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A multifunctional label-free electrochemical impedance biosensor for Hg^{2+}, adenosine triphosphate and thrombin

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ABSTRACT

A multifunctional label-free biosensor for the detection of Hg^{2+}, adenosine triphosphate and thrombin has been developed based on the changing of the electrochemical impedance spectroscopy (EIS) from the modified electrodes when nucleic acid subunits interacting with different targets. The modified electrode consists of three interaction sections, including DNA with T–T mismatch recognizing Hg^{2+} to form T–Hg^{2+}–T complex, split DNA chip against ATP, and DNA domin against thrombin to form G-quadruplex. Upon DNA interaction with thrombin or ATP, an increased charge transfer resistance (R_{ct}) had been detected. However, a decreased R_{ct} against Hg^{2+} was obtained. The R_{ct} difference (ΔR_{ct}) has relationship with the concentration of the different targets, Hg^{2+}, ATP and thrombin can be selectively detected with the detection limit of 0.03, 0.25, and 0.20 nmol L^{-1}, respectively. To separately detect the three analytes existing in the same sample, ATP aptamer, G-rich DNA strands and EDTA were applied to mask ATP, Hg^{2+} or thrombin separately.

1. Introduction

The development of biosensors for specific detection of bioanalyte has attracted particular attention due to their essential roles in clinical diagnostics [1], drug discovery [2] and environmental monitoring [3]. Among various biosensors, DNA-based ones are particularly interesting because of their high sensitivity [4], specificity [5] and easy regeneration capabilities [6]. The structural switching properties of DNA allow the biosensors to mediate target-responsive signal transduction. Over these years, great progresses have been made in developing DNA biosensors for the detection of small molecules [7,8] (e.g. cocaine or adenosine), proteins [9,10] (e.g. thrombin or lysozyme), or metal ions [11,12]. Although most of the biosensors are focused on monitoring single target analyte, many efforts have been taken to develop multifunctional DNA biosensors for the parallel detection of multiple target analytes [13]. For example, Du et al. [14] proposed a electrochemical impedance biosensor based on the integrated aptamer consisting of the adenosine triphosphate aptamer and the thrombin aptamer for the parallel detection of adenosine triphosphate (ATP) and thrombin with the detection limits of 1×10^{-8} and 1×10^{-11} mol L^{-1}, respectively. Deng et al. [15] adopted gold nanoparticles (AuNPs) as the signal amplifier and fabricated a sensitive bifunctional electrochemical biosensor for small molecule and protein. Lin et al. [16] designed a novel DNA-based electrochemical biosensor for the detection of three metal ions based on DNA interaction with Pb^{2+}, Ag^{+} and Hg^{2+}. These biosensors had been applied in real samples detection with satisfied results. However, to the best of our knowledge, no literature about simultaneous detection of metal ions, small molecules and proteins had been reported.

Electrochemical impedance spectroscopy (EIS) is one of the most generally used electrochemical techniques to monitor the properties of electrode surfaces. Any tiny changes on the electrode surfaces can be captured by impedimetric spectroscopy [17–19], which is widely employed in biosensors for monitoring bimolecular interactions or conformational changes. In this work, a sensitive multifunctional label-free electrochemical impedance biosensor is developed for the parallel detection of metal ion, small molecule and protein, and Hg^{2+}, ATP and thrombin were chosen as the example of metal ion, small molecule and protein. The targets could be captured onto the electrodes upon interaction with the sensing elements, in the present of Hg^{2+}, Hg^{2+} can insert into two DNA T–T mismatches, which leads to the formation of a T–Hg^{2+}–T pair, which elimination of the block of the electron-transfer reaction of the redox probe, however when the thrombin and ATP was introduced, the electrode surface formed an analogous insulating layer which retarded the interfacial electron transfer reaction of the redox probe, which further induces the changes of charge-transfer

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resistance (ΔR_{ct}). As a result, three different analytes coexisting in a sample could be detected separately by a single biosensor.

2. Experimental

2.1. Materials and reagents

All oligonucletides were synthesized and purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences of oligonucleotides used in this study are list as follow:

Capture probe DNA (S1): 5′SH-TCAGACTTTTTTTTACCTGGGG-GAG TATGGTTGGTGTGGTTGGACG-3′.

Detection probe DNA (S2): 3′AGTCTGTTTTTTTTTGGAAGGAGG CGT GGTTGGTGTGGTTGGACG5′.

Linker Probe DNA (S3): 5′CCTGCTTTCGTCC3′.

G-rich DNA: 5′GGTTGGTGTGGTTGG3′.

ATP aptamer DNA: 5′ACCTGGGGGAGTATTGCGGAGGAAGGT3′

The sequences of split DNA against ATP and thrombin aptamer were labeled by wave line and straight line, separately.

6-Mercaptohexano (MCH), cysteine, adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), anduridine triphosphate (UTP), bovine serum albumin (BSA), bovine Hemoglobin (BHb) and immunoglobulin G (IgG) were purchased from Sigma (St. Louis, MO) and used as received. The human serum had been got from the Hospital of Fuzhou University. Other chemicals were of analytical reagent grade and were used directly without further purification. Deionized water from a Millipore Milli-Q system was used throughout this work.

2.2. Sensor preparation

The Au electrodes (2 mm in diameter) were pretreated according to previous procedures [20]. Briefly, they were polished with various particle sizes (1.0 and 0.05 μm) of alumina slurry (Al_2O_3) and sonicated with pure water for 5 min and dried in a nitrogen stream to obtain clean gold electrode surfaces. They were electrochemically activated by consecutive potential scanning between −0.3–1.6 V in a fresh 0.5 mol $L^{-1}H_2SO_4$ solution under until stable cyclic voltammogram were obtained. Finally, they were rinsed with pure water and dried with nitrogen before modification. Capture DNA (S1) (10 μL) was dropped on surface of gold electrodes in a closed container at 37 °C for 2 h to form self-assemble monolayer, then the modified electrode was incubated into detection DNA (S2) (10 μL) at 37 °C for 2 h, and finally the electrodes was incubated into the buffer solution of linker DNA DNA (S3) (10 μL) for another 2 h before next experiment, and the final DNA concentration was 3.33 μmol L^{-1}. during the process, the capture DNA was modified onto the electrode surface first through thiol–Au interaction, then capture DNA was hybridized with detection DNA to form partially hybridized DNA (S1+S2), which was further hybridized with linker DNA (S3), finally the film of DNA (S1+S2+S3) was formed and modified to the electrode surface, then the electrode was incubated in 1 mM 6-mercaptohexanol (MCH) solution for 1 h to remove nonspecific DNA adsorption on the gold surface. The modified electrodes were finally incubated with different concentrations of Hg^{2+}, ATP and thrombin for 1, 2 and 1 h, respectively. For each step of immobilization, the EIS of the modified electrode was recorded while $[Fe(CN)_6]^{3-/4-}$ was used as redox probe. All experiments were carried out at laboratory ambient temperature.

2.3. Measurement

All electrochemical measurements were performed on a CHI Model 660D electrochemical analyzer (Chenhua Instruments, Shanghai, China) with a conventional three-electrode system. Au electrode, Ag/AgCl electrode and Pt wire were used as working electrode, reference electrode and counter electrode, respectively. EIS studies were performed in 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol L^{-1} KCl. The AC (alternating current) voltage amplitude was 5 mV, and the voltage frequency was from 100 kHz to 100 mHz. The applied potential was 250 mV vs Ag/AgCl. A Randles equivalent circuit was used to fit the obtained impedance spectra.

3. Results and discussion

3.1. The principle of the biosensor

The protocol of the proposed biosensor is shown in Fig. 1. The capture probe with an alkanethiol moiety at the 5-terminus covalently immobilized on a gold electrode through Au–S bond, then detection DNA was partly hybridized with the capture probe through the linker DNA, result in the formation of the film of DNA (S1+S2+S3) onto the electrode surface. Both the capture and the detection probes consist of DNA fragments that serve as the sensing elements, in which T-rich DNA sequence, aptamer domain and split ATP aptamer recognize Hg^{2+}, thrombin and ATP, respectively. On the one hand, DNA S1 and S2 can bind with Hg^{2+} to form T–Hg^{2+}–T structure and the formed duplex-like DNA can promote the electron transfer, which results in the reduce of the charge transfer resistance R_{ct}. On the other hand, the addition of ATP can induce recombination of DNA S1 and S2 since both of which contain the sequence of split ATP aptamer fragments, this make the enhancement of the rigid structure of DNA S1+S2+S3, thus resulting in an increase of R_{ct}. In addition, DNA S1 and S2 which contain thrombin aptamer could also specifically recognize thrombin. The hybridized parts among DNA strands S1, S2 and S3 are dehybridized to form thrombin-stabilized G-quadruplex. Although DNA strand S3 is free released from the electrode and may provide an easier penetration channel for the redox probe, the macromolecular protein thrombin can be captured on the electrode surface to increase film thickness, thus resulting in a significant enhancement of R_{ct}.

Simple experiments had been performed to verify the feasibility of the proposed biosensor. Fig. 2 shows the EIS at different conditions. The equivalent circuit (Fig. 2, inset) as the model of the working electrode was applied to fit the EIS data [21], where R_s, Z_w and C_d are

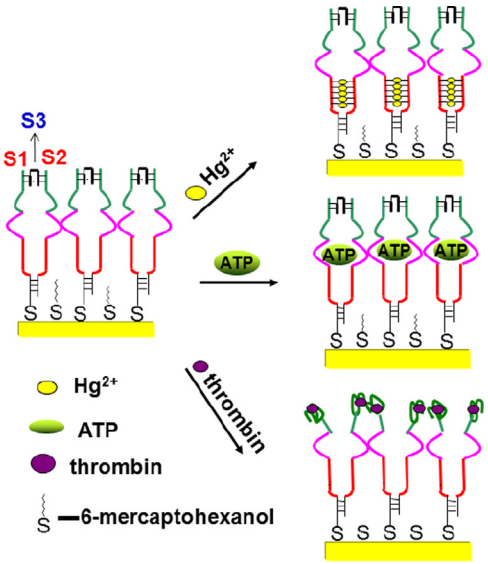

Fig. 1. Principle of the DNA-based EIS biosensor in response to Hg^{2+}, ATP and thrombin.

the electrolyte solution resistance, the Warburg impedance and the double layer capacitance, respectively. The most important parameter is R_{ct}, which reflects the electron transfer kinetics in the surfaces of the different electrodes. As shown in Fig. 2, curve *a* depicts the Nyquist diagram of the DNA S1+S2+S3 film modified gold electrode without analytes with R_{ct} of 2905 Ω. In the presence of Hg^{2+}, R_{ct} decreases to a smaller value (curve *b*, 1405 Ω) due to Hg^{2+} inserting into two DNA T–T mismatch and the formation of DNA(S1)/Hg/DNA (S2) complex as duplex DNA, thus enhancing the electron transfer ability of the redox probe on the electrode surface. Since ATP can induce the association of split aptamer fragments and the formation of DNA(S1)/ATP/DNA(S2) complex, the addition of ATP stabilizes the associated complex so that R_{ct} increases greatly (curve *c*, 5613 Ω). When thrombin is captured on the surface of Au electrode, R_{ct} increases significantly also (curve *d*, 4882 Ω) due to the bind of thrombin to the electrode surface, thus increasing the film thickness.

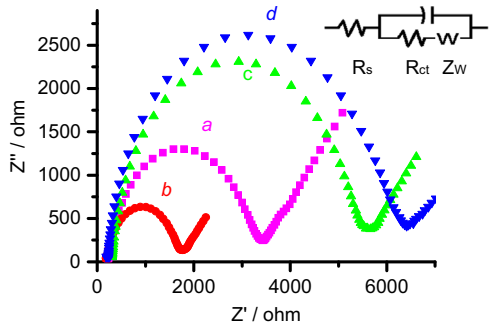

Fig. 2. Representative impedance spectra for the DNA biosensor before (a) and after incubating with 1 μmol L^{-1} Hg^{2+} (b); 1 μmol L^{-1} thrombin (c); 1 μmol L^{-1} ATP (d), respectively. The inset represent the equivalent circuit employed to fit the EIS measured data with 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ as redox probe.

3.2. Performance of the biosensor

In order to further investigate the analytical application, the biosensor was employed to detect Hg^{2+}, ATP and thrombin with various concentrations, respectively. As shown in Fig. 3A and Table 1, ΔR_{ct} decreased with increasing the concentration of Hg^{2+}, in which the ΔR_{ct} exhibited a linear relationship with the logarithm of Hg^{2+} concentration in the range from 5×10^{-7} to 1×10^{-10} mol L^{-1}. On the other hand, with the increase of the concentration of ATP, the formed DNA(S1)/ATP/DNA(S2) complex is progressively increased (Fig. 3B), which resulted in increased ΔR_{ct}. The linear responses of ΔR_{ct} against the logarithm of ATP concentration is from 1×10^{-6} to 1×10^{-9} mol L^{-1}. For thrombin, as shown in Fig. 3C, the increase of thrombin concentration caused the increase of ΔR_{ct}, until no distinct change in ΔR_{ct} was observed when the concentration of thrombin was decreased to 1 nmol L^{-1}. The ΔR_{ct} increased in line with the logarithm of thrombin concentration within a range from 1×10^{-6} to 1×10^{-9} mol L^{-1}. By the application of the R_{ct} difference (ΔR_{ct}), Hg^{2+}, ATP and thrombin were selectively detected with the detection limit of 0.03, 0.25 and 0.20 nmol L^{-1}, respectively, which was lower than the previous reported EIS sensor for Hg^{2+} [11], ATP [14] and thrombin [22]. The reproducibility of the electrochemical sensor

Table 1
The linear relationship between ΔR_{ct} and the logarithm of the concentrations of Hg^{2+}, ATP or thrombin.

	Linear regression equation (ΔR_{ct})	Regression coefficient (R)	Detection limit (mol L^{-1})
Hg^{2+}	303.78log$C$(Hg^{2+})+ 3208.16	−0.9918	3.0×10^{-11}
ATP	785.73log$C$(ATP)+ 7247.91	−0.9928	2.5×10^{-10}
Thrombin	531.46log$C$(thrombin)+ 5202.32	−0.9854	2.0×10^{-10}

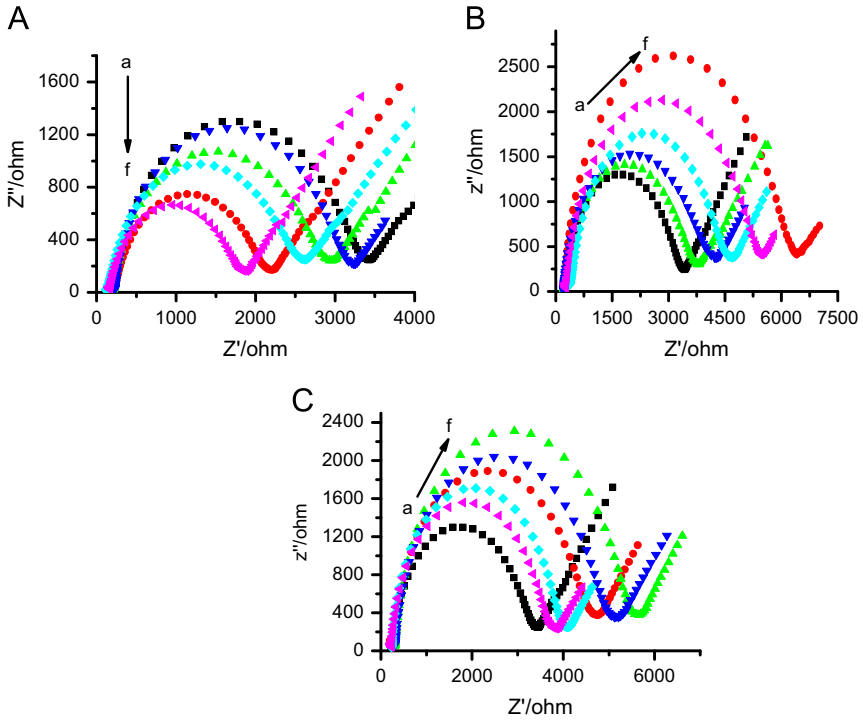

Fig. 3. Impendence spectra upon incubation with various concentrations of Hg^{2+}, ATP and thrombin at ambient temperature, (A) Hg^{2+} (a: 1×10^{-10} mol L^{-1}; b: 1×10^{-9} mol L^{-1}; c: 1×10^{-8} mol $^{-1}$; d: 1×10^{-7} mol $^{-1}$; e: 5×10^{-7} mol $^{-1}$; f: 0 mol L^{-1}); (B) ATP (a: 0 mol L^{-1}; b: 1×10^{-9} mol $^{-1}$; c: 5×10^{-9} mol $^{-1}$; d: 1×10^{-8} mol $^{-1}$; e: 1×10^{-7} mol $^{-1}$; f: 1×10^{-6} mol $^{-1}$); (C) thrombin (a: 0 mol $^{-1}$; b: 1×10^{-9} mol $^{-1}$; c: 5×10^{-9} mol $^{-1}$; d: 1×10^{-8} mol $^{-1}$; e: 1×10^{-7} mol $^{-1}$; f: 1×10^{-6} mol $^{-1}$).

was also investigated. Every five freshly modified electrodes were tested for the targets, respectively. All electrodes showed nearly similar electrochemical response, in which the relative standard deviation is 3.8% for Hg^{2+}, 3.0% for ATP and 4.8% for thrombin detection. The sensor still retained bioactivity after being stored at 4 °C for four weeks and impedance signal reduced less than 5%. These results suggest that the biosensor exhibits good reproducibility.

3.3. Separately detection of analytes

The introduced label-free multifunctional electrochemical biosensor could separately detect three different analytes. As mentioned above, in contrast with a decreased R_{ct} in response to Hg^{2+}, ATP and thrombin could induce an increased R_{ct}. When the signal is reduced upon the addition of the sample, we could first suppose that the sample contains Hg^{2+}, but it is still unable to determine whether the sample is a mixture. In order to exclusively detect Hg^{2+}, ATP or thrombin, masking agents was used to differentiate the electrochemical signals due to different analytes. EDTA, ATP aptamer and G-rich DNA were thus used for masking Hg^{2+}, ATP and thrombin, respectively. The mixture of EDTA and ATP aptamer successfully mask Hg^{2+} and ATP for the detection of thrombin. The mixture of EDTA and G-rich DNA was used for masking Hg^{2+} and thrombin for the detection of ATP, whereas the mixture of G-rich DNA and ATP aptamer was used for masking thrombin and ATP for the detection of Hg^{2+}. As shown in Fig. 4, it is demonstrated that the exclusive detection of Hg^{2+}, ATP and thrombin existing in the buffer solution could be achieved, respectively.

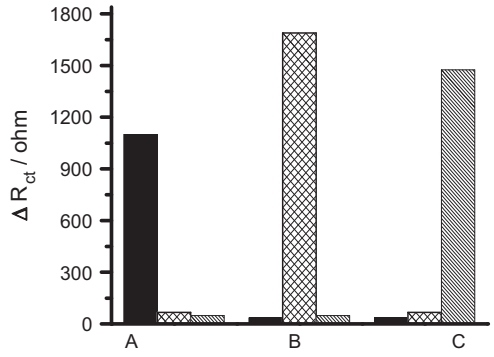

Fig. 4. (A) Specific detection of Hg^{2+}, ATP and thrombin existing in the buffer solution. (A) In the presence of ATP aptamer and G-rich DNA for Hg^{2+} determination; (B) in the presence of G-rich DNA and EDTA for ATP determination; (C) in the presence of ATP aptamer and EDTA for thrombin determination. The concentrations of masking reagents were both 10 μmol L^{-1}, and the targets were 100 nmol L^{-1}.

3.4. Interference study and sample detection

In order to explore the application of this biosensor in practical detection, the specificity of the multifunctional biosensor was also investigated. The interfering experiments were made when the analyte and the interfering species present in the same system. Other metal ions including Mg^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Ca^{2+}, Fe^{3+}, Ag^{+} and Pb^{2+} were used as the interferents. It was revealed that only Hg^{2+} caused a considerable decrease of the R_{ct} (Fig. 5A). This is due to these metal ions could not insert into a T–T base pair to form a stable structure such as T–Hg^{2+}–T, and T–T base pair is only selective to Hg^{2+}. On the other hand, cytidine triphosphate (CTP), guanidine triphosphate (GTP) and anduridine triphosphate (UTP) were used in the control experiments to assess the specificity of the biosensor for the detection of ATP. As shown in Fig. 5B, significant changes in the EIS response were unobserved in presence of other triphosphates, implying that other nontarget molecules do not interact with the biosensor and thus not interfere with the detection of ATP. As for the detection of thrombin, bovine serum albumin (BSA), bovine hemoglobin (BHb) and immunoglobulin G (IgG) were used as the interferents to examine the selectivity of the sensor (Fig. 5C). It was found that the EIS response in the present of other interferents protein, the EIS response for thrombin was not found significant change. These results demonstrate that this biosensor displays a sufficient

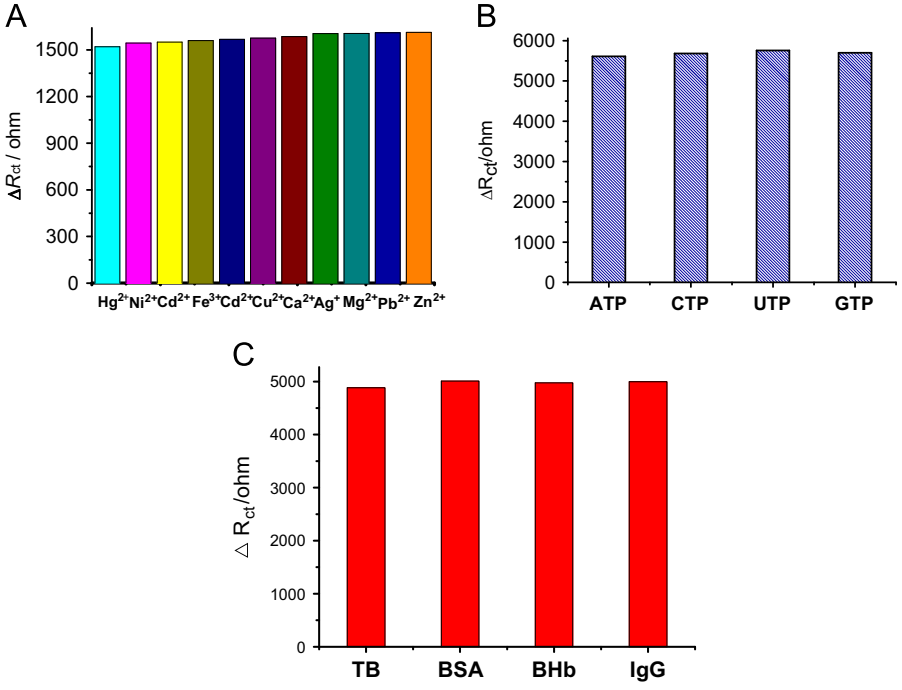

Fig. 5. Selectivity of the electrochemical biosensor. (A) Selectivity of the biosensor toward Mg^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Ca^{2+}, Fe^{3+}, Ag^{+} and Pb^{2+} in the present of ATP and ATP. (B) EIS response for cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP) in the present of ATP and ATP. (C) EIS response for bovine serum albumin (BSA), bovine Hemoglobin (BHb) and immunoglobulin G (IgG) in the present of thrombin and thrombin. All the concentrations of tested molecules were used at 10^{-6} mol $^{-1}$.

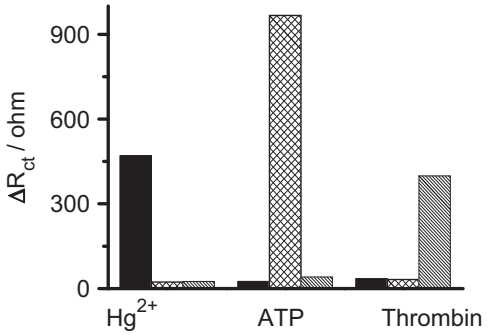

Fig. 6. Specific detection of Hg^{2+}, ATP and thrombin in the human serum in the presence of masking reagents, the concentrations of masking reagents were both 10 μmol L^{-1}, 10^{-8} mol L^{-1} Hg^{2+}, 10^{-8} mol L^{-1}ATP and 10^{-9} mol L^{-1} thrombin.

specificity for the exclusive detection of Hg^{2+}, ATP and thrombin, respectively.

To validate the possibility of the proposed biosensor in a real application, the human serum samples were spiked with different concentrations of Hg, ATP and thrombin, the concentration of Hg^{2+} is 1.5 mg/100 mL (75 nmol L^{-1}) which is defined by American Conference of Governmental Industrial Hygienists (ACGIH). Therefore, the concentrations in human serum were chosen to contain 1×10^{-8} mol L^{-1} Hg^{2+}, 1×10^{-8} mol L^{-1} ATP, and 1×10^{-9} mol L^{-1} thrombin, respectively. As shown in Fig. 6, simultaneous detection of three targets with masking reagents in the human serum was achieved. The results indicated that the biosensor has great potential for practical application.

4. Conclusions

An efficient, label-free DNA-based electrochemical impedance biosensor has been developed for the detection of Hg^{2+}, ATP and thrombin by taking advantage of the unique properties of split DNA fragment/target interaction and the specific aptamer target recognition. Hg^{2+} could interact with T–T mismatch even in the presence of other competitive metal ions to form T–Hg^{2+}–T so that the electron transfer is highly enhanced and the sensor showed high specificity for mercury(II). ATP could bind to partially split aptamer fragment and thrombin interacts with G-rich DNA strands to be preferentially adsorbed on the DNA modified Au electrode, both of which weakens the electron transfer. Based on this principle, we have successfully detected Hg^{2+}, ATP and thrombin at a concentration down to 0.03, 0.25 and 0.2 nmol L^{-1}, respectively. This electrochemical biosensor exhibits obvious advantages over the conventional methods. First, by combining of the unique properties of split aptamer fragment/target interaction and the specific aptamer-target recognition, label-free detection has been developed which makes the fabricating process simple and convenient, it took only 1, 2 and 1 h for the further detection Hg^{2+}, ATP, thrombin, respectively. Second, DNA could be prepared easily at low cost to afford a cheap biosensor. The multifunctional DNA-based biosensor shows good selectivity, reproducibility, and the useful life could retained for weeks for detection, which provides a promising platform for the electrochemical parallel detection of metal ions, small molecules and proteins, respectively. Moreover, the biosensor was applied to analyze targets in human serum with satisfactory results, which provide it could be used in the analysis of targets in complex samples.

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