

Impedimetric Hg^{2+} Detection on Multilayered Reduced Graphene Oxide-Modified Electrode

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Recent efforts toward the development of electrochemical biosensors have emphasized the incorporation of nano-materials, such as carbon nanotubes, metal nanoparticles, and graphene into the electrode. This incorporation, particularly of graphene, significantly improves the sensor sensitivity and selectivity.^{1,2} Graphene, a single-atom-thick two-dimensional sp^2 carbon network material, serves as a good support for biomolecules due to its flat surface, large surface area, and rich π -conjugation structure.³⁻⁷ The combination of graphene and DNA has attracted much interest in the biosensor field. Recently, the interaction between hairpin DNA (hp-DNA) and graphene surface have been used to detect single nucleotide polymorphisms (SNP).^{5,8} Hp-DNA is a secondary DNA structure in which two regions of the same DNA strand form base-pairs in a double helix capped by an unpaired loop. The presence of the unpaired loop permits hp-DNA to readily adsorb onto graphene surfaces.

The development of efficient methods for detection of trace Hg^{2+} is of significant importance for human health and the environment because Hg^{2+} is an extremely toxic heavy metal ion. Numerous strategies including optical and electrochemical methods for the detection of Hg^{2+} have been developed.⁹ Among them, electrochemical impedance spectroscopy (EIS) as a label-free method, is highly attractive for biosensing assay due to its high sensitivity and simplicity.¹⁰

Herein, we report an Hg^{2+} detection on the reduced graphene oxide (RGO) surfaces modified with hp-DNA using EIS. The RGO-modified electrode surface was prepared by the chemical reduction of layer-by-layer (LbL) assembled graphene oxide (GO) and polyethyleneimine (PEI). The hp-DNA used here as the probe DNA included a loop region containing several thymine bases. The hp-DNA was immobilized onto the RGO-modified indium-tin-oxide (ITO) electrode surface. Hg^{2+} may be captured between thymine-thymine (T-T) mismatched base pairs to form a duplex structure stabilized by covalent bonds between Hg^{2+} and the thymine residues (T- Hg^{2+} -T),¹¹ which results in removal of hp-DNA from graphene surface, as shown in Figure 1(a).

EIS was used to monitor the interfacial changes in the electrode surface after exposure to an Hg^{2+} solution. The charge transfer resistance of a redox couple was recorded using the RGO-modified ITO electrode surface after exposure

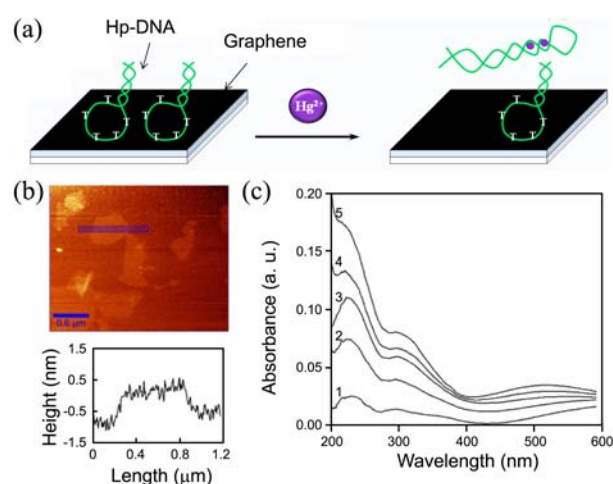


Figure 1. (a) A schematic representation for the interaction of Hg^{2+} with hp-DNA on the graphene surface. (b) AFM image of GO sheets deposited on the aminopropyltriethoxysilane-modified silicon surface (top) and sectional analysis (bottom). (c) UV-vis spectra of LbL assembly of GO and PEI on ITO-coated quartz slide surfaces with different number of bilayers.

to the probe hp-DNA and the subsequent interactions with Hg^{2+} in the buffer solution. To the best of our knowledge, this is the first report of using RGO-modified surface for the electrochemical impedimetric detection of Hg^{2+} . Furthermore, Hg^{2+} detection using hairpin DNA immobilized on the RGO-modified electrode surface has never been reported.

GO was observed by atomic force microscopy (Figure 1(b)), which confirms that individual single-layer GO sheets with a thickness of ~ 1 nm. GO is readily dispersed in water due to its intrinsic oxygen-containing groups, including phenol, carbonyl, and epoxide groups.¹² The presence of these groups enables the easy adsorption of GO onto positively charged surfaces through electrostatic interactions.¹³ GO may be assembled into a multilayer film *via* the layer-by-layer (LbL) assembly methods.^{14,15} A positively charged surface was prepared by coating polyethyleneimine (PEI) onto an ITO-coated glass surface using a dip-coating method. The LbL assembly of GO and PEI on the ITO surface (this electrode is referred to here as GO/ITO) proceeded by alternately immersing the electrode into a PEI solution and a

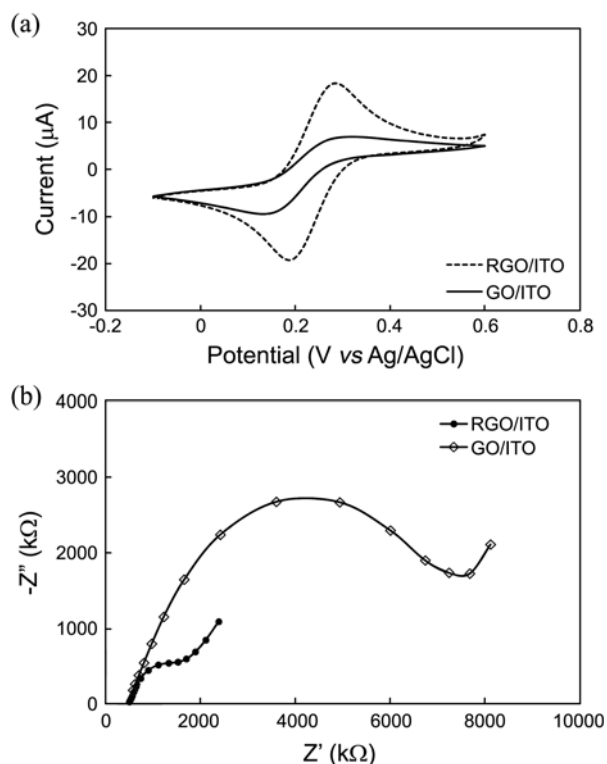


Figure 2. (a) Cyclic voltammograms of GO and RGO-modified ITO surfaces in 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution at a scan rate of 50 mV/s, (b) Nyquist plots for GO and RGO-modified ITO electrode in the same solution.

GO-dispersed solution for a total of 5 alternating cycles. The resulting ITO surfaces obtained from the LbL assembly were characterized by UV-Vis spectroscopy. For this work, we used ITO-coated quartz slide instead of ITO-coated glass. The UV-Vis spectra exhibited a maximum absorption peak at 217 nm as displayed in Figure 1(c), which is consistent with the previous results.¹⁶ The absorbance at the peak increases gradually with increasing number of layers, indicating that the LbL assembly was successfully achieved.

The LbL assembly on the ITO-coated glass surface was then exposed a hydrazine monohydrate solution to chemically reduce GO to reduced graphene oxide (RGO).^{14,15,17} The modified electrode surfaces were characterized by cyclic voltammetry (CV) and EIS before and after the reduction of GO. CV and EIS were carried out using a redox couple of $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$. As shown in Figure 2(a), the GO/ITO surface showed a larger peak separation and a smaller peak current in the CV for the redox couple due to the insulating properties of GO and the repulsive interactions between the negatively charged GO sheets and the redox couple; however, a significant decrease in the peak separation and an increase in the peak current were observed after the reduction of GO to RGO. This behaviour arose from a decrease in the negatively charged species on the modified surface and the recovery of a conjugated system through the reduction of GO to RGO, resulting in a high conductivity and electrode reactivity. The charge transfer resistance of the redox couple on the modified electrodes was also monitored.

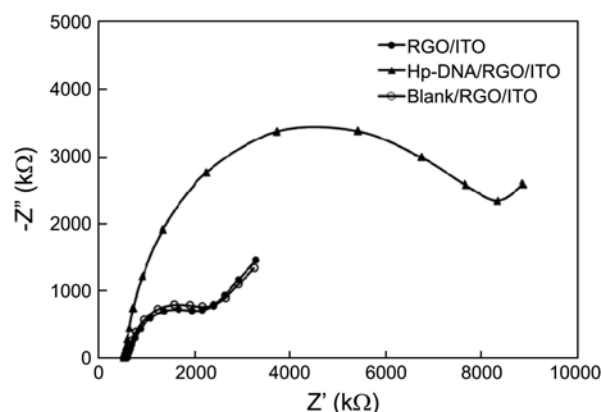


Figure 3. Nyquist plots for GO and RGO-modified ITO electrode in 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution after the modification with the hp-DNA and exposure to a blank buffer solution.

As shown in Figure 2(b), the results were consistent with the CV results. The charge transfer resistance of the RGO-modified ITO surface significantly decreased after the reduction step.

The hp-DNA was immobilized by physical adsorption onto the RGO-modified ITO surface.^{8,18} The EIS technique was used to monitor the charge transfer resistance of the redox couple on the RGO-modified electrode surface after the immobilization of the probe hp-DNA. The introduction of an hp-DNA probe layer sterically hindered the redox couple, $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$, from gaining access to the electrode surface. Electrostatic repulsion between the negative charges on the phosphate backbone of the hp-DNA and the negatively charged redox couple increased the charge transfer resistance (Figure 3). In the negative control experiment, the RGO-modified ITO surface was exposed to a blank buffer solution. As shown in Figure 3, no significant changes in the charge transfer resistance were observed. These results indicated that hp-DNA was efficiently adsorbed onto the RGO-modified ITO surface.

The charge transfer resistance of the redox couple was observed after the immobilization of the probe hp-DNA and the subsequent exposure to Hg^{2+} . Figure 4(a) shows a Nyquist plot showing the imaginary impedance (Z'') and the real impedance (Z') over a range of frequencies, 10 kHz to 0.05 Hz. As shown in Figure 4(a), the charge transfer resistance of the redox couple decreased gradually as the concentration of Hg^{2+} increased. Higher Hg^{2+} concentrations are expected to induce the hybridization and dissociation of hp-DNA from the electrode surface due to the formation of T- Hg^{2+} -T bonds.¹⁴ Figure 4(b) shows the changes in the difference ratio of the charge transfer resistance $(R_{DNA}-R_{ct})/R_{DNA}$ (R_{DNA} : the charge transfer resistance after immobilization of hp-DNA onto the RGO-modified ITO surface, R_{ct} : the charge transfer resistance after exposure to different concentrations of Hg^{2+} in solution) as a function of the logarithm of Hg^{2+} concentration. As shown in Figure 4(b), $(R_{DNA}-R_{ct})/R_{DNA}$ was linearly related to the logarithm of Hg^{2+} concentration over a range of Hg^{2+} concentrations from 1 nM to 1 μ M, with a linear correlation coefficient (R^2) of 0.948, indicating

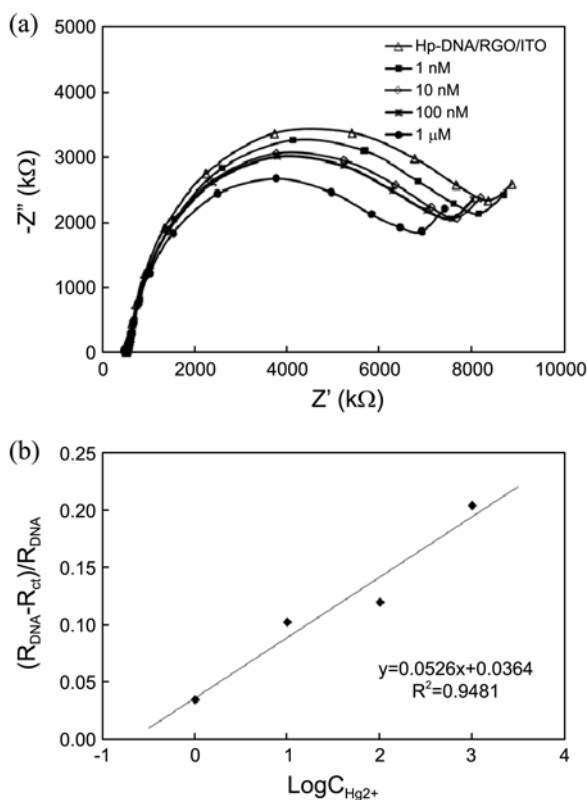


Figure 4. (a) Nyquist plots for hp-DNA-modified RGO surface in 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution after immersing in various concentrations of Hg^{2+} . (b) Plot of a relationship between $(R_{DNA}-R_{ct})/R_{DNA}$ and the logarithm of Hg^{2+} concentration.

that the sensor is highly sensitive, which is comparable to the previous report on electrochemical Hg^{2+} sensor employing amplification procedure.¹⁹

The selectivity of the impedimetric Hg^{2+} sensor was investigated by testing the sensor response to three other divalent ions: Ba^{2+} , Mg^{2+} , or Ni^{2+} . As shown in Figure 5(a), these metal ions did not significantly alter the charge transfer resistance of the redox couple, whereas Hg^{2+} exhibited an obvious decrease in the charge transfer resistance of the redox couple, even at concentrations 10 times lower than the concentration of the other divalent metal ions. Figure 5(b) shows that the difference ratio of the charge transfer resistance was the highest upon exposure of the modified surface to a 100 nM Hg^{2+} solution. This indicated that the other divalent metals did not interact with the hp-DNA on the sensor. Hence, the impedimetric Hg^{2+} sensor based on an RGO-modified ITO surface displayed good selectivity toward Hg^{2+} .

In conclusion, we developed a new strategy for detecting Hg^{2+} using RGO as an electrode material as well as a platform for the probe immobilization. An RGO-modified electrode surface was prepared by chemically reducing GO and PEI layers that had been LbL-assembled on an ITO electrode surface. Hp-DNA was for the first time employed as a probe DNA for the capture of Hg^{2+} . The charge transfer resistance of a redox couple on the probe-immobilized RGO surface decreased with increasing Hg^{2+} concentration due to the release of hp-DNA from the RGO surface. The electro-

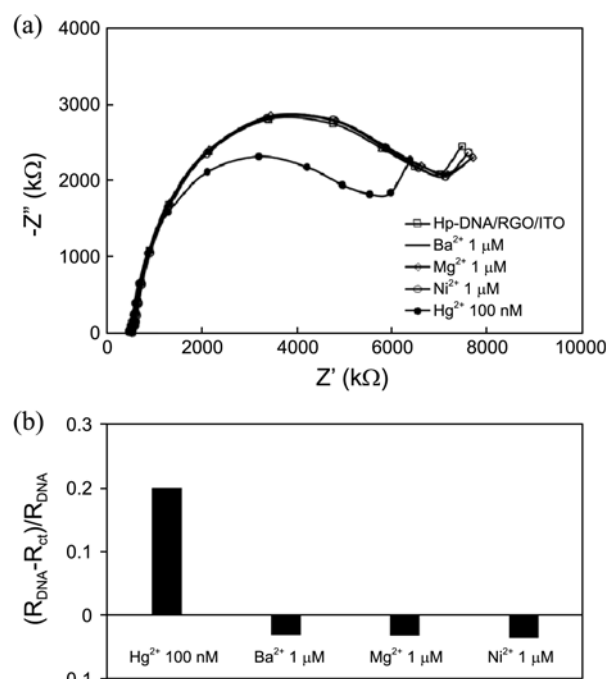


Figure 5. (a) Nyquist plots for various divalent metal ions, including Ba^{2+} , Mg^{2+} , Ni^{2+} , Hg^{2+} (Hg^{2+} : 100 nM, other metal ions: 1 μM). (b) $(R_{DNA}-R_{ct})/R_{DNA}$ change for Hg^{2+} and other divalent ions.

chemical sensor permitted highly sensitive and selective detection of Hg^{2+} . The combination of hp-DNA and LbL assembly of RGO surface is expected to contribute to developing high performance biosensors.

Experimental

DNA oligonucleotides were purchased from Genotech (Korea). The sequence is 5'-CCGGTTTTTTT TTTTTTTT CCGG-3'. Polyethyleneimine (PEI), graphite powder (< 150 μm), hydrazine monohydrate, potassium ferricyanide, potassium ferrocyanide, mercury nitrate (II) were purchased from Sigma Aldrich. Borate buffer (BB; 0.1 M, pH 9), Phosphate buffer (PBS; 0.01 M, 100 mM $NaNO_3$). All buffer and all other aqueous solutions were made with 18.2 M Ω pure water.

Graphite oxide was synthesized from graphite powder by a modified Hummers method. Graphite powder (3 g) was taken in an Erlenmeyer flask and stirred in ice bath. $NaNO_3$ (1.5 g) and concentrated H_2SO_4 (69 mL) were added into the Erlenmeyer flask. Then $KMnO_4$ (9 g) was added slowly and the mixture was stirred for 3 h. 120 mL of deionized (DI) water was added slowly over 30 min. The mixture was stirred for 10 min at 110 $^{\circ}C$. DI water (200 mL) was added, and followed by slow addition of 30% H_2O_2 (3 mL) solution. The color of the suspension changed from brown to yellow. The suspension was filtered, washed with 5% HCl to remove metal ions, and washed with DI water to remove the acid. It was then repeatedly centrifuged and decanted. The graphite oxide was collected and dried in vacuum for 2 days at RT. GO dispersion in water was prepared by bath sonication for

1 h. Finally, the prepared GO dispersion was centrifuged at 3000 rpm for 5 min to remove any unexfoliated graphite oxide (which was extremely small amount) and the upper solution was taken for future experiments.

Prior to the surface modification, ITO-coated quartz or glass slides were cleaned by rinsing with ethanol, acetone, and water. After drying with N₂ gas, the electrodes were immersed in piranha solution (H₂SO₄:H₂O₂ = 3:1) for 5 seconds, washed with sufficient amount of water, and finally dried with N₂ gas. LbL-assembly of GO and PEI on the ITO surfaces were conducted by alternately immersing the ITO electrodes in a 1 mg/mL PEI aqueous solution and a 0.5 mg/mL aqueous dispersion of GO, each for 5 min, and the assembly was performed for 5 cycles. The RGO-modified ITO surfaces (referred to here as RGO/ITO) were prepared by dipping the GO/ITO electrodes into hydrazine monohydrate solution for 20 h.

RGO/ITO electrodes were exposed to 10 μM of hp-DNA for 1.5 h at room temperature followed by washing with water and drying with N₂ gas to obtain hp-DNA/RGO/ITO surface. Afterward, the surfaces were exposed to various concentrations of Hg²⁺ ion solutions and incubated for 30 minutes. For negative control experiment, same procedure was followed without Hg²⁺ ion. To test the specificity of the sensor, other divalent metals including Ba²⁺, Mg²⁺, Ni²⁺ ion were used in place of Hg²⁺. Cyclic voltammetry and EIS were carried out with Ivium Compactstat (B07014) potentiostat interfaced with a PC. An electrochemical cell consisting of the modified ITO electrode as a working electrode in connection to Ag/AgCl and Pt wire as a reference and counter electrode respectively. Electrochemical impedance measurements were recorded between 10 kHz and 0.05 Hz at the formal potential of redox couple. The experiments were carried out at room temperature using 1 mM K₄[Fe(CN)₆]/K₃[Fe(CN)₆] (1:1 molar ratio) as a redox couple in BB (0.1 M, pH 9). Randle equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plot in

the complex plane.

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