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Technical Note

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Insertion Approach: Bolstering the Reproducibility of Electrochemical Signal Amplification via DNA Superstructures

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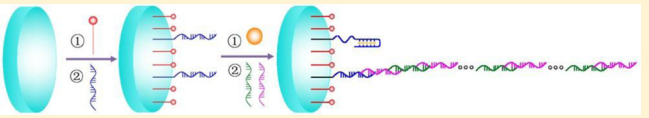
Supporting Information

ABSTRACT: For more than a decade, the backfilling approach for the immobilization of DNA probes has been routinely adopted for the construction of functional interfaces; however, reliably reproducing electrochemical signal amplification by this method is a challenge. In this research, we demonstrate that the insertion approach significantly bolsters the reproducibility of electrochemical signal amplification via DNA superstructures. The combination of the backfilling approach and the DNA superstructure formation poses a big challenge to reliably reproducing electrochemical signal amplification. In order to use the detection of Hg^{2+} as a prototype of this new strategy, a thymine-rich DNA probe that is specific to mercury ion was applied in this study. The presence of Hg^{2+} induces the folding of the DNA probes and inhibits the formation of DNA superstructures. By using electroactive probes ($[Ru(NH_3)_6]^{3+}$) that are electrostatically adsorbed onto the double strands, differential pulse voltammetry (DPV) could quantitatively confirm the presence of Hg^{2+}. A limit of detection (LOD) and a limit of quantification (LOQ) (LOQ) as low as 0.3 and 9.5 pM, respectively, were achieved. Furthermore, excellent selectivity and real sample analysis demonstrated the promising potential of this approach in future applications.



The self-assembly of DNA monolayers on gold electrodes is a topic of great interest for the electrochemical sensor community.[1] For more than a decade, the backfilling approach, which entails the shoehorning of an alkanethiol into the void spaces between constituents of a preassembled DNA monolayer, has become the mainstream.[2−4] Despite the tremendous progress achieved in this field, the reproducibility of electrochemical signals from DNA-modified electrodes still merits further investigation. One concern that prompts additional study is the competitive binding of the alkanethiols and DNA thiol groups to the gold surface, leading to the diffusion of the DNA probes along the surface and the formation of aggregated domains. Consequently, the interprobe distance among the DNAs in the monolayer becomes inhomogeneous, with smaller spacings in the aggregated domains than the average interprobe distance calculated from measurements via the ensemble techniques. The uncontrollable heterogeneity caused by local aggregation[5,6] is unfavorable for the reproducibility of the signal amplification via large nano/microstructures; instead, the signal amplification prefers low probe density. To achieve optimal low-probe density and avoid uncontrollable heterogeneity, strict control over experimental operations to construct reproducible electrochemical biosensors is required.

Suitable signal amplification in these electrochemical sensors has been achieved through the use of DNA superstructures, which consist of long DNA polymers hybridized from two single stranded DNAs that have partially complementary segments.[7] When the backfilling approach is used in combination with a DNA superstructure, electrochemical sensors show large electrochemical signal variations. To improve sensor quality in terms of hybridization efficiency and reproducibility, a monolayer with large void spaces between each DNA probe is required to eliminate the steric impedance and electrostatic repulsion between each superstructure.[8−12] Most recently, monolayers formed via the insertion approach, in which thiol moiety-labeled DNA is inserted into a loosely packed alkane monolayer, have met the above-mentioned requirement and have been systematically investigated by atomic force microscopy (AFM).[13] As compared with the backfilling approach, DNA probes can be inserted into an alkane monolayer at lower surface density,[14] which is highly advantageous for combination with DNA superstructures.

To date, no electrochemical sensor based on the combination of the insertion approach and DNA superstructures has been proposed. In this study, the distinct features of this concept were investigated, using the detection of Hg^{2+} as a sample case. Mercury ions are common heavy metal pollutants in the environment, and although toxic to humans, they can be consumed by bacteria. Further biological accumulation through the food chain imperils human health. Even low concentrations in the kidneys, hematopoietic system, or liver can result in serious consequences.[15−17] Therefore, the establishment of highly selective and sensitive methods to detect Hg^{2+} would be significant. Heretofore, the most commonly used techniques

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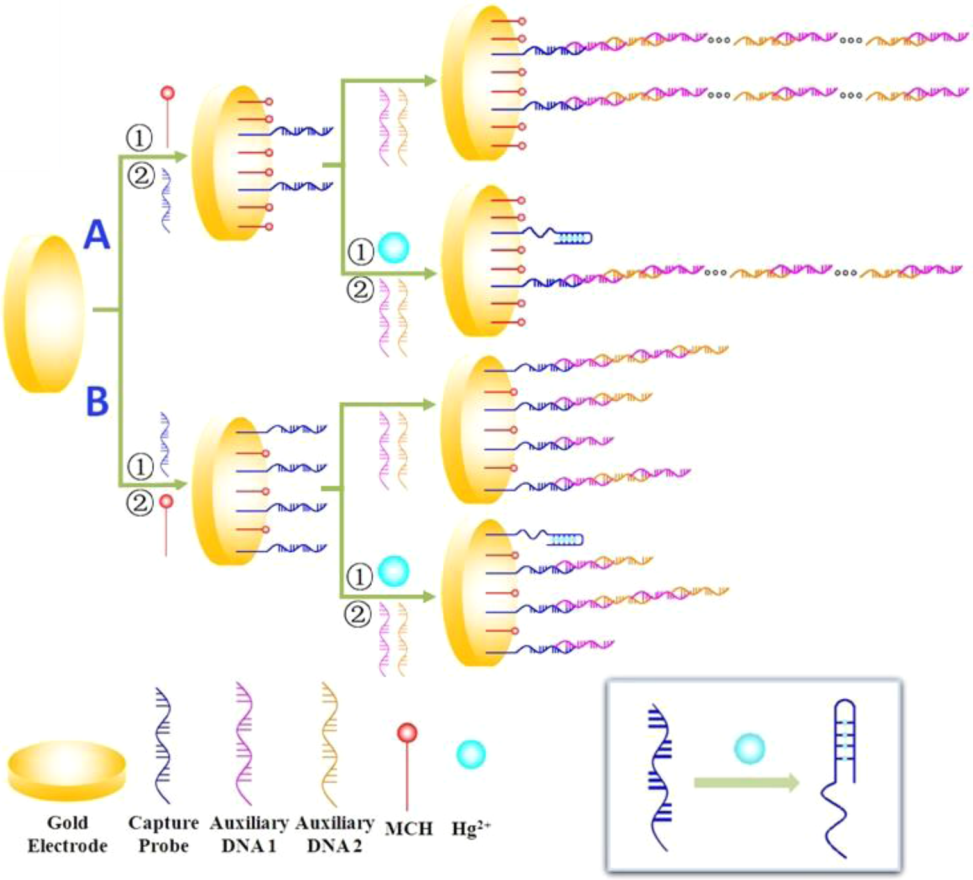
Scheme 1. Schematic Illustration for Detecting Mercury Ions Based on Electrochemical Signal Amplification by DNA Superstructures in Combination with (A) the Insertion Approach and (B) the Backfilling Approach[a]



[a]The capture probe forms thymine-Hg^{2+}-thymine complexes in the presence of Hg^{2+}.

for Hg^{2+} detection have been atomic absorption spectrometry (AAS),[18,19] cold-vapor atomic fluorescence spectroscopy (CV-AFS),[20,21] inductively coupled plasma−mass spectrometry (ICPMS),[22] inductively coupled plasma−atomic emission spectrometry (ICP-AES),[23] and surface-enhanced Raman scattering (SERS)[24] sensing. Although these techniques are sensitive and accurate for assaying Hg^{2+}, most rely on costly instruments with time-consuming procedures and are unsuitable for real field analysis. In recent years, although several electrochemical sensors have been constructed and shown to exhibit high sensitivity, their preparations have been based on the backfilling approach.[25,26]

In this study, we exploit a specific thymine-Hg^{2+}-thymine (T-Hg^{2+}-T) coordination: T-rich DNA probes immobilized on a gold electrode via the insertion approach can fold into hairpin structures in the presence of mercury ions, blocking further DNA polymer extension. The decrease in the current observed by differential pulse voltammetry (DPV) can be used to quantitatively determine the presence of Hg^{2+}. The DNA superstructures that grow from the probes on the electrode significantly amplify the change in the current peak. This new protocol for the construction of electrochemical sensors can provide reproducible electrochemical signals. Label-free detection is another advantage for our proposed scenario, which utilizes an electroactive complex ($[Ru(NH_3)_6]^{3+}$) as a signaling molecule that binds to the anionic phosphate backbone of DNA strands via electrostatic force.

EXPERIMENTAL SECTION

Materials. 6-Mercapto-1-hexanol (MCH), hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from J&K Scientific, Ltd. and used as received. Ethylenediaminetetraacetic disodium salt (Na_2EDTA), magnesium chloride hexahydrate, and sodium chloride were purchased from Beijing Chemical Co., Ltd. All other reagents were of analytical grade. The oligonucleotides were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and purified by HPLC; their sequences are listed in Table S1, Supporting Information. The capture probe was dissolved in immobilization buffer ("I-buffer": 10 mM Tris-HCl, 10 mM TCEP, 0.5 M NaCl, pH 7.4) for 1 h before use, to ensure the absence of probe dimers. All solutions were prepared with Milli-Q water (18.2 MΩ cm^{-1}) from a Millipore system.

Gel Electrophoresis. All the hairpin oligonucleotides were heated to 95 °C for 10 min before use to ensure the absence of dimers. Auxiliary DNA 1 (AD1, 2 μM) was incubated with Auxiliary DNA 2 (AD2, 2 μM) for 3 h in hybridization buffer ("H-buffer": 10 mM Tris-HCl, 0.5 M NaCl, pH 7.4) before gel electrophoresis analysis. The agarose gel concentration was 1%, prepared by using 1× TAE buffer. The agarose gel electrophoresis was run at 50 V/10 cm for 30 min, followed by visualization under UV light and photography with a digital camera.

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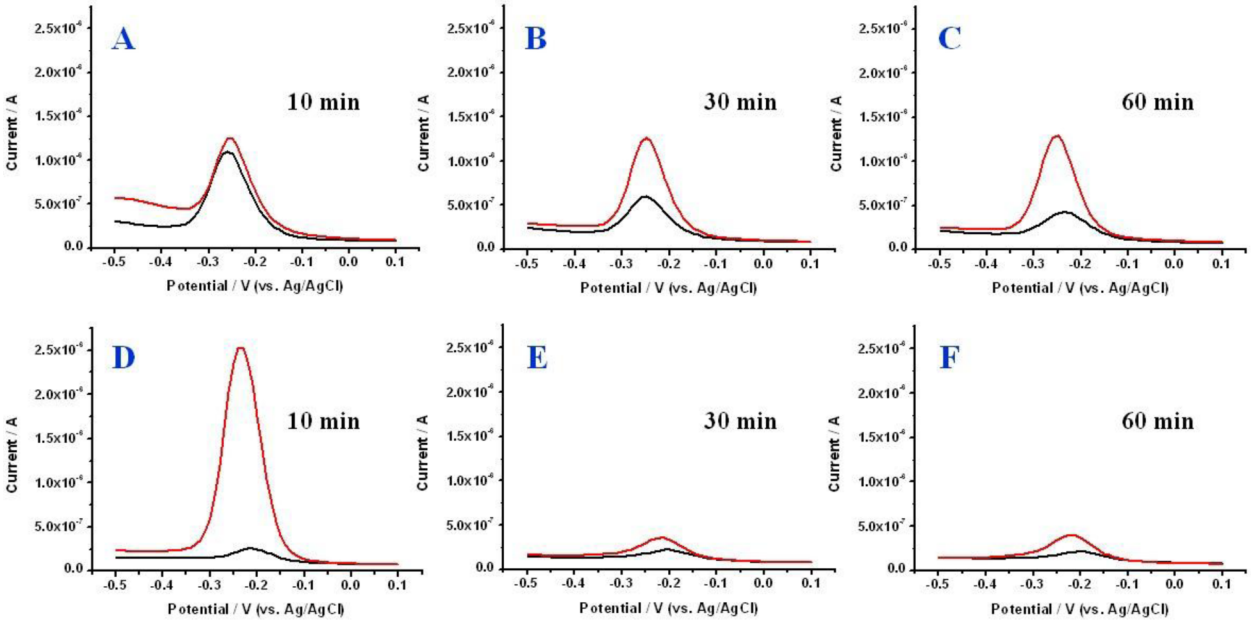

Figure 1. Optimization of the experimental conditions for the backfilling approach (A, B, and C) and the insertion approach (D, E, and F) at a fixed concentration of MCH (50 μM). The red and black lines represent the electrochemical signals corresponding to the capture probe and the DNA superstructure after assembly, respectively.

Electrode Pretreatment. The gold electrode (2 mm diameter) was successively polished with 1.5, 0.5, and 0.05 μm alumina slurries (Tianjin AidaHengsheng Technology Co., Ltd.) until a mirror surface was formed. Then, the electrode was sonicated in ultrapure water followed by ultrapure water and ethanol (1:1 v/v) for 30 s each to remove the residual Al_2O_3 powder. After this treatment, the gold electrode was immersed in freshly prepared piranha solution (1:3 v/v 30% H_2O_2/H_2SO_4) for 10 min and thoroughly rinsed with distilled water. *(Caution: piranha solution is highly caustic and must be used with care.)* Finally, the electrode was cleaned by electrochemical polishing with 30 successive cyclic voltammetry (CV) scans from +0.2 to +1.6 V vs Ag/AgCl in 0.5 M H_2SO_4 at 50 mV/s.[27−29] The prepared electrode was rinsed with ultrapure water and dried with nitrogen.

Sensor Preparation. The capture probe was freshly reduced by mixing 2 μM of the probe with I-buffer for 1 h. For heterogeneous monolayer preparation via the insertion approach, the cleaned gold electrode was immersed in 50 μM MCH solution for the proper time at room temperature, followed by incubation in the DNA probe solution for 16 h at 4 °C. Thereafter, the electrode was rinsed thoroughly with the washing buffer ("E-buffer": 10 mM Tris-HCl, pH 7.4). For the mixed monolayer derived from the backfilling approach, the above process was reversed and the MCH occupied the free interspaces between the DNA probes. The capture probe-functionalized electrode was subsequently incubated with the reaction buffer (50 mM HEPES, 100 mM $NaNO_3$, pH 7.4) containing different concentrations of Hg^{2+} for 1 h at 25 °C. Then, after rinsing the electrode with the E-buffer, the electrode was incubated with H-buffer containing 2 μM each of AD1 and AD2 for 3 h at 25 °C. The electrode was again rinsed with the E-buffer and dried with nitrogen before using for electrochemical detection.

Electrochemical Measurements. For each point in the calibration curve of electrochemical signal versus concentration, four measurements were replicated on the same electrode. We incubated the electrode modified with capture probes into the reaction buffer containing different concentrations of Hg^{2+} for 1 h to form T-Hg^{2+}-T complexes. Then, the electrode was incubated in H-buffer containing 2 μM AD1 and AD2 for 3 h to assemble the DNA superstructure, followed by a brief rinse with E-buffer. The electrode with the assembled DNA superstructure was immersed in the E-buffer containing 5 μM $[Ru(NH_3)_6]^{3+}$. The electrochemical measurements were performed on an electrochemical workstation (CH Instruments, Inc., Shanghai Chenhua Equipment, China) at room temperature using a three-electrode system consisting of the oligonucleotide-modified gold electrode as the working electrode, a silver/silver chloride electrode (Ag/AgCl) as the reference electrode, and a platinum wire as the counter electrode. DPV was carried out in E-buffer within the potential range from 0.1 to −0.5 V with potential increments of 0.01 V. The amplitude and pulse width were both 0.05 V, and the pulse period was 0.1 s.

Electrochemical Detection of Hg(II) in Lake Water. Lake water was collected from Nanhu Park (Changchun, China) and then filtered. Hg^{2+} was spiked into the lake water at different concentrations (0, 1, 10, 100 nM) without dilution. The electrochemical detection of Hg^{2+} was then performed in the same manner as that used for the buffer samples.

RESULTS AND DISCUSSION

The maximum contour length of the DNA superstructure assembled from two partial complementary DNAs can be more than 1 μm.[14] Once elongation is initiated on the surface, sufficient free space is necessary for successful assembly. The traditional DNA probe immobilization approach easily triggers aggregation that leads to irreproducible results when combined with DNA superstructures. The drawing in Scheme 1 illustrates the insertion approach given by pathway A and the backfilling approach represented by pathway B.

DNA Superstructure Characterization via Agarose Gel Electrophoresis. The oligonucleotides were heated to 95° for 10 min before use. Lanes 1, 2, 3, and 4 in the 1% agarose gel were

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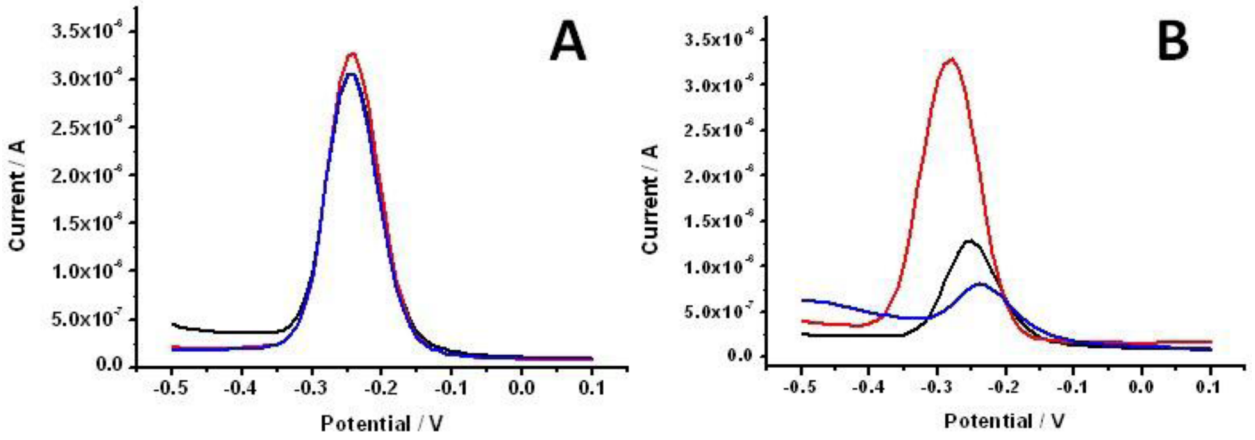

Figure 2. Reproducibility comparison between DPV signals via (A) the insertion approach and (B) the backfilling approach. Three independent experiments for each approach were carried out.

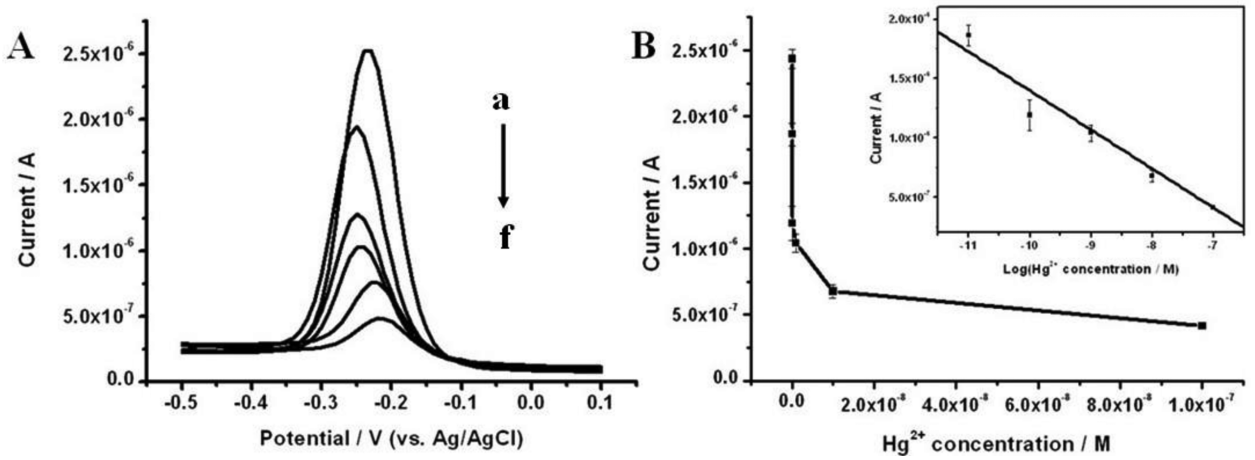

Figure 3. (A) DPV curves of various DNA superstructures assembled on the electrode in the presence of different amounts of Hg^{2+} (from a to f: 0, 10 pM, 100 pM, 1 nM, 10 nM, and 100 nM, respectively). (B) Plots of peak current versus the Hg^{2+} concentration. Inset is the linear relationship between the peak current and the logarithm of the Hg^{2+} concentration. The illustrated error bars represent the standard deviation from four repetitive measurements.

loaded with AD1 (2 μM), AD2 (2 μM), a mixture of AD1 (2 μM) and AD2 (2 μM), and a Trans 5K DNA marker, respectively. As shown in Figure S1, Supporting Information, the bands in lanes 1 and 2 clearly indicate the absence of high molecular weight constituents. Instead, the lane loaded with the mixture of AD1 and AD2 exhibits a continuous broad band that demonstrates a wide distribution of DNA superstructures. Assembly over 3 h allowed DNA superstructure formation with a maximum length above 500 base pairs, which was shown to be sufficient for signal amplification. The experimental conditions adopted for DNA assembly in solution were the same as those used for assembly on the electrode surface. Although the growth of the DNA superstructure in buffer solution concurs with the assembly process on the electrode, it has little influence on the reproducible assembly of DNA superstructures on the gold electrode.

Advantage of the Insertion Approach versus the Backfilling Approach. It has long been a concern that MCH addition into the preassembled DNA monolayer can displace the preexisting DNA probes tagged with thiol moieties and lead to irreproducible results. In the absence of signal amplification, the backfilling approach is good enough for many applications. However, for building a highly sensitive sensor, signal amplification is necessary, which entails enough free space around the DNA probes to allow the subsequent assembly. Furthermore, reproducibility is also another important criterion for sensor quality. In our case, a DNA superstructure more than 100 nm long was assembled on the gold electrode in a way that the requisite space between the DNA probes was large enough to achieve sufficient length.

Initially, to find the best conditions for both approaches, we fixed the DNA probe concentration at 2 μM and adjusted the concentration and the immobilization time for MCH. As shown in Figure 1A–C, a more than 30 min immobilization of 50 μM MCH that backfilled the interspaces between the preassembled DNA probes provided higher electrochemical signal amplification than a 10 min immobilization. The failure to observe a distinct change in the peak current before and after DNA superstructure assembly meant that a 10 min immobilization (Figure 1A) was insufficient to remove the nonspecifically absorbed DNA probes. We also utilized 1 mM MCH, which has been routinely used in previous studies;[14,30−33] the results are shown in the Supporting Information (Table S2 and Figure S2). Unfortunately, the electrochemical signal amplification (Figure S2, Supporting Information) did not appear to increase after DNA superstructure assembly. Furthermore, the reproducibility

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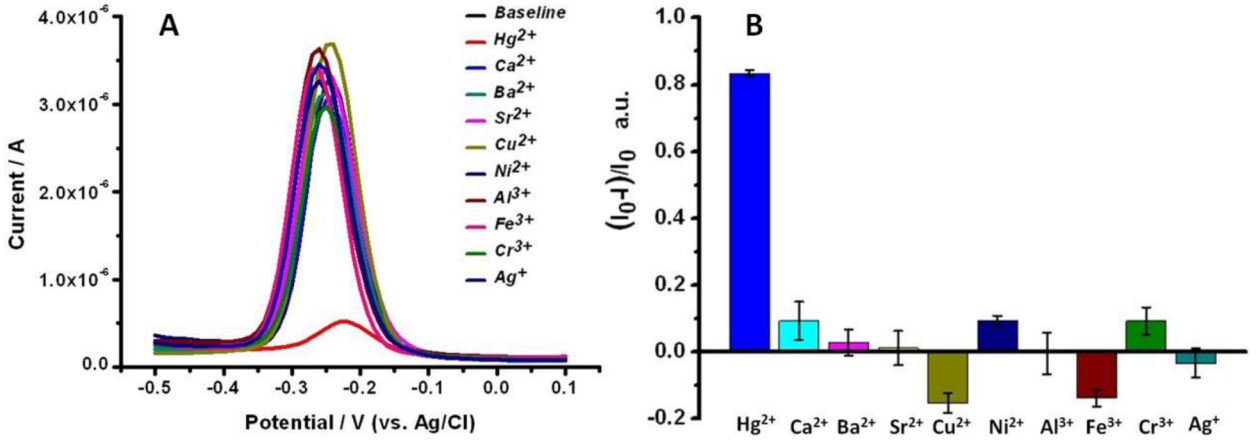

Figure 4. (A) DPV curves of various DNA superstructures assembled on the electrode in the presence of different metal ions. (B) Histograms of the peak currents of DPV curves of DNA superstructures in the presence of different metal ions. The concentration of each metal ion was both 100 nM. The illustrated error bars represent the standard deviation of four repetitive measurements.

based on the backfilling approach was much worse than previous expectations would suggest, as demonstrated by Figure 2B.

Driven by the impetus to solve the poor reproducibility and worse signal amplification problems posed by the backfilling approach, the insertion approach, which has been shown to allow sufficient space between DNA probes, was very appealing. As shown in Figure 1D, the electrochemical signal amplification corresponding to the difference in the peak currents was the largest with a 10 min immobilization in 50 μM MCH. According to the chronocoulometry method (Figure S3, Supporting Information) under the same experimental conditions, the DNA surface densities with the insertion approach and backfilling approaches are 1.03152×10^{12} and 2.4156×10^{12} molecules/cm^2, respectively. For longer immobilization times, the efficiencies of DNA probe binding and the subsequent DNA superstructure assembly were significantly suppressed. The number of defect sites after monolayer assembly was critical to the amount of the immobilized DNA probes via the insertion approach. Longer immobilization times (Figure 1E,F) and a higher concentration of MCH (Figure S2D, Supporting Information) were not favorable for this approach. By comparing the two approaches under the same experimental conditions (Figure 2A,B), it can be concluded that the reproducibility of the insertion approach was much superior to the backfilling approach.

Electrochemical Detection of Hg^{2+}. The electrochemical detection of Hg^{2+} was performed by incubating the capture probe-modified gold electrode for 1 h in aqueous solutions of Hg^{2+} with defined concentrations. After the DNA probes folded into loop structures via the formation of T-Hg^{2+}-T, the electrode was incubated in a DNA solution containing AD1 and AD2. The free DNA probes on the gold electrode acted as seeds to initiate DNA assembly on the surface. The assembly process occurred simultaneously on the electrode and in solution. By using $[Ru(NH_3)_6]^{3+}$ as a signaling molecule that could bind to the anionic phosphates of the DNA strands through electrostatic interactions, the DPV peak current was used to quantitate the Hg^{2+} concentration. As the Hg^{2+} concentration increases (Figure 3A), the DPV peak current correspondingly decreases. As shown in Figure 3B, the calibration curve at target ion concentrations from picomolar to submicromolar shows a linear relationship between the current peak and the log value of the Hg^{2+} concentration. A limit of detection (LOD) and limit of quantification (LOQ) as low as 0.3 and 9.5 pM, respectively, could be obtained based on this insertion approach. Although the absolute value of the voltage corresponding to the peak current was shifted toward to higher value as the Hg^{2+} concentration decreased, this feature did not degrade the quality of our sensor that combines the insertion approach and with signal amplification via DNA superstructure formation.

Selectivity of Hg^{2+} Detection. The specificity of this sensor for Hg^{2+} detection was evaluated using a variety of environmentally relevant metal ions, including Ca^{2+}, Ba^{2+}, Sr^{2+}, Cu^{2+}, Ni^{2+}, Al^{3+}, Fe^{3+}, Cr^{3+}, and Ag^+. We first investigated whether these metals could individually influence the detection sensitivity of Hg^{2+}. As shown in Figure 4A, the peak currents in the DNA superstructures showed no change in the absence or presence of other metal ions. The other metal ions presented only slight or negligible effects on the peak current of this sensor's detection system, indicating its outstanding specificity and selectivity (Figure 4B) for Hg^{2+} against other metal ions.

Recovery in Sample Analysis. To investigate whether this method was applicable to natural samples, we tested lake water spiked with three different concentrations of Hg^{2+}: 1, 10, and 100 nM. Possibly interfering materials present in the lake samples did not influence Hg^{2+} detection via the described method. As shown in Table S3, Supporting Information, the recovery of Hg^{2+} from spiked lake water samples demonstrates that the detection of Hg^{2+} in natural waters is quite feasible. Therefore, this sensor may be of great value for Hg^{2+} assays in real sample applications.

CONCLUSIONS

In this study, we adopted the insertion approach instead of the backfilling approach to build up an electrochemical sensor. We demonstrated that the insertion approach significantly improves the reproducibility of electrochemical signal amplification via DNA superstructure assembly. Using the detection of Hg^{2+} as a prototypical example, we demonstrated that DPV could quantitatively confirm the presence of Hg^{2+}. LOD and LOQ values as low as 0.3 and 9.5 pM, respectively, were achieved. Furthermore, excellent selectivity and real sample analysis demonstrate the system's promising potential in future applications. Other targets such as small molecules, DNAs, and proteins might also be detected by the combination of the insertion approach and signal amplification via DNA superstructure assembly. Along with our results based on the

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combination of the insertion approach and rolling circle amplification (Figures S4−S7, Supporting Information), we can envision many potential applications of this method beyond the scope of this study, including its integration with other amplification systems that require sufficient spacing.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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