Oligonucleotide-Based Fluorescence Probe for Sensitive and Selective Detection of Mercury(II) in Aqueous Solution

Cheng-Kang Chiang, Chih-Ching Huang, Chi-Wei Liu, and Huan-Tsung Chang*

Department of Chemistry, National Taiwan University, 1, Section 4, Roosevelt Road, Taipei 106, Taiwan

In this paper we unveil a new homogeneous assay—using TOTO-3 and the polythymine oligonucleotide T33—for the highly selective and sensitive detection of Hg^{2+} in aqueous solution. The fluorescence of TOTO-3 is weak in the absence or presence of randomly coiled T33. After T33 interacts specifically with Hg^{2+} ions through T–Hg^{2+}–T bonding, however, its conformation changes to form a folded structure that preferentially binds to TOTO-3. As a result, the fluorescence of a mixture of T33 and TOTO-3 increases in the presence of Hg^{2+}. Our data from fluorescence polarization spectroscopy, capillary electrophoresis with laser-induced fluorescence detection, circular dichroism spectroscopy, and melting temperature measurements confirm the formation of folded T33–Hg^{2+} complexes. Under optimum conditions, the TOTO-3/T33 probe exhibited a high selectivity (~265-fold) toward Hg^{2+} over other metal ions, with a limit of detection of 0.6 ppb. We demonstrate the practicality of this TOTO-3/T33 probe for the rapid determination of Hg^{2+} levels in pond water and in batteries. This approach offers several advantages, including rapidity (~15 min), simplicity (label-free), and low cost.

Heavy-metal pollution is an important environmental issue because of its adverse effect on human health. Mercury(II) is one of the most potently toxic metal ions; it affects many different areas of the brain and their associated functions, resulting in symptoms such as tremors, vision problems, deafness, and losses of muscle coordination, sensation, and memory. In addition to the brain, inorganic mercury can damage the heart, kidney, stomach, and intestines. The U.S. Environmental Protection Agency (EPA)’s estimate of annual total global mercury emission from all sources—both natural and human-generated—is ca. 7500 tons per year. Thus, techniques for the detection and/or removal of Hg^{2+} are required to protect our environment and health.

Although many techniques such as atomic absorption/emission spectroscopy, inductively coupled plasma mass spectrometry (ICP-MS), and selective cold vapor atomic fluorescence spectrometry have been applied widely to detect Hg^{2+} in environmental samples, they require expensive and sophisticated instrumentation and/or complicated sample preparation processes and their complexity makes them unsuitable for use in in-field Hg^{2+} analyses. Alternative techniques based on fluorescent probes using small molecules, DNAzymes, oligonucleotides, polymer–protein complexes, and nanoparticles have been demonstrated for the detection of Hg^{2+}. Nevertheless, each of these approaches exhibits some feature that limits its practical use, be it poor aqueous solubility, cross-sensitivity toward other metal ions, short emission wavelengths, and/or weak fluorescence intensities.

In this study, we developed a simple and rapid fluorescence approach—using the polythymine oligonucleotide T33 and a double-strand-chelating dye TOTO-3—for the sensitive and selective detection of Hg^{2+} in aqueous solutions. TOTO-3 is a weakly fluorescent unsymmetrical cyanine dye that exhibits a more than 1000-fold enhancement in its fluorescence upon binding to double-stranded DNA, with excitation and emission wavelengths centered at 620 and 660 nm, respectively.

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Hg$^{2+}$ is based on the fluorescence increase that occurs as a result of the strong interaction between TOTO-3 and the folded T$_{33}$ structure induced by Hg$^{2+}$.

**EXPERIMENTAL SECTION**

**Chemicals.** Tris(hydroxymethyl)aminomethane (Tris), the metal salts, and all the other reagents were purchased from Aldrich (Milwaukee, WI). OliGreen, TOTO-3, and YOYO-3 were obtained from Molecular Probes (Portland, OR). The N,N-dimethyl-2,7-diazapyrenium dication was synthesized and purified according to an established procedure. All of the DNA samples (T$_{7}$, T$_{15}$, T$_{33}$, T$_{80}$, and 6-FAM–T$_{33}$) were purchased from Integrated DNA Technology (Coralville, IA).

**TOTO-3/T$_{33}$-Based Sensor for Hg$^{2+}$.** A stock solution of TOTO-3 (0.2 $\mu$M) was prepared in DI water. Aliquots of this TOTO-3 solution (50 $\mu$L) were added separately to 5 mM Tris–HCl (pH 7.4) solutions containing Hg$^{2+}$ (0–10 $\mu$M) and the unmodified T$_{33}$ oligonucleotide (10 nM) to give final volumes of 500 $\mu$L. After equilibration at ambient temperature for 15 min, the fluorescence intensities of the mixtures were measured using a Cary Eclipse fluorescence spectrophotometer (Varian, CA), equipped with a 10 k$\Omega$ resistor to a 24 bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a PC. Capillaries (i.d., 75 $\mu$m; o.d., 365 $\mu$m; total length, 40 cm; effective length, 30 cm; Polymeric Technologies, Phoenix, AZ) were dynamically coated overnight with 5.0% poly(vinyl pyrrolidone) (PVP; $M_w$ 1.3 $\times$ 10$^5$) and then with 0.5% poly(ethylene oxide) (PEO; $M_w$ 8.0 $\times$ 10$^4$) for 12 h prior to use in DNA separations. Before conducting CE separations, aliquots of Hg$^{2+}$ (0–1.0 $\mu$M) were added separately to 5 mM Tris–HCl solutions (pH 7.4) containing 6-FAM–T$_{33}$ (10 nM) and fluorescein (30 nM) and equilibrated for 10 min. The mixtures were injected hydrodynamically at the cathode end into the capillary at a 20 cm height for 10 s; separations were conducted at ~5 kV. After each run, the capillary was sequentially washed and filled with 5 mM Tris–HCl (pH 7.4) containing Hg$^{2+}$ (0–10 $\mu$M). For measurement of the fluorescence polarization spectra, 5 mM Tris–HCl solutions (pH 7.4) containing 6-FAM–T$_{33}$ (50 $\mu$L) and Hg$^{2+}$ (0–120 $\mu$M) were reacted for 15 min and then the anisotropy of each solution was recorded using a Cary Eclipse fluorescence spectrophotometer (Varian, CA) equipped with a manual polarizer accessory (Varian, CA). Circular dichroism (CD) spectroscopy measurements were conducted using a J-815 spectropolarimeter (JASCO, Inc., Easton, MD). Melting temperature ($T_m$) was determined by measuring the fluorescence intensities of the mixtures of TOTO-3 and T$_{33}$ in the absence and presence of Hg$^{2+}$. We define the temperature at which the fluorescence of TOTO-3 reaches 50% of its original value as $T_m$.

**Analysis of Real Samples.** A pond water sample from the National Taiwan University campus was filtered through a 0.2 $\mu$m membrane and analyzed using ICP-MS. Aliquots of the pond water (250 $\mu$L) were spiked with standard solutions (50 $\mu$L) containing Hg$^{2+}$ at concentrations over the range of 0.01–1.0 $\mu$M. Next, 50 mM Tris–HCl solution (pH 7.4, 50 $\mu$L), T$_{33}$ solution (100 nM, 50 $\mu$L), TOTO-3 solution (200 nM, 50 $\mu$L), and water (50 $\mu$L) were added to the mixtures to give final volumes of 500 $\mu$L. The mixtures were equilibrated for 15 min. The samples from three button-type alkaline manganese batteries were prepared according to the standard method published by the National Electrical Manufacturers Associations. Briefly, these samples were digested in a mixture of HCl and HNO$_3$ (2:1, v/v) for 18 h and then directly basified through the addition of 0.5 N NaOH and filtered through a 0.2 $\mu$m membrane. The solutions (10 $\mu$L) were then diluted to 10 mL with 5 mM Tris–HCl solution (pH 7.4) prior to analysis using both ICP-MS and the TOTO-3/T$_{33}$ probe. The quantitation of Hg$^{2+}$ in these samples was obtained by applying a standard addition method.

**RESULTS AND DISCUSSION**

**Sensing Strategy.** Scheme 1 depicts the mechanism underlying this TOTO-3/T$_{33}$ probe’s approach to sensing Hg$^{2+}$. In the absence of Hg$^{2+}$, T$_{33}$ exists in aqueous solution in a random-coil structure. Because the interactions between the randomly coiled T$_{33}$ and TOTO-3 are weak, the fluorescence of such a mixture is weak. In the presence of Hg$^{2+}$, however, T–Hg$^{2+}$·T bonding results in T$_{33}$ changing its random-coil conformation to that of a folded structure. Because TOTO-3 has a high affinity ($K_b$ = 1.0$^{10}$ M$^{-1}$) for double-stranded DNA (dsDNA), TOTO-3–DNA complexes form preferably in the presence of Hg$^{2+}$. As a result of reduced collision and forming stiffer structures, TOTO-3–DNA complexes fluoresce more strongly than does free TOTO-3. Thus,

![Scheme 1. Schematic Representation of the Function of a Hg$^{2+}$ Sensor That Operates based on Modulation of the Fluorescence of the Complex Formed between TOTO-3 and T$_{33}$](image-url)


(10) EPBA, BAT, and NEMA. Battery industry standard analytical method for the determination of mercury, cadmium and lead in alkaline manganese cells using AAS, ICP-AES and cold vapor; European Portable Battery Association (EPBA), Battery Association of Japan (BAT), and National Electrical Manufactures Association (NEMA): Brussels, Belgium, Tokyo, Rosslyn, VA, respectively, April, 1998.

the fluorescence intensity of the complexes formed between TOTO-3 and T33 increases in the presence of Hg$^{2+}$. Evidence for Folded T33 Forming in the Presence of Hg$^{2+}$. Curve a in Figure 1 indicates that the fluorescence at 660 nm of TOTO-3 (20 nM) in 5 mM Tris-HCl solution (pH 7.4) is very weak when excited at 620 nm. The fluorescence intensity of TOTO-3 in the presence of 10 nM T33 is slightly higher (curve b), supporting the notion that it interacts weakly with random-coil T33. After adding 1.0 $\mu$M Hg$^{2+}$ to this mixture of TOTO-3 and T33, a rapid (<10 s) and significant increase (8.3 fold) in fluorescence intensity occurred (curve c). In the absence of T33, we did not observe any change in the fluorescence spectrum of TOTO-3 (20 nM) after adding Hg$^{2+}$ (1.0 $\mu$M). To confirm the specificity of T33 toward Hg$^{2+}$, we tested a control DNA sample having the sequence GCC TTA ACT GCA GTA CTG GTG AAA TTG CT. We expected this control DNA to have difficulty folding in the presence of Hg$^{2+}$, mainly because it has a less of a chance to form T-Hg$^{2+}$-T bonds than does T33. We observed only slight changes (<10%) in the fluorescence intensity of the mixture of TOTO-3 and the control DNA after adding the same amount of Hg$^{2+}$.

We further applied CE to support the notion of the formation of folded T33 in the presence of Hg$^{2+}$. Because the fluorescence of mixtures of TOTO-3 and random-coil T33 is weak, we could not observe random-coil T33 when using CE in conjunction with laser-induced fluorescence (LIF) detection. In other words, the changes in the electrophoretic mobility of the complexes of T33 and TOTO-3 in the presence of Hg$^{2+}$ were difficult to observe using CE-LIF. Thus, we use a 5’ end labeled 6-FAM-T33 for the CE experiment. Upon increasing the concentration of Hg$^{2+}$, the migration time for 6-FAM-T33 decreased, while that for the internal standard (fluorescein) remained almost unchanged (Figure 2A). We suspect that once 6-FAM-T33 interacted with Hg$^{2+}$, its conformation changed from a random coil to a folded structure leading to increased electrophoretic mobility (i.e., a decrease in migration time). On the basis of the plot in Figure 2B, we calculated the binding constant ($K_b$) for the interaction between T33 and Hg$^{2+}$ to be $6.1 \times 10^6$ M$^{-1}$. For comparison, we note that a value of $K_b$ of $4.2 \times 10^5$ M$^{-1}$ has been reported for Hg$^{2+}$ interacting with two oligonucleotides having two binding sites for Hg$^{2+}$ (i.e., each has two T units). The formation of folded DNA molecules having many T residues in the presence of Hg$^{2+}$ has been proven using CD and
nuclear magnetic resonance spectroscopy. The CD spectra of T33 (Figure S1, Supporting Information) indicated T33 (500 nM) changed from random-coil conformation to that of a folded structure upon increasing the Hg2+ concentration from 0 to 15 μM. To further support the formation of folded 6-FAM-T33 in the presence of Hg2+, we recorded fluorescence polarization spectra of 6-FAM-T33 in the presence of various concentrations of Hg2+ (Figure 2C). Because of the relatively low sensitivity of fluorescence polarization spectroscopy, we used higher concentrations of 6-FAM-T33 and Hg2+ than those used in the CE-LIF experiments. The anisotropy of 6-FAM-T33 (50 nM) increased from 0.026 to 0.224 upon increasing the Hg2+ concentration from 0 to 120 μM, consistent with the putative changes in the structure of 6-FAM-T33. The anisotropy in folded 6-FAM-T33 is higher than that in random-coil 6-FAM-T33 mainly because of the former’s stiffer structure. We further conducted Tm measurements to support our reasoning. Upon increasing the temperature, the fluorescence intensity of TOTO-3-poly-T complexes (10 nM) decreased as a result of breaking in the T-Hg2+-T bonding (Figure 2D). Upon increasing Hg2+ concentration, Tm increased and reached a plateau at the concentration of Hg2+ of 750 nM.

Effect of the Length of Poly-T and pH. Having observed that Hg2+ induced the formation of folded DNA structures, we suspected that the sensitivity of our analytical system would be dependent on the length of the DNA strand. We employed five poly-T ss-DNA samples of various lengths, T7, T15, T33, T50, and T80 (each 10 nM), to test our hypothesis. After plotting the values of (Ip − Is)/Ip for the TOTO-3-poly-T complexes against the concentration of Hg2+ (Figure S2, Supporting Information), we found that Hg2+ induced significant positive responses when the number of bases of poly-T was greater than 15. The degree of Hg2+-induced fluorescence enhancement of the complex of TOTO-3 and T7 was very small, mainly because of the difficulty of forming a folded structure from T7. The values of (Ip − Is)/Ip for the complexes formed between TOTO-3 and T90, T30, and T31 in the presence of Hg2+ were similar; they all increased upon increasing the Hg2+ concentration and reached a plateau at a concentration of 500 nM.

We also explored the effect of pH of TOTO-3/T33 solution on sensing Hg2+. As depicted in Figure S3 (Supporting Information), the values (Ip − Is)/Ip for TOTO-3-poly-T complexes were optimized at pH 7.4. The affinity of TOTO-3-DNA was reported optimized at pH 6–8, while Hg2+ binds directly to N3 of thymidine in place of the imino proton and bridges two thymidine residues to form the T–Hg2+–T pair.13 Thus, 5 mM Tris–HCl buffer (pH 7.4) was selected for the following studies.

Impact of Fluorophore. We investigated the effect of the TOTO-3 concentration on the sensing of 1 μM Hg2+ in the presence of 10 nM T33. The values of (Ip − Is)/Ip for the complexes of TOTO-3, T33, and Hg2+ increased upon increasing

![Figure 3](image-url)
the value we calculated from the CE-LIF data. The limit of detection (LOD) at a signal-to-noise ratio (S/N) of 3 was 3 nM (0.6 ppb), which is below the maximum level of mercury permitted by the U.S. EPA for drinking water. Thus, our present approach provides a sensitivity toward Hg$^{2+}$ that is 1 order of magnitude better than that previously reported for a T-T mediated sensor.7d

Next, we investigated the selectivity of our new approach for Hg$^{2+}$ over other metal ions (Li$^+$, Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Al$^{3+}$, Cr$^{3+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Cd$^{2+}$, Ag$^+$, and Au$^{3+}$; each 1.0 µM) under the optimum conditions. As indicated in Figure 4B, the TOTO-3/T$_{33}$ probe was highly selective (265-fold or more) for Hg$^{2+}$ over the other metal ions. We performed a series of competition experiments to test the practicality of our TOTO-3/T$_{33}$ sensor for the selective detection of Hg$^{2+}$. The tolerance concentrations of various metal ions (within a relative error of ±5%) for the sensing of Hg$^{2+}$ (100 nM) using the TOTO-3/T$_{33}$ probe were 1.0 µM for Au$^{3+}$, 5.0 µM for Ag$^+$, Cd$^{2+}$, and Pb$^{2+}$, and 10.0 µM for Cu$^{2+}$, Co$^{2+}$, Fe$^{3+}$, and Ni$^{2+}$. These results suggest that the metal ions we tested should not interfere with the determination of Hg$^{2+}$ when applying our developed probe.

Next, we investigated the effect that some chelating agents, including citrate, nitrilotriacetic acid, ethylene diamine tetracetic acid (EDTA), and iminodiacetic acid, have on the sensing capability of TOTO-3·T$_{33}$ complexes toward Hg$^{2+}$. Only EDTA forms strong complexes with Hg$^{2+}$ has a significant effect on the sensing. When using TOTO-3·T$_{33}$ complexes containing Hg$^{2+}$ (200 nM), the fluorescence intensity decreased 71% and 85% in the presence of 200 nM and 2 µM EDTA, respectively (data not shown).15 These results suggest that the TOTO-3·T$_{33}$ Sensor can be made renewable by using EDTA and conducting dialysis.

To evaluate the resistance of our sensor to endogenous nuclease degradation, we conducted sensing Hg$^{2+}$ ions (0–1.2 µM) using TOTO-3·T$_{33}$ (10 nM) in the presence of DNase I (100 nM). A linear plot ($R^2 = 0.98$) of the signal enhancement ratios (I$_{F0}$ – I$_{F}$)/I$_{F0}$ of the TOTO-3·T$_{33}$ complexes against the concentration of Hg$^{2+}$ (50–200 nM) is exhibited in Figure S4 (Supporting Information). This result indicated Hg-mediated folded structure resisted DNase I digestion.

Detection of Hg$^{2+}$ in Real Samples. As indicated in Figure 5, the intensity of the fluorescence of the TOTO-3·T$_{33}$ complexes increased upon increasing the spiked concentration of Hg$^{2+}$ in pond water over the range of 25–200 nM ($R^2 = 0.98$). The recoveries of these measurements were valued at 97–108%. The LOD at an S/N ratio of 3 for Hg$^{2+}$ in the presence of the complicated pond water matrix was 10 nM (2.0 ppb). We also applied our TOTO-3/T$_{33}$ probe to the detection of Hg$^{2+}$ in battery

samples. Table 1 lists the concentrations of Hg$^{2+}$ that we determined in three different types of batteries using both our developed probe and ICP-MS. On the basis of F-test, the results using our present approach are in good agreement with those obtained using ICP-MS. Note, however, that the sample preparation and analysis time when using the TOTO-3/T33 probe was less than 15 min; this assay provides the additional advantages of simplicity, low cost, and high throughput.

CONCLUSION

We have developed a homogeneous Hg$^{2+}$ assay using TOTO-3 and T33. Upon interaction with Hg$^{2+}$, T33 changes its conformation from a random coil to a folded structure, leading to an increase in the fluorescence intensity, electrophoretic mobility, and fluorescence anisotropy for the TOTO-3·T33 complex. This probe is highly sensitive and selective for Hg$^{2+}$. Although we have demonstrated the detection of Hg$^{2+}$ ions only, we suspect that this probe strategy—using DNA samples of various lengths and sequences—will also be applicable to systems for the detection of metal ions such as Pb$^{2+}$ and Cu$^{2+}$.

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SUPPORTING INFORMATION AVAILABLE

CD spectra of the T33 (500 nM) and TOTO-3 (1 µM) complexes after the addition of Hg$^{2+}$ ions (0–20 µM) (Figure S1). Fluorescence responses of mixtures of TOTO-3 (20 nM) and poly-T (10 nM) after the addition of Hg$^{2+}$ ions (Figure S2). Fluorescence responses of mixtures of TOTO-3 (20 nM) and T33 (10 nM) after the addition of Hg$^{2+}$ ion (200 nM) in various pH value buffers (5 mM) (Figure S3). Relative fluorescence $[(I_F - I_{F0})/I_{F0}]$ of the T33·TOTO-3 complexes in the presence of DNase I (100 nM) at 660 nm against Hg$^{2+}$ concentration (0–1.2 µM) (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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