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Guanine chemiluminescent Biosensor capable of rapidly sensing mercury in a sample

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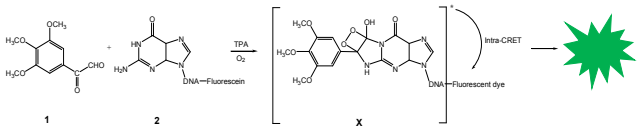
Using DNA aptamer and guanine chemiluminescene detection, we developed a highly sensitive biosensor for the rapid quantification and monitoring Hg^{2+} in drinking water. Random coli structure of DNA aptamer was transformed to hairpin-link thymin (T)-Hg^{2+}-T complex, closed by the hybridization of guanine and cytocine of DNA aptamer, in the presence of Hg^{2+} in drinking water. The brightness of light, emitted from the reaction between guanine of DNA aptamer and 3,4,5-trimethoxyl phenylglyoxal hydrate (TMPG) in the presence of tetra-n-propylammonium hydroxide (TPA) and O_2, was exponentially decreased with the increase of Hg^{2+} due to the transformation of DNA aptamer. The sensitivity of biosensor was dependent on the incubation time for forming hairpin-link T-Hg^{2+}-T complex closed by the hybridization of guanine and cytosine of DNA aptamer. The sensitivity of biosensor was the highest when DNA aptamer was incubated with Hg^{2+} for 20 min at room temperature (21 ± 2 °C). The sensitivity of biosensor generated with incubation longer than 20 min was not as good as that operated with a 20-min incubation because the dehybridization of guanine and cytosine of DNA aptamer is predominant after 20 min. The limit of detection (LOD = CL_0 - 3σ) of biosensor operated with a 20-minute incubation was as low as 2.11 nM. Also, the accuracy, precision, and recovery of time-dependent biosensor were good within statistically acceptable error range.

Introduction

Due to the virulent toxicity of Hg^{2+}, a number of analytical methods using various optical detections (e.g., absorbance,[1, 2] fluorescence,[3, 4] chemiluminescence[5, 6]) were developed for public health and environment.

It is well-known that random coil structure of single strand DNA added in solution containing Hg^{2+} is transformed to hairpin-link structure with the interaction between thymine (T) and Hg^{2+} to form T-Hg^{2+}-T complex.[7-9] Thus, various types of DNA aptamers[7, 8, 10] designed using the chemical and physical interaction between T and Hg^{2+} were applied for the quantification of Hg^{2+} in human and environmental samples.



Scheme 1. Guanine chemiluminescence. 1: TMPG, 2: DNA aptamer conjugated with fluorescein, X: high-energy intermediate

3,4,5-trimethoxylphenyl-glyoxal (TMPG) reacts with guanine to emit dim light.[11, 12] Light emitted from the reaction of TMPG and guanine in the presence of tetra-n-propylammonium hydroxide (TPA) and O_2, was enhanced.[13, 14] Recently, light emitted from the reaction between guanines of DNA aptamer and TMPG was applied as a new chemiluminescence detection method of biosensor capable of rapidly quantifying anlytes in a sample.[12-16] As shown in Scheme 1, dim light emitted from the reaction of TMPG and guanine of DNA aptamer can transfer energy to fluorescein (or 6-FAM) labelled with DNA aptamer to emit bright green light based on the principle of intra chemiluminescence resonance energy transfer (Intra-CRET).[13-15] The CRET in guanine chemiluminescence is similar to that in peroxyoxalate chemiluminescence reaction.[17-19] Using Intra-CRET between fluorescent dye (e.g., fluorescein, 6-FAM) and high-energy intermediate formed from the reaction between TMPG and guanine of DNA aptamer in the presence of TPA, biomarkers in human samples were quantified for the early diagnosis of prostate cancer[14] and the monitoring of blood coagulation.[13] Guanines of DNA aptamer not bound with a specific biomarker rapidly reacted with TMPG to form high-energy intermediates, whereas guanines of DNA aptamer-bound the biomarker did not react with TMPG. Thus, relative CL intensity of guanine chemiluminescence was proportionally decreased with the increase of biomarkers in a sample.[13, 14]
Using the complexation of T and Hg^{2+} as well as the rapid chemical reaction of guanine and TMPG in the presence of TPA, we designed appropriate DNA aptamers to develop a highly sensitive biosensor capable of rapidly quantifying Hg^{2+} in a sample.

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Experimental

Chemical and materials

Three different types of T-Hg^{2+}-T hairpin DNA sequences (e.g., A1, A2, A3) capable of forming a T-Hg^{2+}-T complex were designed based on the previous research results reported by other research groups.[7, 8, 10] These T-Hg^{2+}-T hairpin DNA sequences were denoted as DNA aptamer in this report even though they had not been discovered by systematic evolution of ligands by exponential enrichment (SELEX).[20] We purchased them from Alpha DNA.

A1: 5'-fluorescein-GGGGTTCTTCCCCTTGTTCCCC-3'
A2: 5'-fluorescein-GCGCTTCTTCCCCTTGTTGCGC-3'
A3: 5'-fluorescein-GGTTCTTCCCCTTGTTCC-3'

In order to study whether guanine-cytosine base pair formed from the hybridization of target (H1) and complementary (H2) DNAs emits light in guanine chemiluminescence reaction, H1 and H2 were purchased from Alpha DNA.

H1: 5'-fluorescein-GGGGAAAA-3'
H2: 5'-TTTTCCCC-3'

3,4,5-trimethoxyl phenylglyoxal hydrate (TMPG, 97 %) was purchased from Matrix Scientific (Columbia, SC, USA). $FeCl_2$ (99 %), $FeCl_3$ (99 %), Tetra-n-propylammonium hydroxide (TPA, 40 % w/w aqueous solution), and deionized H_2O (HPLC grade) were purchased from Alfa Aesar (Ward Hill, MA, USA). N,N-Dimethylformamide (DMF) and 10 ×PBS were purchased from EMD (Billerica, MA, USA). Graphene oxide (GO) was purchased from Graphene Supermarket (Calverton, NY, USA). 7.0 mM $Hg(NO_3)_2$ stock solution was purchased from VWR.

Free single-strand DNA aptamer removal using Fe_3O_4-graphene nanoparticle

In order to remove free single-strand DNA remaining after the binding of Hg^{2+} and DNA aptamer, Fe_3O_4-graphene nanoparticles were synthesized based on our previous reports.[13, 14, 21] The mixture (0.5 ml) of $FeCl_2$ (2.5 mg) and $FeCl_3$ (7.5 mg) in water was added to a 1.5ml-centrifugetube containing graphene oxide (GO, 0.5 ml, 1 mg/ml). Ammonium hydroxide (NH_4OH, 20 µl, 30%) was also dispensed into the tube. The microcentrifuge tube was then incubated for 60 minutes at 85 °C. Fe_3O_4-graphene nanoparticles washed using a magnetic separator (Bioclone,Inc) were stored in a refrigerator. Free single-strand DNA aptamers were rapidly immobilized on the surface of Fe_3O_4-graphene nanoparticle due to the π-π static interaction between single strand DNA and graphene oxide, whereas the complex of DNA aptamer-bound Hg^{2+} remained in aqueous solution.

Determination of incubation time for the quantification of Hg^{2+}

Hg^{2+} (40 nM, 100 µl) in water was mixed with mercury aptamer (1 µM, 100 µl) in PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer) at room temperature (21 ± 2 °C). We selected PBS as buffer solution based on the previous reports to enhance the sensitivity of guanine chemiluminescence detection.[15] Light emitted from the mixture with the addition of guanine chemiluminescence reagents (e.g., TMPG, TPA) was immediately measured with a luminometer (Lumat 9507, Beththold, Inc, Germany). Also, the remaining mixture in the absence of guanine chemiluminescence reagents was incubated at room temperature. Then, light emitted from the mixture incubated at 10 min intervals for up to 60 min was measured. With relative CL intensities measured after the different incubations, we determined the best incubation time to quantify trace levels of Hg^{2+} in a sample.

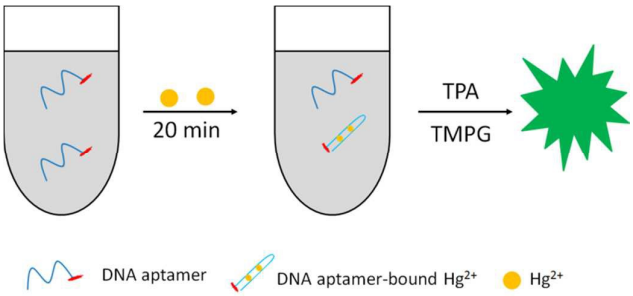


Fig. 1 Quantification of Hg^{2+} using DNA biosensor with guanine chemiluminescence detection.

Quantification of Hg^{2+}

As shown in Fig. 1, Hg^{2+} in a sample was rapidly quantified with the simple procedure without time consuming washing of the mixture before adding guanine chemiluminescence reagents. A certain concentration of Hg^{2+} (100 µl) in water was mixed with A1 (0.6 µM, 100 µl) in PBS buffer (pH 7.4). The mixture was incubated for 20 min at room temperature. After the incubation, the mixture (20 µl) was mixed with 20 mM TPA (10 µl) in a borosilicate test tube. Then the test tube was inserted into the luminometer. Then, light emitted in the test tube with the addition of 4 mM TMPG (100 µl) in DMF through a syringe pump of the luminomeer was measured for 20 seconds. Then, Hg^{2+} concentration in the sample was determined with the linear calibration curve obtained with 6 different standards (0 ~ 400 nM).

Results and discussion

Quenching effect of Hg^{2+} in guanine chemiluminescence reaction

Before the development of with guanine chemiluminescence detection capable of quantifying Hg^{2+}, we studied whether Hg^{2+} is a quencher in guanine chemiluminescence reaction using single strand DNA (H1), a guanine-rich and thymine-free oligo. Fig 2 shows that light emitted in guanine chemiluminescence reaction in the presence of 1,800 nM Hg^{2+} is the same as that in the absence of Hg^{2+} within the statistically acceptable error range. However, the relative CL intensity in the presence of Hg^{2+} higher than 1,800 nM Hg^{2+} was lower than that in the absence of Hg^{2+}. The results indicate that Hg^{2+} higher than 1,800 nM acts as a quencher of

guanine chemiluminescence generated in the reaction condition of Fig. 2.

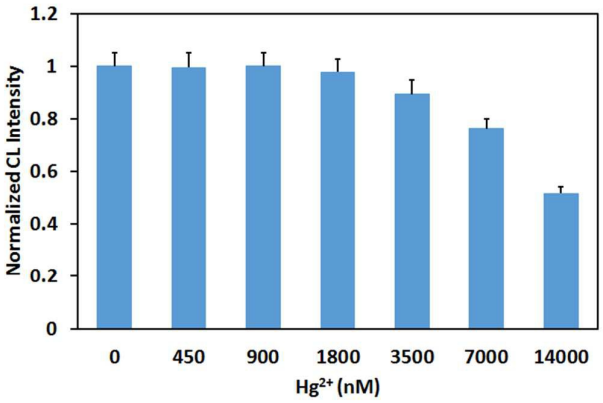


Fig. 2 Quenching effect of Hg^{2+} in guanine chemiluminescence reaction. Condition: [H1] = 1 μM in PBS (pH 7.4), [TPA] = 20 mM in H_2O, [TMPG] = 5 mM in DMF.

Determination of DNA aptamer to quantify Hg^{2+} in a sample

As shown in Fig. 3, relative CL intensities in the absence of Hg^{2+} using A1 and A2 were higher than that in the presence of only 40 nM Hg^{2+} even though the concentration of Hg^{2+} is out of the concentration range of Hg^{2+} capable of acting as a quencher in guanine chemiluminescence (see Fig. 2). Thus, these results indicate that single strand DNA bound with Hg^{2+} does not emit light or emits relatively dim light when guanine chemiluminescent reagents are added in the solution as shown in Fig. 1. Also, Fig. 3 shows that the binding rate between A1 and Hg^{2+} is faster than that between A2 and Hg^{2+}. Based on these results, we select A1 to develop a highly sensitive biosensor capable of rapidly quantifying Hg^{2+} in a sample.

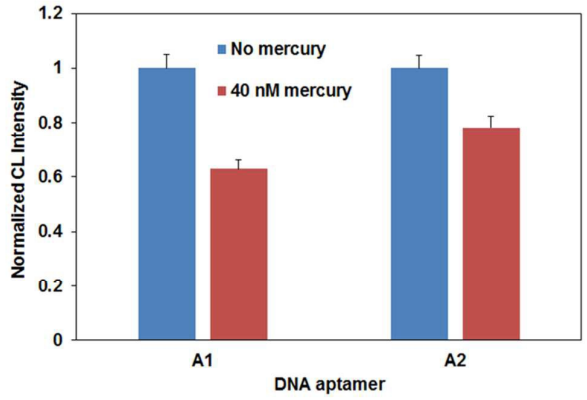


Fig. 3 CL emission of DNA aptamer (1 μM in PBS) in the absence and presence of Hg^{2+} (40 nM in deionized water). The mixture containing DNA aptamer and Hg^{2+} was incubated for 20 min at room temperature.

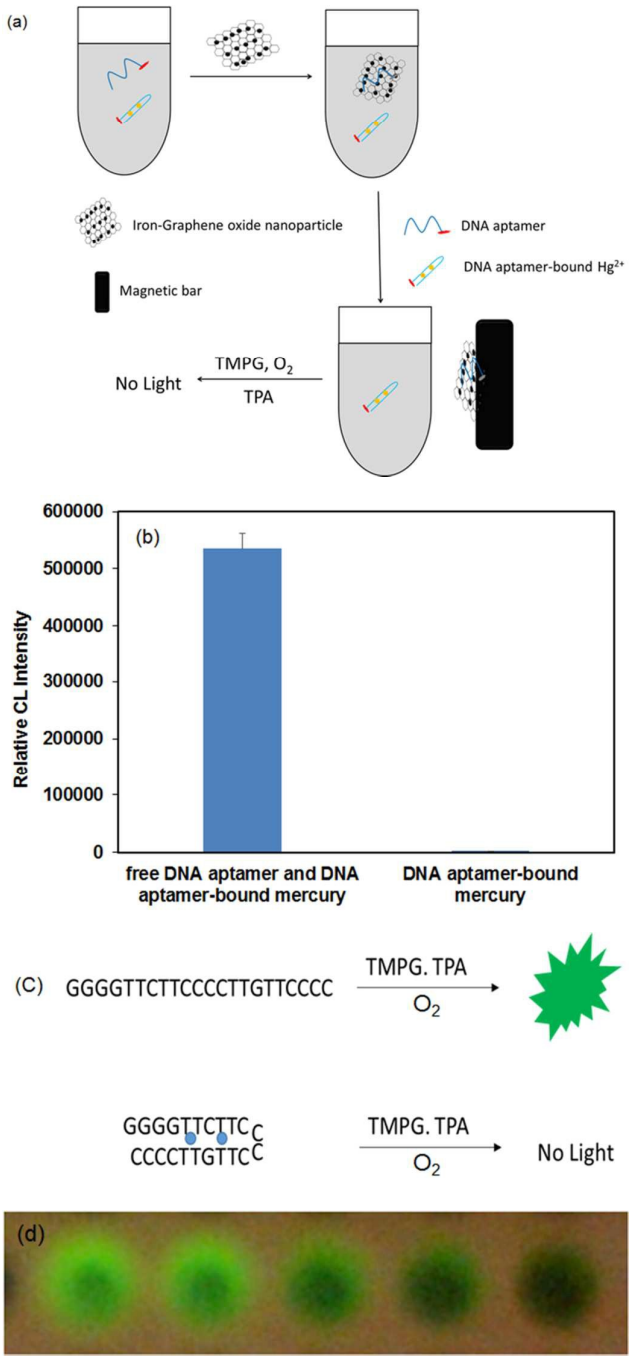
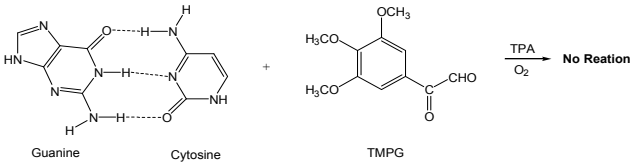


Fig. 4 (a) No emission of DNA aptamer-bound Hg^{2+}, (b) CL emissions of mixture (free DNA aptamer and DNA aptamer-bound Hg2+) and DNA-aptamer-bound Hg^{2+} only, (c) Differences between free DNA aptamer and DNA aptamer-bound Hg^{2+} in guanine chemiluminescence, (d) CL emission in the absence and presence of Hg^{2+} in guanine chemiluminescence. [Hg^{2+}] from left: 0, 5, 20, 100, 400 nM, [DNA aptamer] = 1 μM.

Determination of incubation time for the quantification of Hg^{2+}

In order to understand the results shown in Fig. 3, first, free DNA aptamers remaining after the reaction to capture $Hg2^{+}$ for 20 min were removed using Fe_3O_4-graphene nanoparticles we synthesized[13, 14, 21] and a magnetic bar based on π-π static interaction between single strand DNA and graphene oxide

(see Fig. 4(a)). Then, we studied whether DNA aptamer-bound Hg^{2+} can emit light with the addition of guanine chemiluminescent reagents. As shown in Fig. 4(b), the solution containing DNA aptamer-bound Hg^{2+}does not emit light, whereas the other solution containing the mixture of free DNA aptamer and DNA aptamer-bound Hg^{2+} emits bright green light. Based on the results, plausible reaction mechanisms shown in Fig. 4(c) can be proposed. Fluorescein excited from high-energy intermediate formed from the reaction of guanine of free DNA aptamer and TMPG emit bright green light based on the principle of intra-CRET. However, guanines of DNA aptamer-bound Hg^{2+} do not react with TMPG as shown in Scheme 2. because primary and second amines of guanine are already exhausted due to the hybridization of guanine and cytosine of DNA aptamer to stably maintain T-Hg^{2+}-T complex Thus, relative CL intensity measured after the hybridization was lower than that recorded before the hybridization as shown in Fig. S1. Fig. 4(d) shows that the brightness of light emitted in guanine chemiluminescence reaction is dependent on the concentration of Hg^{2+}.



Scheme 2. No reaction between guanine-cytosine base pair and TMPG.

Determination of incubation time for the quantification of Hg^{2+}

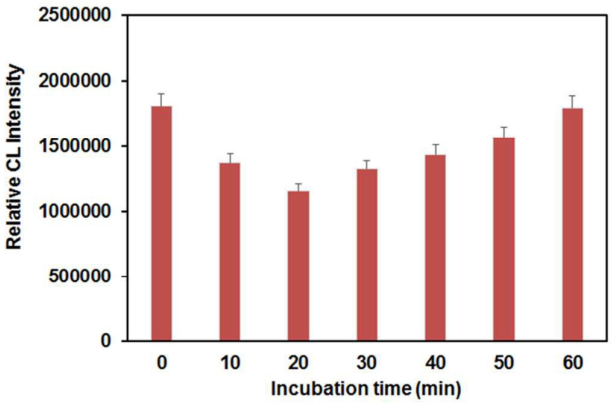


Fig. 5 Time-dependent biosensor with guanine chemiluminescence detection for the quantification of Hg^{2+}.

Fig. 5 shows that the sensitivity of biosensor capable of quantifying Hg^{2+} in a sample is dependent on the incubation time of DNA aptamer and Hg^{2+} before adding guanine chemiluminescent reagents. With the increase of incubation time to up to 20 min, relative CL intensity was decreased because of the increase of DNA aptamer-bound Hg^{2+} concentration. With longer incubation time than 20 min, however, relative CL intensity began to be enhanced. Relative CL intensity measured after a 60-min incubation was similar to that immediately measured when DNA aptamer is mixed with Hg^{2+}. Fig. 5 indicates that hairpin-link T-Hg^{2+}-T complex closed with the hybridization of guanine and cytosine (see Fig. 4(c)) is decomposed with the dehybridization of guanine and cytosine under this condition. Thus, it is possible that hairpin-link T-Hg^{2+}-T complex closed with the hybridization of guanine and cytosine is predominantly formed until 20 min of incubation. After 20 min, however, the decomposition of hairpin-link T-Hg^{2+}-T complex is dominant. Thus, we selected the 20-min incubation to develop a more sensitive biosensor capable of quantifying Hg^{2+} in a sample. Fig. S2 shows that the stability of hairpin-link T-Hg^{2+}-T complex is dependent on the hybridization of T-Hg^{2+}-T hairpin-DNA. The results indicate that the hairpin-link T-Hg^{2+}-T complex formed with A1 is more stable than those generated with A2 and A3. Additionally, it is expected that the biosensor using A1 will be more sensitive than those using A2 and A3.

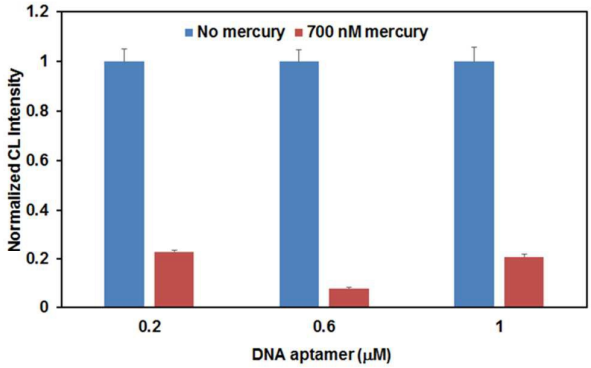


Fig. 6 Effect of DNA aptamer for the quantification of Hg^{2+} in a sample. Incubation time was 20 min at room temperature.

Determination of DNA aptamer concentration

Fig. 7 shows that the sensitivity of biosensor is dependent on the concentration of A1. The best concentration of A1 for developing a more sensitive biosensor was 0.6 μM under this condition. This is because the ratio (CL_0/CL_{700} = 12.7) of CL intensities measured in the absence (CL_0) and presence (CL_{700}) of 700 nM Hg^{2+} in 0.6 μM A1 solution are the highest. This is because (1) 0.2 μM A1 is too low to capture 700 nM Hg^{2+} within 20 min and (2) the concentration of free A1 remaining after the 20-min incubation using 1 μM A1 is higher than that using 0.6 μM A1.

Selectivity of DNA aptamer

Fig. 7 shows that biosensor using DNA aptamer (e.g., A1) can selectively quantify Hg^{2+} existing in a sample containing various contaminants. Also, relative CL intensity of tap water spiked with Hg^{2+} (20 nM) was the same as that in deionized water containing Hg^{2+} (20 nM). These results indicate that Hg^{2+} in tap water containing more impurities can be rapidly quantified without any pre-treatment to remove interferences. Additionally, the results shown in Fig. S3 is a

proof that the biosensor can selectively trace levels of Hg^{2+} in the presence of excess impurities.

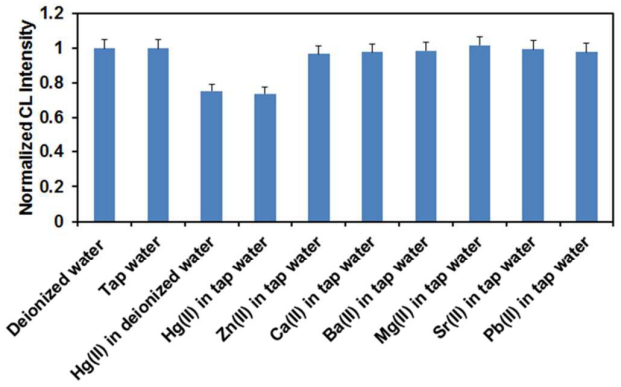


Fig. 7 Selectivity of DNA aptamer capable of binding Hg^{2+} in a sample. Condition: [Hg^{2+}] = 20 nM, [DNA aptamer] = 0.6 µM in PBS buffer (pH 7.4). The concentration of each metal spiked in tap water was 100 nM.

Quantification of Hg^{2+} in tap water

As shown in Fig. 8(a), relative CL intensity was exponentially decreased with the increase of Hg^{2+} ions. Also, we obtained a wide linear calibration curve (0 ~ 400 nM) using inverse numbers of relative CL intensities measured in the absence and presence of Hg^{2+} (see Fig. 8(b)). The limit of detection (LOD = CL_0 - 3σ) of biosensor calculated using the linear calibration curve was as low as 2.11 nM (0.4 ppb). σ is the standard deviation of CL_0. Thus, we expect that the biosensor can be applied as a new tool for the quantification and monitoring of Hg^{2+} in drinking water based on the regulation of US EPA (i.e., Maximum contaminant level of Hg^{2+} in drinking water is 2 ppb. http://water.epa.gov/drink/contaminants/basicinformation/mercury.cfm#four).

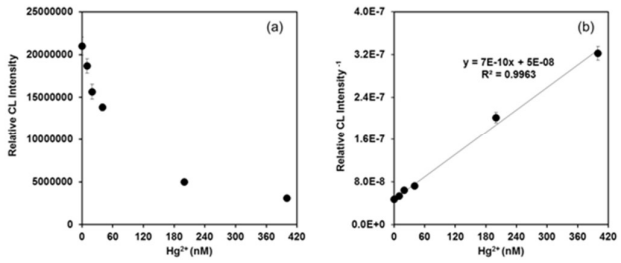


Fig. 8 Calibration curves for the quantification of Hg^{2+} in tap water. Condition: [Hg^{2+}] = 0, 10, 20, 40, 200, and 400 nM in deionized water, [DNA aptamer] = 0.6 µM in PBS. The mixture of Hg^{2+} and DNA aptamer was incubated for 20 min at room temperature.

Table 1 shows that the accuracy, precision, and recovery of biosensor developed in this research are good within the statistically acceptable error range.

Table 1. Accuracy, precision, and recovery of biosensor capable of sensing Hg^{2+} in drinking water (n = 5).

Sample 1 (nM)	Sample 2 (nM)	Expected (nM)[a]	Measured (nM)	Recovery (%)
20	10	15	14.2 ± 0.3	94.7
40	50	45	46.6 ± 1.6	102.8
100	200	150	145.8 ± 2.7	97.2

[a] Expected = (sample 1 + sample 2)/2

Conclusions

We developed a time-dependent biosensor capable of rapidly quantifying trace levels of Hg^{2+} in drinking water using DNA aptamer and guanine chemiluminescence detection. The random coli structure of DNA aptamer in the presence of Hg^{2+} was transformed to a hairpin-link structure with the formation of T-Hg^{2+}-T complex. Also, guanines of DNA aptamer hybridized with cytosine to form relatively stable hairpin hairpin-link structure. Thus, the brightness of light generated from the reaction between TMPG and guanine of DNA aptamer not bound with Hg^{2+} was exponentially decreased with the increase of Hg^{2+} concentration. The concentration of T-Hg^{2+}-T complex was dependent on the incubation time of DNA aptamer and Hg^{2+} in PBS. The formation of T-Hg^{2+}-T complex was predominant until 20 min of incubation. Then, T-Hg^{2+}-T complex was decomposed with the dominant dehybridization of guanine and cytosine after a 20-min incubation. Thus, the sensitivity of biosensor operated with 20 min incubation was the best. In conclusion, we confirmed that the biosensor can be applied as a monitoring system capable of rapidly quantifying Hg^{2+} in drinking water based on the regulation of US EPA.

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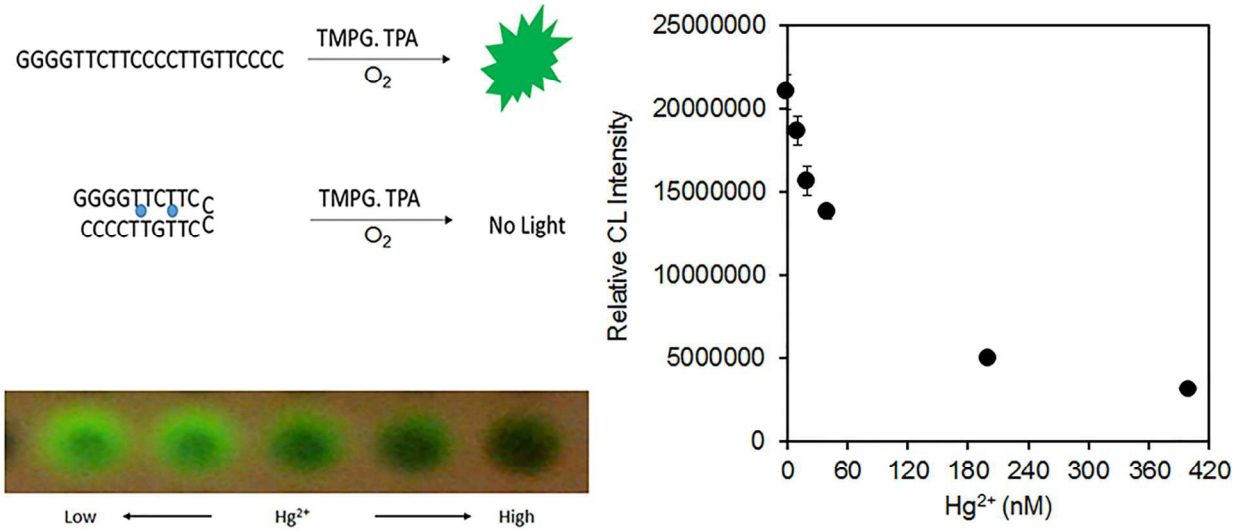
Graphical Abstract



Using DNA aptamer (T-Hg^{2+}-T hairpin-DNA) and guanine chemiluminescene detection, a highly sensitive biosensor was developed for the rapid quantification and monitoring of Hg^{2+} in drinking water.