As seen in Fig. 3c, the proportionally decreasing signal as the concentration of Hg^{2+} was increased established a dose-response curve for quantification of Hg^{2+} over a concentration range from 0 to 6 μM . Two dose-response curves are shown employing two different concentrations, 10 and 20 nM, of fluorescence-labeled complementary DNA. The data indicate that the quantification range and the detection limit can be tuned by adjusting the con-

centration of the labeled cDNA used for competitive reactions. The detection limits for Hg^{2+} decreased from 5.0 nM at 20 nM cDNA to 2.1 nM at 10 nM cDNA based on a signal-to-noise ratio of 3. Comparison of the signals and detection limits with the two different cDNA concentrations indicated that cDNA concentration lower than 10 nM is not expected to further improve the detection without compromising the desired signal-to-noise ratio. The quantification of Hg^{2+} over concentration ranges from 0 to 600 nM and 0 to 2 μM , respectively, were observed at 10 nM and 20 nM cDNA levels. Therefore, our novel sensor can be applied for direct detection of Hg^{2+} in drinking water with the ability to meet even the most stringent EPA requirements (10 nM). The detection limit observed is comparable to those of the other sensing methods, such as the “turn-on” fluorescence (3.2 nM) (Wang et al., 2008), ratiometric (50 nM) (Nolan and Lippard, 2007), electrochemical based on gold nanoparticle amplification (0.5 nM) (Zhu et al., 2009), piezoelectric (1 nM) (Manganiello et al., 2002), and surface plasmon resonance (5 nM) (Wang et al., 2010). Although more sensitive detection of Hg^{2+} (10 pM) has been achieved using a microcantilever sensor (Xu et al., 2002) and a nanosensor (Chen et al., 2007), the sensor developed here is simpler and faster (only 6 min, including measurement and regeneration). Typically, the measurement time of SPR and electrochemical biosensors is about 1–2 h. Although the sensing process of the “turn-on” fluorescence sensor is less than 5 min, the pre-incubation reaction time of fluorescence-labeled and quencher-labeled strand DNAs needed before detecting Hg^{2+} is approximately 1 hour.

3.4. Selectivity of the sensing system

To investigate potential interference from other metal ions, we evaluated the sensor's response to 20 μM Ca^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} , Sn^{2+} , Cr^{2+} , Mn^{2+} , Ni^{2+} , and Pb^{2+} . As seen in Fig. 4, the sensor exhibits no significant response (<8%, as compared to a blank control) to these metal ions. This high selectivity must be due to a selective binding of Hg^{2+} to T–T mismatches, resulting in the formation of stable T– Hg^{2+} –T complexes.

3.5. Reusability and stability of the sensor

The regeneration performance of the sensing interface is an important issue for practical implementation of biosensors (Homola, 2003). Therefore, the stability and reusability of the DNA probe covalently immobilized to the sensing surface was evaluated over a large number (>100) of assays. As seen in Fig. 3a and b, after each assay a complete removal of Hg^{2+} and cDNA from the sensor surface was achieved using a 0.5% SDS solution (pH 1.9). After 100 successive assays, less than a 5% loss of performance was observed.

Using ^{15}N NMR, Ono and co-workers found that Hg^{2+} binds directly to the N3 of the thymidine ring in place of the imino pro-

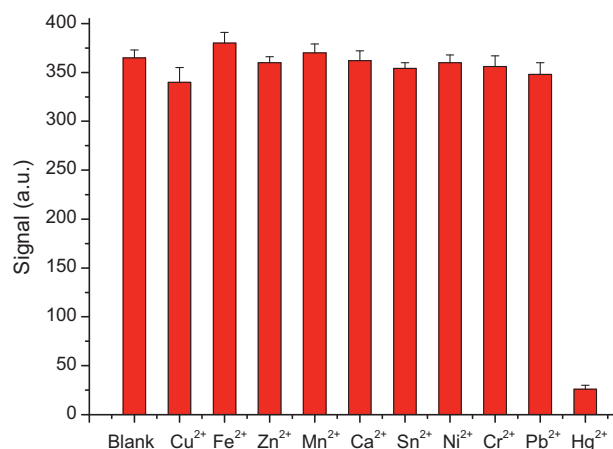


Fig. 4. Comparison of sensing signal to various 20 μM metal ions.

ton and bridges two thymidine residues to form the T– Hg^{2+} –T pair (Tanaka et al., 2007). The putative Hg^{2+} -mediated T–T base pair (T– Hg^{2+} –T pair) is at least as stable as normal Watson-Crick base pairs. Lu and co-workers obtained a dissociation constant of 471 nM for the structure presented here through fitting to Hill plot (Wang et al., 2008). To our best knowledge, this is the first time that an optical DNA biosensor for Hg^{2+} detection can be reused so many times, which indicates that the structure (T– Hg^{2+} –T pair) is reversible.

We also investigated the storage stability of the proposed sensor system. After performing seven daily measurements over 10 days of continuous analysis, a decrease in the average maximum signal response in the absence of analyte was less than 10% for cDNA. This slight drop in fluorescence signal did not affect the DNA biosensor's specific response: all measurements were normalized with respect to the blank signal at the beginning of the daily analysis, and signal shifts in the blank and sample measurements were generally the same. After each serial determination of a competitive standard curve, a blank solution containing only the corresponding cDNA was injected to test possible shifts from the baseline. After the fiber probe was stored in $1 \times \text{PBS}$ buffer at 4°C over 30 days, the DNA biosensor could still be used to assay for Hg^{2+} without significant change in fluorescence response.

3.6. Spiked environmental water samples analysis

To evaluate possible matrix effects, we tested the sensor developed herein with water samples using lab tap water, commercially available bottled water, and tertiary effluent from a wastewater treatment plant. These environmental samples were spiked with Hg^{2+} from its stock solutions at concentrations of 20, 100, and 200 nM, followed by measuring the Hg^{2+} content as described

Table 1

Detection results of water samples spiked with Hg^{2+} .

Origin ^a	Hg^{2+} added to the samples (nM)	Hg^{2+} determined by biosensor (nM)	CV (%)	Recovery (%)
Tap water	20	20.1	8.1	100
	100	88.4	7.4	89
	200	223	13.6	111
Pinery Wastewater Plant	20	23.8	7.8	119
	100	70.8	6.5	71
	200	253.3	9.4	127
Bottled water	20	18.6	1.4	93
	100	99.2	4.2	99
	200	249.3	7.0	125

^a All samples were collected on 15th of September 2010, and the initial concentration of Hg^{2+} in these water samples was undetectable (<2.1 nM). CV: coefficient of variation (CV, %; $n = 3$).

above. The results obtained, shown in Table 1, reveal good consistencies between the actual and the measured Hg^{2+} concentrations. These data confirm that the proposed sensing system is applicable for Hg^{2+} detection with enough precision and accuracy even in real environmental sample matrices.

4. Conclusions

A DNA-based evanescent wave optical biosensor for rapid and sensitive Hg^{2+} detection was developed by immobilizing a thymine–thymine (T–T) containing DNA probe onto an optical fiber sensor, which binds specifically to mercury ions to form a T– Hg^{2+} –T complex. Based on the structure-competitive detection mode, the biosensing assay approach for Hg^{2+} detection in water samples features high sensitivity, adequate specificity, rapidity, and ease of use. The robustness of the aminated DNA sensing surface and the acceptable surface regeneration procedures allow at least 100 assay cycles with no significant loss of performance. The performance of the biosensor with spiked real water samples showed good recovery, precision and accuracy, indicating low susceptibility to water matrix effect. The high specificity of the sensor was demonstrated by evaluating its response to a number of potentially interfering metal ions. All of these observations demonstrate that the portable biosensor presented here could be extended toward the on-site monitoring of the other metal ions or trace pollutants in environmental matrices with the use of different probes modified by DNA or aptamers.

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