Strategic Approaches for Highly Selective and Sensitive Detection of Hg$^{2+}$ Ion Using Mass Sensitive Sensors

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Here we present a quartz crystal microbalance (QCM) sensor for the highly selective and sensitive detection of Hg$^{2+}$ ion, a toxic chemical species and a hazardous environmental contaminant. Hg$^{2+}$ ion can be quantitatively measured based on changes in the resonance frequency of QCM following mass changes on the QCM sensor surface. The high selectivity for Hg$^{2+}$ ion in this study can be obtained using a thymine-Hg$^{2+}$-thymine pair, which is more stable than the adenine-thymine base pair in DNA. On the other hand, gold nanoparticles (AuNPs) and their size-enhancement techniques were used to amplify the QCM signals to increase the sensitivity for Hg$^{2+}$ ion. With this strategic approach, the proposed QCM sensor can be used to quantitatively analyze Hg$^{2+}$ ion with high selectivity and sensitivity. The detection limit was as low as 98.7 pM. The sensor failed to work with other metal ions at concentrations 1000-times higher than that of the Hg$^{2+}$ ion. Finally, the recovery does not exceed 10% of the original value for the detection of Hg$^{2+}$ ion in tap and bottled water. The results indicate acceptable accuracy and precision for practical applications.

Keywords Hg$^{2+}$ ion sensor, stem-loop oligonucleotides, quartz crystal microbalance (QCM), selectivity, sensitivity, signal amplification

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Introduction

Environmental pollution caused by heavy metals is one of the serious threats around the world worldwide resulting in countless deaths and disabilities among humans. The most common toxic heavy metals, including arsenic, cadmium, mercury, chromium, and lead, are associated with health and environmental hazards. Mercury is a widespread heavy metal contaminant derived from diverse anthropogenic and natural sources. Mercury ion (Hg$^{2+}$), one of the most common and stable contaminants, is a hazardous environmental contaminant and extremely toxic to humans. It can cause damage to the brain, kidneys, nervous system and immune system. The Hg$^{2+}$ ion is a highly toxic chemical species that can easily penetrate biological systems via the food chain or high-dose exposure. Diseases such as Hunter-Russell syndrome, acrodermatitis, Alzheimer’s disease, and Minamata disease are attributed to mercury exposure. Therefore, Hg$^{2+}$ ion has been listed as a priority pollutant by many countries and international agencies. The maximum allowable levels of Hg$^{2+}$ ion in drinking water as defined by the World Health Organization (WHO) and the United States Environmental Protection Agency (EPA) are 30 and 10 nM, respectively. Therefore, a simple and inexpensive method of Hg$^{2+}$ ion sensing with high sensitivity and specificity is required. Various traditional methods for the analytical detection of Hg$^{2+}$ ion have been proposed, including atomic absorption and fluorescence spectrometry, inductively coupled plasma mass spectroscopy, gas chromatography and high-performance liquid chromatography. However, these approaches are not suitable for routine Hg$^{2+}$ ion detection because they require complicated and expensive equipment, and involve tedious sample preparation. On the other hand, the use of biosensors for the detection of Hg$^{2+}$ ion has the advantage of high selectivity provided by biological recognition and high detection sensitivity due to the application of signal transduction technologies, such as optical, electrochemical, and piezoelectric biosensors.

Piezoelectric biosensors, especially quartz crystal microbalance (QCM) biosensor, are attractive alternatives for the detection of Hg$^{2+}$ ion. They represent versatile techniques, for successful and practical application of Hg$^{2+}$ ion biosensor. QCM biosensors measure mass change per unit area on the sensor surface with exquisite sensitivity, speed and reliability. In addition, their simple construction offers experimental and cost efficiency. Changes in the resonance frequency are directly proportional to mass changes, which facilitates real-time detection of biochemical molecules on the sensor surface without any labeling requirement. Despite these advantages, QCM biosensors are well known for their low sensitivity and reproducibility in the presence of low sample concentrations of target analytes. Furthermore, the problem of signal distortion due to undesirable and non-specific adsorption on the sensor surface always remains to be resolved.

In this study, we designed a QCM biosensor with high sensitivity and specificity for the simultaneous detection of Hg$^{2+}$ ion. The specific interactions between Hg$^{2+}$ ion and the nitrogen atom of thymine (thymine-Hg$^{2+}$-thymine pair) were combined with the QCM surface for highly selective Hg$^{2+}$ ion detection.

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It is well known that thymine-thymine (T-T) mismatched spots in DNA duplexes selectively capture Hg$^{2+}$ ion to form T-Hg$^{2+}$-T complex.\textsuperscript{26} The stability of the T-Hg$^{2+}$-T pair is much higher than that of the A-T pair, which is attributed to the specific interaction between Hg$^{2+}$ and nitrogen atoms of thymine. Hence, this specific interaction has been extensively used to design DNA sensors for Hg$^{2+}$ ion detection and to distinguish Hg$^{2+}$ ion from other heavy metal ions.\textsuperscript{20–28} On the other hand, gold nanoparticles (AuNPs) and their size enhancement were used to amplify QCM signals to increase the detection sensitivity of Hg$^{2+}$ ion. We used a gold staining process to increase the detection sensitivity by utilizing a reducing agent, NH$_2$OH•HCl, to reduce the HAuCl$_4$ to metallic gold, which was deposited on the surface of AuNPs attached to the oligonucleotides.\textsuperscript{29} The size-enhanced gold nanoparticles following gold staining increase the sensitivity by maximizing the frequency changes of the QCM sensor.

**Experimental**

Reagents and apparatus

3-(Glycidoxypropyl)-trimethoxysilane (3-GPTMS), 6-amino-1-hexanol, gold(III) chloride trihydrate (chloorauric acid, HAuCl$_4$) and hydroxylamine hydrochloride (NH$_2$OH•HCl) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 5′-Aminohexyl-labeled oligonucleotides (5′-H$_2$N-(CH$_2$)$_6$-CGGCTGCTTTTTTCAGCGG-3′, stem-loop structure) and 3′-mercaptohexyl-labeled oligonucleotides (5′-HS-(CH$_2$)$_6$-GGCCTGTITTTTGCAC) were obtained from Bioneer (Daejeon, Korea). The cation sources mentioned in the text contain chloride as anion: mercury(II) chloride, cobalt(II) chloride, cadmium(II) chloride, nickel(II) chloride, lead(II) chloride, copper(II) chloride, silver(I) chloride, manganese(II) chloride, iron(II) chloride, and zinc(II) chloride, which were also obtained from Sigma-Aldrich Chemical Co. Absolute ethanol was purchased from Junsei Chemical Co. (Tokyo, Japan). Saline sodium citrate (SSC) buffer (20X) was also purchased from Sigma-Aldrich and was diluted with deionized water to 1X solution before use. Doubly distilled water was obtained from the Milli-Q Water Purification System (18 MΩ cm) and used to make all aqueous solutions including buffer dilution.

Instrumentation

A QCM measurement system comprising detection and fluidic modules yielded real-time sensor responses during the Hg$^{2+}$ ion detection assays. The mass loading effect on the surface of 9 MHz AT-cut quartz crystal resonators (5 mm in diameter) coated with silicon dioxide (SiO$_2$) was recorded by QCM922A from Princeton Applied Research (SEIKO EG&G Co., Ltd.). The fluidic module was comprised with an ISM597 ISMATEC peristaltic pump (Zürich, Switzerland), a in-house made fluidic cartridge, and a silicone gasket. Figure 1 shows the QCM detector and in-house made fluidic modules. It contained fluidic connectors to allow diffusion across QCM devices. The flow of the sample and buffer solution into reaction chambers in the fluidic block was aided by the peristaltic pump. The volume of the reaction chamber formed by the silicon gasket was 20 L, and the flow rate was maintained at 1.0 mL/min. After each run, the reaction chambers and the silicone rubber gaskets were thoroughly rinsed with doubly distilled water and 0.1% Tween 20 (Sigma-Aldrich, MO, USA) in a pH 7.0 phosphate-buffered solution. Fluid was introduced into or out of the chamber using silicon peristaltic pump tubing (0.030 in. i.d., Thomas Scientific, Swedesboro, NJ). The cost-effectiveness of this fluidic platform is very favorable due to the high effectiveness of the multi-use possibility. This fluidic platform can also be used semi-permanently when accompanied by careful management, such as washing with dilute hydrochloric acid after the entire assay process including gold staining enhancement. However, the QCM sensor chip and silicone gasket are disposable, which can be used only 2 – 3 times by a piranha solution (98% sulfuric acid/30% hydrogen peroxide = 3/1, v/v) treatment after proceeding the assay, including gold staining enhancement.

Preparation of oligonucleotide-modified AuNPs

Gold nanoparticles, ca. 19 nm in diameter, were prepared by the citrate reduction of chloroauric acid (HAuCl$_4$) and capped with a 3′-mercapto-terminated single stranded oligonucleotide (5′-HS-(CH$_2$)$_6$-GGCCTGTITTTTGCAC) according to a procedure reported previously.\textsuperscript{31} To prepare oligonucleotide-modified AuNPs, 1.0 mL of AuNPs was mixed with 5.0 μL of Tween 20 and 1.0 mL of 1.0 μM mercapto-terminated oligonucleotide and left for 24 h at room temperature. The mixture was centrifuged for 15 min at 6000 rpm. Finally, the supernatant was decanted, and the red precipitate re-dispersed in 1.0 mL of doubly distilled water. We repeated this process twice.

Surface modification of QCM sensor chip

A SiO$_2$–coated AT-cut QCM resonator (SEIKO EG&G) was washed sequentially with doubly distilled water, absolute ethanol and doubly distilled water again. The QCM sensor chip
was placed in a plasma cleaner for 10 min followed by incubation in 5% (vol/vol) 3-GPTMS in methanol for 1 h. The sensor chip was rinsed with methanol for 2 min and dried under nitrogen. The 3-GPTMS-modified sensor was then baked at 110°C for 1 h in the oven followed by washing with methanol and drying under nitrogen. The 5'-amino-modified stem-loop oligonucleotides (5'-H2N-(CH2)6-CCGCTGCTTTTTTCAGCGG-3') were attached to the 3-GPTMS-modified QCM sensor surface according to the following protocol: The 5'-amino-modified oligonucleotides were dissolved in 100 mM sodium phosphate buffer at pH 8.5 and 25 μM concentration. After dropping the 10 μL of oligonucleotide solution, the QCM sensor chips were incubated for 24 h in a humidity chamber at 37°C. The excess epoxide groups were deactivated for 30 min at 37°C in a solution of 50 mM ethanolamine in 100 mM Tris buffer pH 8.5. The sensor chips were washed with doubly distilled water, in a solution containing 1X SSC and finally with doubly distilled water. The sensor chips were desiccated for storage at room temperature until use.

Detection of Hg2+ ion by QCM

We performed all experiments in triplicate at room temperature (25°C). The QCM analysis of Hg2+ ion was conducted by mixing 5.0 μL solutions of oligonucleotide-modified AuNPs, 20 μL of various Hg2+ ion concentrations in doubly distilled water, and 15 μL of pH 7.0 phosphate-buffered solution. Then, the mixed solutions were added into the stem-loop oligonucleotide-modified QCM sensor surface and the sensor chip followed by incubation for 30 min at room temperature. After washing with a phosphate-buffered solution for 1 min, a gold staining solution consisting of 50 μL gold(III) chloride trihydrate (10 mM) and 50 μL hydroxylamine hydrochloride (20 mM) was introduced to the reaction chamber for 2 min, which resulted in a catalytic deposition of metallic gold onto the AuNPs captured to oligonucleotides on the sensor surface. Then, the sensing surface was rinsed again with phosphate-buffered solution briefly. In these whole processes, the changes of the mass on the sensor surface were detected as changes in the resonance frequency in real time.

Results and Discussion

Sensing and signal amplification in Hg2+ detection

The sensing and signal amplification principle for the detection of Hg2+ ion based on the surface-attached stem-loop oligonucleotide and oligonucleotide-modified AuNPs in combination with gold staining strategy is illustrated in Scheme 1. Initially, the surface-attached oligonucleotides (5'-H2N-(CH2)6-CCGCTGCTTTTTTCAGCGG-3') carry a stable stem-loop structure that is complementary to the six bases at both ends. In the presence of AuNP-oligonucleotide conjugates and Hg2+ ion, the surface-attached oligonucleotides and AuNP-oligonucleotide conjugates with four T-T mismatches formed stable double-stranded oligonucleotides via six complimentary pairs and four T-Hg2+-T pairs, and the signal was enhanced by gold staining. In the absence of Hg2+ ion, however, no double-stranded oligonucleotides formed between the surface-attached oligonucleotides and the AuNP-oligonucleotide conjugates because the stem-loop structure of the surface-attached oligonucleotides was stabler than the double-stranded oligonucleotides formed. Thus, a highly specific and sensitive detection method for Hg2+ ion can be developed based on such signal detection and amplification strategy.

Dependence of QCM sensor response on Hg2+ ion concentration

Figure 2 represents the QCM sensor response to the addition of 10 nM Hg2+ ion with AuNP-oligonucleotide conjugates and the subsequent addition of gold staining reagents. As expected, the addition of Hg2+ ion and AuNP-oligonucleotide conjugates to the surface immobilized by the stem-loop oligonucleotide resulted in a decrease in frequency. The frequency decreased over time as gold (O) deposition on the captured AuNPs increased the mass significantly over the sensor surface, according to Sauerbrey’s equation. Notably, the major contribution to the decrease in frequency during the entire reaction occurred during the gold staining step. In the absence of gold staining, the signal is faint and undetectable due to the poor sensitivity.

We investigated the variation in the resonance frequency with the formation of AuNP-conjugated double-stranded oligonucleotides combined with gold staining as a function of
applied Hg^{2+} ion concentration in the phosphate-buffered solution at pH 7.0. Measurements were performed in triplicate for each Hg^{2+} ion concentration, the results are presented in Fig. 3. In the blank experiment (0 M Hg^{2+} ion concentration), we observed the frequency change upon the addition of a solution containing AuNP-oligonucleotide alone without Hg^{2+} ion to the stem-loop oligonucleotides attached to the sensor surface. In the absence of Hg^{2+} ion, no significant changes in the frequency were observed (223.8 ± 32.9 Hz), suggesting that the proposed detection method was effective only in the presence of Hg^{2+} ion. As the concentration of Hg^{2+} ion increased from 1.0 pM to 10 μM, the frequency changes also increased logarithmically with a correlation coefficient of 0.981. We calculated a detection limit as low as 98.7 pM, which is defined as three times the standard deviation of duplicate measurements of a blank sample. In addition, percent coefficient of variation (%CV), a measure of reproducibility, was less than 10% for all concentrations. Here, %CV is defined as the percent ratio of the standard deviation to the mean. It shows the extent of variability in relation to the mean of the population. The results revealed that the Hg^{2+} ion sensor designed in this study had a detection limit and linearity comparable to those of recently reported sensors.33–39 Table 1 shows the reported LOD values for detecting Hg^{2+} ion with the reported methods.

| Detection method                  | Sensor         | LOD/nM | Reference |
|----------------------------------|----------------|--------|-----------|
| Thymine-Hg^{2+}-thymine pair     | Fluorescence   | 35.0   | 27        |
| Thymine-Hg^{2+}-thymine pair     | Electrochemiluminescence | 0.002 | 6         |
| Thymine-Hg^{2+}-thymine pair     | Electrochemical| 0.01   | 28        |
| Thymine-Hg^{2+}-thymine pair     | Chemiluminescence | 12.0  | 29        |
| Thymine-Hg^{2+}-thymine pair     | Electrochemical| 0.06   | 30        |
| Thymine-Hg^{2+}-thymine pair     | Fluorescence   | 3.0    | 31        |
| Thymine-Hg^{2+}-thymine pair     | Fluorescence   | 0.035  | 32        |
| Thymine-Hg^{2+}-thymine pair     | SERS           | 5.0    | 33        |
| Thymine-Hg^{2+}-thymine pair     | QCM            | 0.099  | This work |

**Hg^{2+} ion selectivity of the QCM sensor**

To evaluate the Hg^{2+} ion selectivity of the proposed method, the QCM resonance frequency changes induced by other metal ions (Co^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Cu^{2+}, Ag^{+}, Mn^{2+}, Fe^{3+}, Zn^{2+}) were analyzed. Figure 4 represents the results of experiments using various metal ions, which indicated that the proposed method for Hg^{2+} ion detection was essentially unaffected by the presence of other metal ions, even if the concentrations of other metal ions were 1000-times higher than that of Hg^{2+} ion (1.0 μM versus 1.0 nM Hg^{2+}). As is well known, the selective binding of Hg^{2+} ion to the thymine-thymine (T-T) mismatched base pairs in double-stranded oligonucleotides plays a key role in differentiating it from other metal ions. Therefore, the excellent detection selectivity for Hg^{2+} ion is attributed to the highly specific interaction of T–Hg^{2+}–T, which induces structural changes in the stem-loop oligonucleotide structure to Hg^{2+}-specific double-stranded oligonucleotides (containing several T-T mismatched base pairs).

**Detection of Hg^{2+} ion in real water samples**

We demonstrated that the proposed QCM-based Hg^{2+} ion sensor has high sensitivity and selectivity under laboratory conditions. However, the real-world application of this QCM-based Hg^{2+} ion sensor was confirmed using tap water and commercial bottled water samples prepared with Hg^{2+} ion concentrations of 0, 25, 50, and 100 nM, respectively and the sample solutions were analyzed using the QCM sensors and the results are summarized in Table 2. The values of the frequency changes of tap water and bottled water without Hg^{2+} ion were almost similar to those of distilled water, indicating that Hg^{2+} ion was not present in the two real samples. We used the standard curves displayed in Fig. 3 to compare the Hg^{2+} ion concentrations of the eight sample solutions prepared above and calculated the recovery rates. As shown in Table 2, the recovery yields were calculated and the average values in real tap water.
sensitivity of the Hg$^{2+}$ ion. Through this strategic approach, our enhancement to amplify the QCM signal for increased detection of duplexes. In addition, AuNPs were subjected to size optimization that is a highly toxic and hazardous heavy metal pollutant. Hg$^{2+}$ is a well-known toxic metal and the development of a highly selective and sensitive detection method for it is of great importance. The proposed QCM sensor quantitatively analyzed Hg$^{2+}$ ion with high selectivity compared with other metal ions in this study. The ion can be quantitatively measured by frequency changes based on the change in mass due to the AuNPs attached to the sensor surface. The oligonucleotide and gold staining of the sensor surface. The on the change in mass due to the AuNPs attached to the sensor surface. The oligonucleotide and gold staining of the sensor surface. The on the change in mass due to the AuNPs attached to the sensor surface. The oligonucleotide and gold staining of the sensor surface. The on the change in mass due to the AuNPs attached to the sensor surface. The oligonucleotide and gold staining of the sensor surface.

Figure 4: Selectivity of the proposed QCM-based Hg$^{2+}$ ion sensor. The concentration of Hg$^{2+}$ ion is 1.0 nM and that of the other metal ions (Co$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, Cu$^{2+}$, Ag$^{+}$, Mn$^{2+}$, Fe$^{3+}$, Zn$^{2+}$) is 1.0 μM.

Table 2: Detection of Hg$^{2+}$ ion in tap water and bottled water samples using the proposed QCM-based sensors and ICP-MS methods.

| Sample          | Hg$^{2+}$, added/ nM | Hg$^{2+}$, found/ nM | Recovery, % | QCM | ICP-MS | QCM | ICP-MS |
|-----------------|---------------------|---------------------|-------------|-----|--------|-----|--------|
| Tap water       | 0                   | <0.1                | —           | —   | —      | —   | —      |
|                 | 25                  | 27.2                | 26.1        | 108.8 | 104.4 | —   | —      |
|                 | 50                  | 54.2                | 52.8        | 108.4 | 105.6 | —   | —      |
|                 | 100                 | 107.8               | 104.7       | 107.8 | 104.7 | —   | —      |
| Bottled water   | 0                   | <0.1                | —           | —   | —      | —   | —      |
|                 | 25                  | 26.8                | 25.8        | 107.2 | 103.2 | —   | —      |
|                 | 50                  | 53.9                | 52.1        | 107.8 | 104.2 | —   | —      |
|                 | 100                 | 107.1               | 103.9       | 107.1 | 103.9 | —   | —      |

The recovery rates of the two methods appear to have good correlation and the recoveries of the proposed QCM-based Hg$^{2+}$ ion sensors were within 10% indicating acceptable accuracy and precision for practical application.

Conclusions

In this study, we successfully demonstrated a QCM-based sensor for highly selective and sensitive detection of Hg$^{2+}$ ion that is a highly toxic and hazardous heavy metal pollutant. Hg$^{2+}$ ion can be quantitatively measured by frequency changes based on the change in mass due to the AuNPs attached to the oligonucleotide and gold staining of the sensor surface. The high Hg$^{2+}$ ion selectivity compared with other metal ions in this sensor system was obtained using a T-Hg$^{2+}$-T pair, known to be more stable than the adenine-thymine base pair in DNA duplexes. In addition, AuNPs were subjected to size optimization to amplify the QCM signal for increased detection sensitivity of the Hg$^{2+}$ ion. Through this strategic approach, our proposed QCM sensor quantitatively analyzed Hg$^{2+}$ ion with high selectivity and sensitivity. The limit of detection was 98.7 μM and the sensor failed to work with other metal ions even if their concentrations were 1000 times higher than that of Hg$^{2+}$ ion. Finally, in the detection of Hg$^{2+}$ ion spiked in the tap water and bottled water, the recovery did not exceed 10% of the original value, which indicated the accuracy and precision for practical application in Hg$^{2+}$ ion detection.

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