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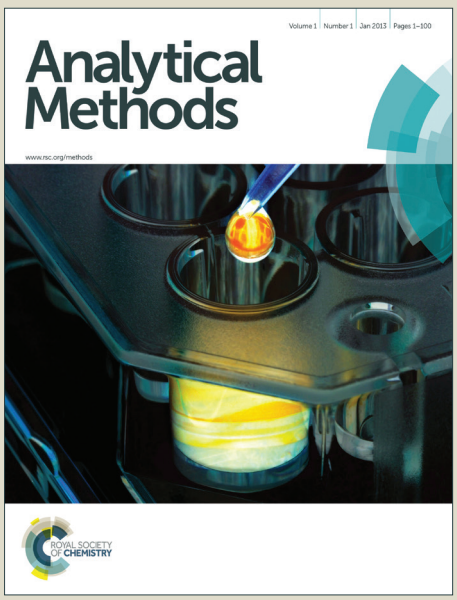

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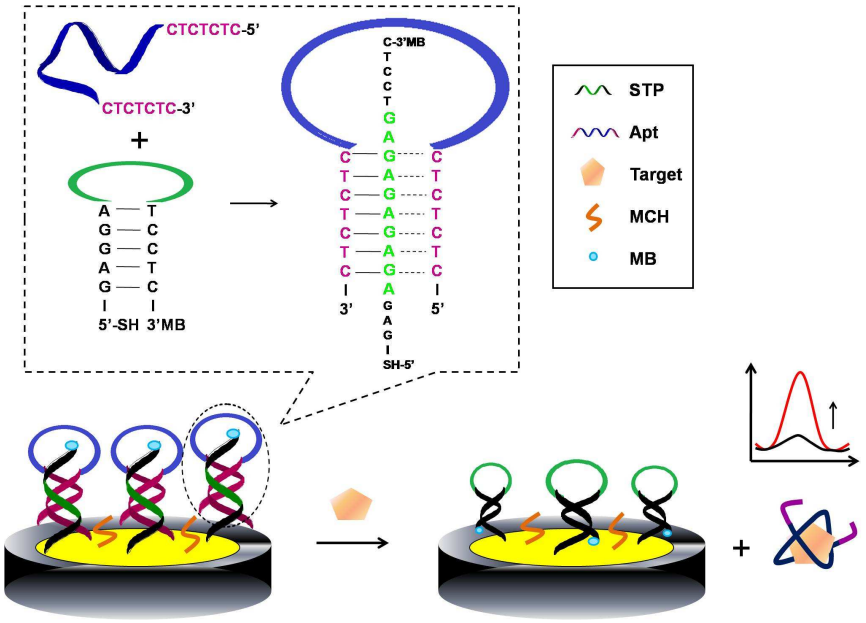
TOC:

A signal on aptamer-based electrochemical sensing platform was demonstrated by taking advantages of triple-helix molecular switch, which contained a label-free target specific aptamer sequence and a hairpin-shaped structured signal transduction probe.



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ARTICLE TYPE

A signal on aptamer-based electrochemical sensing platform using triple-helix molecular switch

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Aptamer is a useful molecular recognition tool for the design of various biosensors owing to their diversified structures, good biocompatibility, high affinity, and specificity for binding a wide range of non-nucleic acid targets, from small molecules to whole cells. Herein, we developed a signal on aptamer-based electrochemical sensing platform by taking advantages of triple-helix molecular switch. In this sensing platform, two tailored DNA probes were involved for constructing triple-helix molecular switch. One was a label-free target specific aptamer sequence (Apt) flanked by two arm segments, called as a recognition probe. The other, serving as a signal transduction probe (STP), was designed as a hairpin-shaped structure and labeled with methylene blue (MB) and sulfydryl group at the 3' and 5'-end, respectively. The target specific Apt contained triple-helix DNA (Apt-THDNA) was formed by binding two arm segments of the Apt with the loop sequence of STP, and then self-assembled onto the gold electrode via Au–S bonding. Due to the "open" configuration of STP in the Apt-THDNA, the methylene blue modified on the STP was far away from the electrode. It was eT OFF state. Formation of aptamer/target complex disassembled the Apt-THDNA and remained the STP section on the gold electrode. Subsequently, the remained STP on the gold electrode could form a hairpin-shaped configuration, mediating methylene blue approach onto the gold electrode surface to generate redox current. It was eT on state. The developed facile signal on aptamer-based electrochemical sensing platform using triple-helix molecular switch showed a linear response to concentration of Human α-thrombin (Tmb) range from 10 to 100 nM. The detection limit of Tmb was determined to be 4.5 nM. Furthermore, the universality of the sensing platform was investigated by virtue of altering the Tmb aptamer sequence to adenosine triphosphate (ATP) aptamer sequence. A linear response to concentration of ATP range from 100 to 500 nM and a 60 nM detection limit were obtained.

1. Introduction

Aptamers are DNA or RNA sequences selected *in vitro* for their ability to bind specific targets, ranging from small organics[1a,b,c] to proteins[2a,b,c,] and even whole cells.[3] They can recognize their targets with high affinities and specificities often matching or exceeding those of antibodies.[4] Due to the chemical stability and the high flexibility, aptamers are being recognized as attractive molecular tools to designing sensors for bio-analytical applications.[5a,b,c ,d] Up to now, many studies on the development of a variety of aptamer based biosensor technologies, including mechanical, [6a,b] fluorescent, [7a,b] electrochemical[8a,b,c,d,e] and so on, have been reported. Among the reported various sensors, the aptamer based fluorescent sensors obtained very rapid development. Based on conformational alteration of the aptamer during the aptamer-target binding event, many molecular engineering strategies such as molecular beacon, double helix DNA molecular switches, and triple-helix DNA molecular switches have been developed for constructing convenient aptamer-based fluorescent sensing strategies.[9a,b] For example, aptamer based molecular beacon fluorescent probes was firstly designed by Tyagi et el.[10] to recognize and report the targets successfully with low background signal. And then, Ho et el.[11] designed a strategy using double helix DNA molecular switch without labeling the aptamer. Recently, Yang et el.[12] successfully developed an aptamer based universal fluorescent biosensor based on a triple-helix DNA molecular switches to detect multiple targets. In this strategy, a distinct signal was obtained in the case of aptamer sequence which was not labeled and was not involved in the hybridization.

Compared with fluorescent aptamer sensors, electrochemical aptamer sensors have also been achieved great development

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because they possess the advantages of simple operation, easy of miniaturization, cost-effectiveness, sensitivity, and fast response.[13a,b] Like the molecular beacon and double helix DNA molecular switches, they are usually employed to develop and design the aptamer based electrochemical sensors. Nevertheless, in some ways, these strategies suffer from some drawbacks. For the molecular beacon model, it needs to design a labeled aptamer as signal reporter.[14a,b,c,d] The covalent attachment of electroactive molecules at a location of an aptamer tends to significantly reduce the aptamer's affinity and specificity toward the targets.[15a,b] For the double helix DNA molecular switches model, the probe function by switching structures from DNA/DNA duplex to DNA/target complex,[16a,b] which will hinder the recognition and binding capacity of an aptamer toward its target. On the other hand, the current electrochemical application of the triple-helix DNA just involves to the detection of single stranded or double stranded DNA.[17a,b] There are still lack of aptamer based triple-helix DNA electrochemical sensing strategies for the detection of proteins and small molecule.

Considering these several aspects, we developed a signal on aptamer-based electrochemical sensing platform. Different from the established aptamer based electrochemical sensors, a triple-helix molecular switch was employed in this electrochemical sensing platform. The triple-helix molecular switch contained two portions: a label-free target specific aptamer sequence (Apt) flanked by two arm segments, called as a recognition probe; a signal transduction probe (STP) designed as a hairpin-shaped structure and labeled with methylene blue (MB) and sulfydryl group at the 3' and 5'-end, respectively. The two arm segments of the Apt could hybridize with the loop sequence of STP and form a target specific Apt contained triple-helix DNA (Apt-THDNA). Via Au–S bonding. The Apt-THDNA was then modified on the electrode. When target was absent, the Apt-THDNA kept intact on the electrode with STP in its "open" configuration. The MB was far from the electrode. It was eT OFF state. In the presence of target, the Apt recognized and captured the target. Subsequently, the Apt was released from the Apt-THDNA, leading to new signal readout with MB closing to the surface of gold electrodes. In this sensing platform, the capture DNA probe and signal probe were separated, and aptamer was not labeled with signal molecule.The universality of the approach was achieved by virtue of altering the aptamer sequence without change of the triple-helix structure. Human α-thrombin (Tmb) was used as the first model to show the feasibility and applicability of electrochemical sensing platform. Furthermore, the universality of the sensing platform was demonstrated by virtue of altering the Tmb aptamer sequence to adenosine triphosphate (ATP) aptamer sequence. The results demonstrated that this sensing platform was not only selective but also convenient and universal.

2. Experimental

2.1 Chemicals and materials

Human α-thrombin (Tmb), adenosine triphosphate (ATP), tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 6-Mercapto-1-hexanol (MCH) were obtained from Sigma–Aldrich (St. Louis, USA). All other chemical reagents were obtained from Reagent and Glass Apparatus Corporation of Changsha and were used without further purification or treatment. Ultrapure water (Milli-Q 18.2 MΩ, Millipore System Inc.) was used for all the experiments. All work solutions were prepared with the sodium phosphate buffer (0.01 M). For the experiment of Tmb, the sodium phosphate buffer contained 20 mM KCl and 2.5 mM $MgCl_2$ (pH 6.2). While for ATP, the sodium phosphate buffer contained 300 mM NaCl, 2.5 m $MgCl_2$, and 0.1 nM (Ethylene Diamine Tetraacetic Acid (EDTA))(pH=6.2).

The DNA probes were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai China). The sequence of the DNA probes used in this work was as follows, signal transduction probe (STP): 5'-SH-$(CH_2)_6$-GAGGAGAGAGAGAGATCCTC-Methylene Blue-3', recognition probe for Tmb: 5'-CTCTCTCGGTTGGTGTGGTTG GCTCTCTC-3', recognition probe for ATP: 5'-CTCTCTCTACC TGGGGGAGTATTGCGGAGGAAGGTCTCTCTC-3'.

2.2. Electrochemical measurements

The electrochemical measurements were carried out on CHI660A electrochemical workstation (Shanghai Chenhua Instrument Corporation, China) at room temperature. A conventional three-electrode system was used for the measurement, which involved a gold disk (2 mm in diameter) working electrode, a platinum wire counter electrode and a saturated calomel reference electrode (SCE). In this paper, all the electric currents were with respect to SCE. Alternating current voltammetry (ACV) was employed in 0.01 M sodium phosphate buffer (pH 6.2) containing 0.3 M NaCl over the potential range -0.05 to -0.45 V with amplitude of 25 mV AC potential, a frequency of 20 Hz. Electrochemical impedance spectroscopy (EIS) measurements were carried out in the electrolyte solution of 0.01 M sodium phosphate buffer (pH 6.2) containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.3 M NaCl. Before experiments, the electrolyte buffer was thoroughly purged with high-purity nitrogen to avoid the interference from the reduction of oxygen.

2.3. Formation of aptamer contained triple-helix DNA.

The aptamer contained triple-helix DNA (apt-THDNA) was formed by pre-hybridization of aptamer sequence flanked by two arm segments with STP based on Watson-Crick and Hoogsteen base pairings. Before hybridization, 5 μl of 20 μM STP was first reduced for 1 h in a solution of 0.5mM of 45 μl TCEP to reduce the disulfide.[17] Then, 50 μl of 1 μM reduced STP and 50 μl of 2 μM aptamer sequence flanked by two arm segments were added into the centrifuge tube respectively, heating at 95 °C for 5 min. The DNA mixture solution was then cooled down slowly to room temperature followed by incubation at room temperature for 3 h to make them be thoroughly hybridized with each other. Thereafter, a sulfydryl group modified apt-THDNA structure was obtained. Tmb aptamer contained THDNA complex was formed in the sodium phosphate buffer containing 20 mM KCl and 2.5 mM $MgCl_2$, pH 6.2. While ATP aptamer contained THDNA complex was formed in the sodium phosphate buffer containing 300 mM NaCl, 2.5 mM $MgCl_2$, and 0.1 nM EDTA, pH 6.2.

2.4. Fabrication of apt-THDNA modified gold electrode

Before modification, the gold electrode (2 mm diameter) was dipped in freshly prepared piranha solution (H_2SO_4/H_2O_2, 7:3 by volume) for 20 min and rinsed with ultrapure water thoroughly.

Then the gold electrode was polished carefully with alumina powder of 0.3 and 0.05μm, followed by sequentially sonication for 10 min each in ultrapure water, ethanol, and ultrapure water. The electrode was then scanned in 0.1 M H_2SO_4 between -0.2 V and 1.55 V at 100 mV/s until a reproducible cyclic voltammogram (CV) was obtained. After being washed with ultrapure water and dried with purified nitrogen, 20 μL of 1.0 μM apt-THDNA solution was dropped on the pre-cleaned Au electrode surface and incubated for 16 h at 4 ℃ 100% humidity. After the incubation step, the electrode was washed with 0.01 M sodium phosphate buffer (pH 6.2) to remove the nonspecific adsorption. Then the electrode was treated with 1 mM MCH solution for 10 min to block the remaining bare region and thoroughly rinsed with ultrapure water. The obtained electrode was stored at 4 ◦C when it was not in use.

2.5. Electrochemical detection of target

A series of standard target solutions contained 0.01 M sodium phosphate buffer (pH 6.2) were prepared. Then the various target solutions at a specific concentration were dropped on the apt-THDNA modified gold electrode and incubated for 1 h at 25□. After incubation, the electrode was thoroughly rinsed with 0.01 M sodium phosphate buffer (pH 6.2). The electrochemical signal was detected with ACV measurements.

2.6. Reproducibility of the electrochemical sensing platform

By selecting ATP as target model, the reproducibility of electrochemical sensing platform has been explored. The hybridization of STP with Apt and the incubation of Apt-THDNA with the target ATP were repeated. After incubating the Apt-THDNA molecular switch with the target ATP, the Au electrode was washed with phosphate buffer and subsequently immersed in the 2.0 μM Apt solution, heating at 95 °C for 5 min and cooling slowly to room temperature for 3.0 h. The Apt-THDNA was formed again. Then it was incubated with 2.0 μM ATP solution again. The process was further repeated two times.

3.1. Target-responsive electrochemical sensing platform and experimental principle

In this work, we developed a signal on aptamer-based electrochemical sensing platform by taking advantages of triple-helix molecular switch. The principle was displayed in Figure 1. Herein, two tailored DNA probes were used for constructing triple-helix molecular switch. One was signal transduction probe (STP), designed as a hairpin-shaped structure and labeled with methylene blue (MB) and sulfydryl group at the 3' and 5'-end, respectively. The other was recognition probe (denoated as Apt), containg target specific aptamer sequence and two arm segments. Firstly, the target specific Apt contained triple-helix DNA (Apt-THDNA) was formed in a certain ionic strength and slightly acidic conditions by pre-hybridization of two arm segments of the Apt with the loop sequence of STP by Watson−Crick and Hoogsteen base pairings. Then the sulfydryl group contained Apt-THDNA was self-assembled onto gold via Au–S bonding. In the Apt-THDNA, the STP was in an "open" configuration. The MB colud not contact with electrode surface to generate electrochemical signals. It was eT OFF state. When target specical to the Apt was present in solution, formation of aptamer/target complex was produced. The Apt-THDNA was then disassembled and remained the STP section on the gold electrode. The remained STP was then formed a hairpin-shaped configuration, mediating MB onto the electrode surface to generate redox current. It was eT on state. The produced electrochemical signal was related to the concentration of target. Therefore, taking advantages of the triple-helix molecular switch, targets specical to the Apt could be detected and the false positives could be effectively reduced. Human α-thrombin (Tmb) was used as the first model to show the feasibility and applicability of electrochemical sensing platform. Furthermore, the universality of the sensing platform was demonstrated by virtue of altering the Tmb aptamer sequence to adenosine triphosphate (ATP) aptamer sequence.

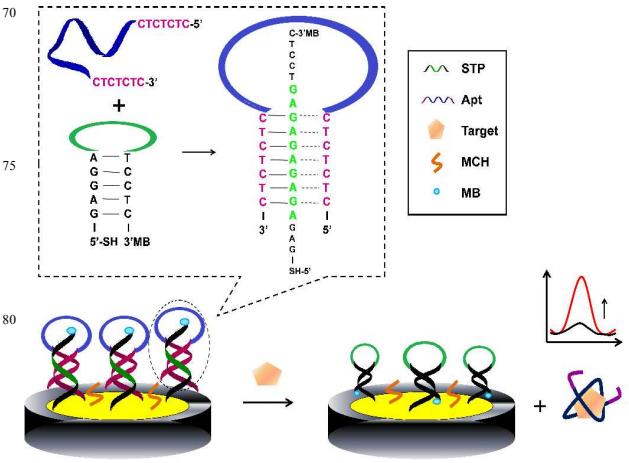

Figure 1 Schematic representation of the signal on aptamer-based electrochemical sensing platform using by using triple-helix molecular switch.

3.2. Characterization and feasibility investigation of the electrochemical assay

To investigate the characterization of the electrochemical assay, the EIS measurements, as an effective method for investigation of the interface properties of modified electrodes, were performed for each step of immobilization. Tmb was chose to be the first type of model target and dissolved in 0.01 M sodium phosphate buffer (pH 6.2) containing 20 mM KCl and 2.5 mM $MgCl_2$. The Apt probe contained the aptamer sequence specfic to Tmb. The corresponding results of differently modified electrodes in 0.01 M sodium phosphate buffer (pH 6.2) containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.3 M NaCl were shown in Figure 2A. The bare gold electrode exhibited a very small semicircle domain (curve 1), attributed to a free electron-transfer process. After the Apt-THDNA was self-assembled onto the gold electrode followed by blocking with MCH, the electron-transfer resistance (Ret) increased (curve 2), which could be ascribed to the electrostatic repulsion between the negative-charged phosphate skeletons of DNA and MCH with $[Fe(CN)_6]^{3-/4-}$. When the target Tmb was added and incubated with the Apt-THDNA/MCH modified electrode, it was found that the gold electrode leaded to a significant decrease of the electron-transfer resistance (Ret) (curve 3) corresponding to the departure of Apt from the Apt-THDNA. These experimental results not

only proved that the surface of gold electrode has been successfully modified with the Apt-THDNA but also demonstrated the Tmb could effectively competed with the Apt to form Tmb/Apt complex to separate from the Apt-THDNA.

Alternating current voltammetry (ACV) was then used to explore the feasibility of the electrochemical assay. As shown in Figure 2B, the STP (1μM) modified gold electrode exhibited a high peak of ACV signals (curve 1). It was because that the STP was in a hairpin-shaped structure. The labeled MB on the STP came close to the gold electrode. However, the Apt-THDNA modified gold electrode (MCH blocked) only exhibited a low ACV background response signal (curve 2), which was ascribed to the far distance between MB and gold electrode surface by the "open" configuration of STP in the Apt-THDNA. After the Apt-THDNA modified gold electrode was treated with Tmb (1μM), the ACV signals recovered obviously (curve 3). Although the recovered ACV signals was some lower than that of STP modified gold electrode (curve 1). The reason for the difference between curve 1 and curve 3 might be due to the two kinds of reasons. One was that there was still a little Apt-THDNA on the gold electrode. The other was that although STP got the same concentration in curve 1 and 3, the final modification density on the electrode may be different. Anyway, these EIS measurements and ACV results confirmed that the triple-helix molecular switch based electrochemical assay could be carried out.

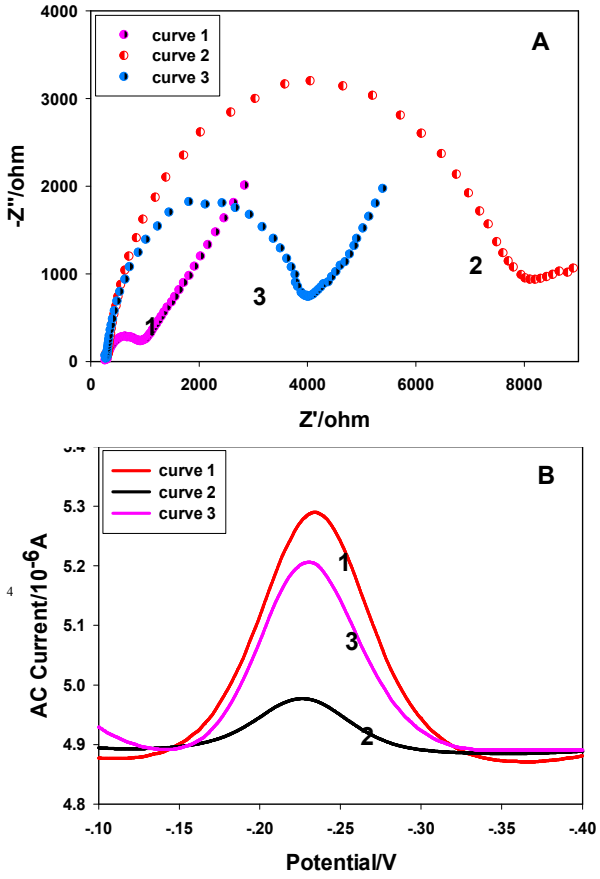

Figure 2 Feasibility and characterization investigation of the electrochemical assay. (A): Faradaic impedance spectra corresponding to bare Au electrode (curve 1), the Apt-THDNA modified electrode followed by incubation with MCH (curve 2) and the Apt-THDNA/MCH modified electrode with addition of Tmb solution (curve 3). (B): Corresponding ACV of STP modified electrode followed by incubation with MCH (curve 1), the Apt-THDNA modified electrode followed by incubation with MCH (curve 2), the Apt-THDNA modified electrode followed by incubation with MCH and further treated with Tmb solution (curve 3).

3.3. Optimization of experimental conditions

3.3.1 Effect of incubation temperature of target with Apt-THDNA

The optimization of experimental conditions was investigated based on the change of the ACV peak current ratio of signal to background (S/B). As described in the principle, the target could compete with the Apt to form Tmb/Apt complex. Therefore, only the STP was stayed on the electrode, mediating MB onto the electrode surface to generate current signal. However, high temperature maybe also dissociate the Apt-THDNA, as reported in the triple-helix molecular switch based fluorescence sensing platform.[12] Therefore, an optimum incubation temperature is very important in this electrochemical assay. As indicated in Figure S1-A, two groups of experiments was assigned to the optimization. One group was operated in the presence of Tmb, and another group was in the solution without Tmb. We can see that the S/B values in the group with Tmb were obviously higher than those without Tmb. With increasing the incubation temperature from 20-37℃, the S/B values in the group with Tmb increased gradually (curve a). In the group without Tmb, the S/B values did not change obviously when the incubation temperature was increased from 20-30□. However, we discovered that the S/B values also increased during 30-37□ (curve b). This transition occurred at~30□. The results indicated that Apt-THDNA is stable at lower temperatures, and the MB on the STP could not close to the gold electrode. However, at high temperatures, the Apt-THDNA could dissociate into a randomly coiled Apt and STP, keeping the STP on the gold electrode with a hairpin-stem structure and restoring the electrical signal. By consideration the stability of the Apt-THDNA and the competition binding of target with Apt, an incubation temperature of 25 □ was chosen in the following experiments.

3.3.2 Effect of incubation time of target with the Apt-THDNA

To achieve the optimal response of the assay, the incubation time of target with the Apt-THDNA modified on electrode should be optimized. As shown in the Figure S1-B, with increasing incubation time, the S/B values were increased and tended to a maximum when the time was up to 60 min. Thus an incubation time of 60 min was chosen in the following experiments.

3.3.3 Effect of the concentration ratio of STP/Apt

The Apt-THDNA is the key part of the electrochemical assay. The Apt-THDNA was formed thorough hybridization of two arm segments of the Apt with the loop sequence of STP by Watson−Crick and Hoogsteen base pairings. Therefore, the concentration ratio of STP/Apt was able to impact the formation efficiency of the Apt-THDNA and further influence the background signal of the DNA sensor. The effect of the concentration ratio of STP/Apt was then investigated. The concentration of STP in the investigation is 1 μM. Figure S1-C

showed the effect of the concentration ratio of STP/Apt on response current. The S/B values were increased by varying the concentration ratio of STP/Apt from 1:1 to 1:2.5. When the concentration ratio was 1:2, the S/B value tended to a maximum. Thus the ratio of 1:2 was chosen as the concentration ratio of STP/ Apt for the following experiments.

3.4. Tmb detection

Under the optimal conditions (Apt-THDNA was formed with 1 μM STP and 2μM Apt; the Tmb was incubated with the Apt-THDNA modified on the gold electrode for 60 min at 25□.), a series of Tmb solutions with concentration from 10 nM to 1.2 μM were detected to demonstrate the applicability of the designed Apt-THDNA molecular switch based electrochemical assay. Figure 3A showed the related ACV response. The ACV signals increased with the increasing Tmb concentration within the range of 10 nM to 1.2 μM. Figure 3B showed the calibration curve for analyzing the Tmb. The peak current increased linearly with Tmb at lower concentration and then slowed down at higher concentration. The line arrange of the Tmb assay was from 10 to 100 nM with a correlation coefficient of 0.9974, the regression

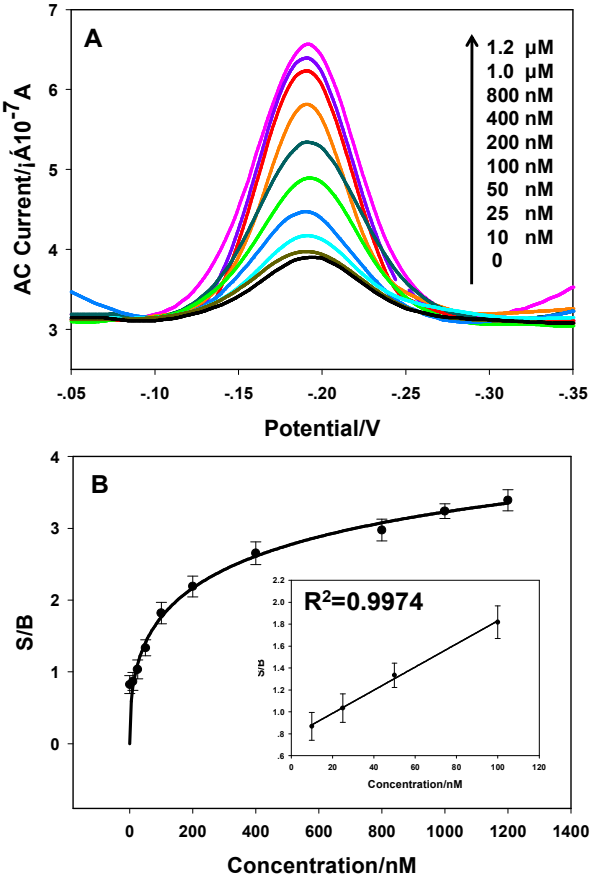

Figure 3 (A) Typical ACV responses of the Apt-THDNA molecular switch based electrochemical assay with a series of concentrations of thrombin (Tmb): 0, 10, 25, 50, 100, 200, 400, 800, 1200 nM. (B) The response curve obtained with different concentrations of thrombin from 10 to 100 nM.

equation was I (A)=0.0106x+0.7747 (x is the concentration of Tmb (nM), and I was the response peak current) (inset of Figure 3B). The detection limit was 4.5nM calculated according to the signal/noise of 3.

The selectivity of the Apt-THDNA molecular switch based electrochemical assay was determined by challenging it with nonspecific target proteins (lysozyme (Lys), BSA and IgG) with a concentration of 1.5 μM, respectively. The ACV responses of the Apt-THDNA molecular switch based electrochemical assay to all the proteins investigated were summarized in Figure S2. Among the proteins studied, Tmb showed the highest S/B value compared with the other three kinds of proteins. It was obvious that the specific recognition and binding of Tmb and its aptamer sequence in the Apt-THDNA endowed the electrochemical assay with the high selectivity.

3.5. ATP detection

In order to show the general applicability of our strategy, we used the same principle to design an Apt-THDNA molecular switch based electrochemical assay for specific detection of ATP. By altering the Tmb aptamer sequence to ATP aptamer sequence, the Apt-THDNA for ATP detection was firstly formed in the sodium phosphate buffer containing 300 mM NaCl, 2.5 mM $MgCl_2$, and 0.1 nM EDTC pH=6.2. Subsquently, the Apt- for molecular switch based electrochemical assay for ATP THDNA detection ATP detection was modified on the gold electrode using the same procedure. The feasibility of the Apt-THDNA was shown in Figure S3. As expected, the Apt-THDNA modified electrode showed a low peak current. After ATP was added, the the ACV signals recovered obviously. The ACV response to different concentrations of ATP was illustrated in Figure 4A. The increased ACV peak currents were observed in the presence of

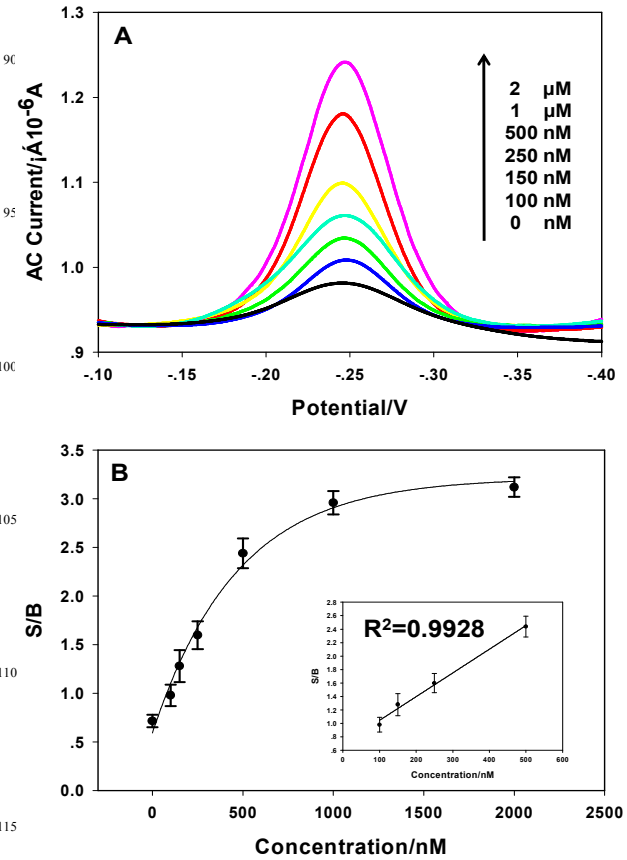

Figure 4 (A) Typical ACV responses of the electrochemical assay with a series of concentrations of adenosine triphosphate: 0, 100, 150, 200, 250, 500, 1000, 2000 nM. (B) The response curve obtained with different concentrations of ATP from 100 to 500 nM.

increasing concentration of ATP within the range from 100 nM to 2 μM. Figure 4B showed the calibration curve for analyzing the ATP. Linear relationship between the peak current and the logarithmic concentration of ATP was observed in the range of 100 to 500 nM with a correlation factor 0.9928. The linear regression equation was I (A) =0.0035x+0.6923 (here, x was the concentration of ATP (nM), and I was the response peak current). Meanwhile, the detection limit was 60 nM as calculated according to the rule of three times standard deviation over the background signal. We found that ATP analogues, such as UTP, CTP and GTP, did not induce similar structural switching, thus failing to the ACV peak current signal changes (Figure S4). The success of Tmb and ATP detection, which represent protein and small biomolecule, respectively, unequivocally supports the feasibility and universal property of the Apt-THDNA molecular switch based electrochemical sensing platform.

In addition, the reproducibility of electrochemical sensing platform has been demonstrated by using ATP as target model. The experimental results were shown in Fig. S5. It was demonstrated that the reproducibility of electrochemical sensing platform was not so good. The reason for this might be due to the following reasons. One was that the Au-S bond might be partly disrupted under the high temperatures of heating. [18a,b] Then, some modified STP on the electrods was leaved. On the other hand, the steric hindrance in the hybridization of STP with Apt on the surface was larger than that of in the solution.

Conclusion

In summary, we have developed a signal on aptamer-based electrochemical sensing platform by taking advantages of triple-helix molecular switch. This platform acquires good advantages differential from other aptamer based electrochemical senors. First, by separating the capture probe and signal transduction element, there is no need to make any modification on the original aptamer. In addition, the aptamer containing triple-helix also achieves the same stability as designs with completely or partially complementary complementary oligo yet leaves more aptamer sequence free, thereby keeping the binding affinity of the aptamer. Furthermore, by virtue of altering the aptamer sequence, the universality of the approach is achieved. So we expect that this triplex-stranded DNA probe based universal electrochemical sensor may offer a new electrochemical protocol in sensitive and selective detection of analytes and high-throughput drug screening.

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