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# A highly sensitive gold nanoparticle-based electrochemical aptasensor for theophylline detection

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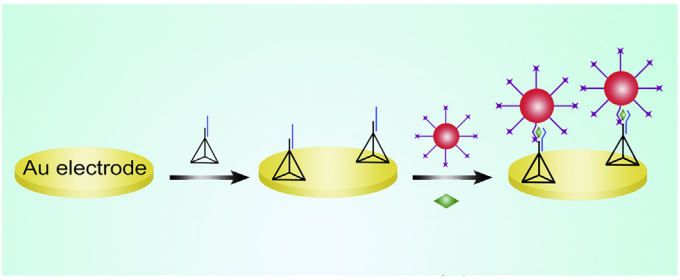
## HIGHLIGHTS

- An electrochemical aptasensor for theophylline analysis is developed.
- DNA tetrahedron is modified on the electrode sensing interface.
- Gold nanoparticles-assisted amplification is involved.
- The developed aptasensor is highly selective for the detection of theophylline.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Theophylline is a common bronchodilator for the treatment of diseases like asthma, bronchitis and emphysema. However, it should be strictly used and monitored due to its toxicity when the concentration is above certain levels. In this work, an electrochemical biosensor for theophylline detection is proposed by recognition of RNA aptamer and gold nanoparticle (AuNP)-based amplification technique. First, RNA aptamer is splitted into two single-stranded RNA probes. One is hybridized with DNA tetrahedron and the resulted nanostructure is then immobilized onto a gold electrode; the other is modified on the surface of AuNPs which is also labeled with methylene blue (MB) as electrochemical species. The recognition process between the two RNA probes and theophylline causes the localization of AuNPs and the enrichment of MB on the electrode interface. A significant electrochemical response is thus generated which is related to the concentration of initial theophylline. This proposed aptasensor shows excellent sensitivity and selectivity which could also be applied in quantitatively detection of theophylline in serums samples.

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## 1. Introduction

Theophylline is a popular drug for the treatment of many diseases such as chronic obstructive pulmonary disease (COPD), neonatal apnea, and asthma, which has been clinically used for more than 80 years [1]. Abundant evidences have indicated that low-dose theophylline possesses anti-inflammatory and immunomodulatory effects in certain diseases like COPD [2,3]. Moreover,

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the cost of theophylline is low, thus it is widely used in developing countries. However, some adverse effects of theophylline exist when the serum concentration is above 20 μg/mL [4]. A narrow therapeutic range is from 5 to 15 μg/mL [5]. Therefore, there is a considerable interest in biological analysis. Although a number of analytical methods have been developed for the determination of theophylline [6–8], unfortunately, some disadvantages do exist including poor time resolution, poor limit of detection, complicated operation and poor detection stability. Furthermore, large sample volumes, complex solvents, expensive instruments may be required in most cases.

Electrochemical techniques have the merits of high sensitivity, low cost and rapid process, which are ideally suited for theophylline assays [9–11]. For instance, Ferapontova et al. developed an electrochemical aptasensor for theophylline using methylene blue (MB) as electrochemical species [12]. RNA aptamer was immobilize on the electrode surface, the conformation of which was changed upon theophylline binding. Accordingly, MB is getting closer to the electrode surface and the electrochemical response was increased to probe the level of target theophylline. However, this electrochemical sensor was preliminary without any signal amplification elements, which could only respond theophylline with a relative high concentration. Kumar et al. constructed a non-enzymatic electrochemical method for the determination of theophylline [13]. Voltammetric behavior was recoded on a para amino benzene sulfonic acid modified glassy carbon electrode, which standed for the oxidation of theophylline. Nevertheless, the detection can be easily interfered by other electroactive species and the selectivity may be limited. Therefore, there is still urgent need to develop more advanced electrochemical methods for theophylline analysis.

Nanomaterials sized between 1 and 100 nm have unique physical or chemical properties [14–16]. They have been applied in a number of analytical methods with excellent electrochemical behaviors such as silver nanoparticles and copper nanoparticles [17,18]. In this contribution, gold nanoparticles (AuNPs) with high specific surface area and excellent electrical conductivity are utilized for amplified electrochemical sensing of theophylline [19]. Briefly, DNA tetrahedron nanostructure has been immobilized on the electrode surface in order to improve molecular recognition efficiency and avoid redundant electrode modification steps [20]. Since split aptamers may help reduce the background and guarantee the high selectivity [21–24], the recognition event occurs herein is conducted between theophylline and two split RNA aptamers on DNA tetrahedron and AuNPs respectively, instead of using a single-stranded RNA aptamer [12]. In addition, AuNPs process huge specific surface area for RNA probe immobilization. As a result, the attached MB molecules on the RNA probe could be enriched to a great extent. Moreover, AuNPs have excellent electrical conductivity, thus, significant electrochemical signal can be obtained [25]. The developed electrochemical method for theophylline shows high sensitivity with the AuNP-based signal amplification. The linear range is obtained from 0.1 to 80 μM with the limit of detection (LOD) as low as 0.07 μM.

## 2. Experimental

### 2.1. Materials and chemicals

Chloroauric acid ($HAuCl_4$) and trisodium citrate were purchased from Shanghai Jiushan Chemicals Co., Ltd. (Shanghai, China). Ethylenediaminetetraacetic acid (EDTA), tris(2-carboxyethyl)phosphinehydrochloride (TCEP) and diethypyrocarbonate (DEPC) were obtained from Sigma (USA). 20 bp DNA Ladder, DNA probes and RNA probes were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China), the sequences of which were listed in Table 1. Other reagents were of analytical grade and were used as received. Double-distilled water used to prepare all solutions was purified with a Millipore system under 18 MΩ cm resistivity and then treated with DEPC.

### 2.2. Preparation of RNA probe b modified AuNPs

AuNPs were synthesized by the citrate reduction of $HAuCl_4$ according to a previous report [26]. A typical process was as follows. 3.5 mL of 1% (w/v) trisodium citrate was freshly prepared and added to 100 mL of 0.01% (w/v) $HAuCl_4$ under violent stirring. The solution was boiling for 15 min and the stirring was stopped after another 30 min. Subsequently, the prepared AuNPs were cooled down to room temperature, which were then purified by three cycles of centrifugation at 12, 000 rpm for 30 min. The functionalization of AuNPs with RNA probe b was achieved by gold–sulfur chemistry [27]. Briefly, 4 μM RNA probe b was added to 1 mL of AuNPs with the concentration of 10 nM. After 16 h, the RNA-AuNPs conjugates were "aged" in salts (0.1 M NaCl, 10 mM phosphate, pH 7.0) for 24 h, which made the RNA molecules on the surface of AuNPs vertical and ordered. Next, the RNA modified AuNPs were purified by centrifuging at 12, 000 rpm for 30 min.

### 2.3. Gold electrode treatment and modification

The substrate gold electrode (3 mm diameter) was soaked in piranha solution (98% $H_2SO_4$: 30% $H_2O_2$ = 3:1) for 5 min (*Caution: Piranha solution was highly corrosive and reacts violently with organic matter!*). After rinsed with double-distilled water, the electrode was carefully polished to a mirror-like surface on P3000 silicon carbide paper and then 1, 0.3 0.05 μm alumina slurry, respectively. Subsequently, it was sonicated for 5 min in both ethanol and double-distilled water in order to remove the residual alumina powder. The electrode was soaked in nitric acid (50%) for 30 min and then electrochemically cleaned with 0.5 M $H_2SO_4$ to remove any remaining impurities. The pretreated electrode was ready for further modification.

Tetrahedral nanostructure was formed through partially hybridization events between four single-stranded DNA probes (DNA probe a, b, c, d) and RNA probe a. Briefly, each strands with the concentration of 5 μM was prepared in the 10 mM Tris-HCl buffer solution (pH 7.0) containing 10 mM TCEP and 50 mM $MgCl_2$, respectively. The five solutions were heated to 95 °C for 2 min to

**Table 1**
Sequences of nucleic acids used in this work.

| Name | Sequence (from 5′ to 3′) |
|---|---|
| DNA probe a | CCAGCCGAAAACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA |
| DNA probe b | SH-$C_6$-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC |
| DNA probe c | SH-$C_6$-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC |
| DNA probe d | SH-$C_6$-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT |
| RNA probe a | CGGCUGGGGCGAUACCAGCCGAAA |
| RNA probe b | SH-$C_6$-CCAGCCGAAAGGCCCUUGGCAGCGUCGGG-MB |

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unfold any possible secondary structures, which were then cooled to room temperature. Then, the five strands were blended together with equivalent volume (20 μL) to form nanostructured tetrahedron spontaneously at room temperature. 10 μL of the tetrahedron (1 μM) was dipped on the pretreated gold electrode for 8 h. After that, it was thoroughly rinsed using 10 mM Tris–HCl (pH 7.4) and dried with nitrogen for further use.

### 2.4. RNA aptamer recognition

Standard theophylline solutions with a series of concentrations (1, 50, 200, 400, 500, 600, 800, 1000, 2000, 5000 μM) were prepared which were then mixed with RNA probe b modified AuNPs. The volume ratio was 1: 9. After that, the modified gold electrode was incubated with the above solutions for 1 h. Subsequently, the electrode was rinsed and ready for electrochemical measurement.

### 2.5. Electrochemical measurements

Electrochemical experiments were performed on a CHI 660D electrochemical workstation (CH Instruments, China). Three electrode system was employed, which was consisted of a saturated calomel reference electrode (SCE), a platinum auxiliary electrode and tetrahedron modified gold electrode as the working electrode. Buffer for electrochemical impedance spectroscopy (EIS) experiments was 5 mM $[Fe(CN)_6]^{3-/4-}$ with 1 M $KNO_3$. The parameters were as follows. Bias potential, 0.21 V; amplitude, 5 mV; frequency range, 0.1 to 100 000 Hz. Buffer for square wave voltammetry (SWV) was 20 mM Tris–HCl buffer (pH 7.5). The modulation amplitude was 25 mV, the frequency was 80 Hz and the step potential was 4 mV.

### 2.6. Gel electrophoresis analysis

To verify the successful formation of DNA tetrahedron, different DNA samples were prepared and analyzed by polyacrylamide gel electrophoresis. It was carried out in the solution (90 mM Tris-boric acid and 1 mM EDTA, pH 8.0) at 110 V for about 50 min. Next, the gel was stained with 4S Red Plus, which was then photographed under UV light by Gel DocTM XR + Imaging System (Bio-Rad, USA).

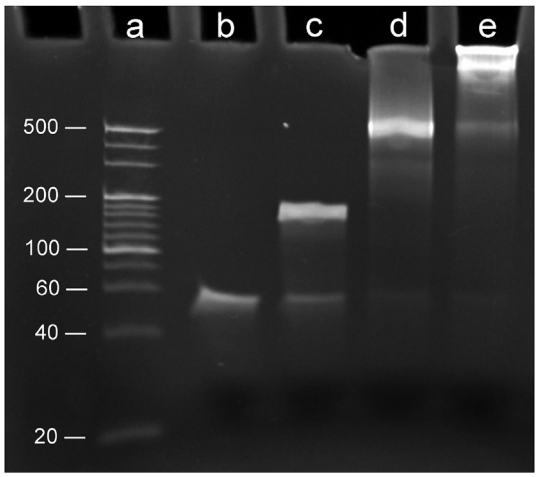


**Fig. 1.** Polyacrylamide gel electrophoresis analysis of the formation of DNA tetrahedron. The lanes represent (a) 20 bp DNA Ladder, (b) DNA probe b, (c) DNA probe b and c, (d) DNA probe b, c and d, (e) DNA probe a, b, c and d. The concentration of each DNA strand is 0.5 μM.

## 3. Results and discussion

### 3.1. DNA tetrahedron characterization

DNA tetrahedron is formed by partially hybridization between several single-stranded nucleic acid probes, which is characterized by polyacrylamide gel electrophoresis. DNA structures formed by one, two, three and four strands are shown in Fig. 1, the molecular weights of which are in good agreement with expected values. In the lane of DNA tetrahedron formed by DNA probe a, b, c and d, no band is presented at the position of 55 bp, indicating that nearly all single-stranded DNA probes are consumed for the construction of DNA tetrahedron. Due to the three thiol groups on the bottom of DNA tetrahedron, it can be firmly attached on the electrode surface via gold-sulfur chemistry [28]. The density of the DNA tetrahedron on the surface of gold electrode is $2.72 \times 10^{12}/cm^2$, which is determined by quantitative measurement of hexaammineruthenium (III) absorbed on DNA [29]. This three-dimensional DNA nanostructure is able to provide a nanoengineered interface for spatial control and enhanced nucleic acid probe accessibility [30]. In addition, “backfilling” process (e.g., 6-mercapto-1-hexanol treatment) can be eliminated, which makes the experimental procedure more succinct and shortens the experiment time [31].

### 3.2. Sensing principle

Detailed sensing strategy is illustrated in Scheme 1, which involves the self-assembly of tetrahedral nanostructure, RNA aptamer recognition and AuNP-mediated signal amplification. RNA probe a is localized on the top of DNA tetrahedron by partial hybridization between DNA probe a and RNA probe a. On the other hand, numerous RNA probe b molecules which have been previously modified with thiol groups at the 5′ end could also be attached on AuNPs due to the specific interaction. In the presence of theophylline, RNA probe a and b recognize the target and a sandwich structure is formed on the electrode. Since the event localizes AuNPs with numerous RNA probe b, the labeled MB molecules at the 3′ end provide intense electrochemical signal for sensing purpose.

### 3.3. Characterization of AuNPs

Bare AuNPs and RNA probe b modified AuNPs are characterized by transmission electron microscopy (TEM), the images of which show that the sizes are about 15 nm and the nanoparticles disperse well in water (Fig. S1). In addition, the UV-vis spectra of bare AuNPs and RNA probe b modified AuNPs contain peaks around 520 nm, which are in consistent with previously reported results (Fig. S2) [32].

### 3.4. Electrochemical characterization of gold electrode interface

EIS has been performed to characterize the gold electrode interface during each modification steps, and the results are shown in Fig. 2. A typical nyquist diagrams usually contains a semicircle portion and a straight line. The diameter of the semicircle could be used to indicate the interfacial charge transfer resistance. In the nyquist diagram of bare gold electrode, no semicircle portion is observed, which stands for the excellent electron transfer capability of the electrode (curve a) [33]. However, the attached tetrahedral nanostructure is negatively charged, which repels the electrochemical species $(Fe(CN)_6^{3-/4-})$. As a result, a semicircle appears with the tetrahedron modification (curve b). After treatment with target theophylline, the diameter of the semicircle increases slightly (curve c). Further loading of RNA probe b modified AuNPs

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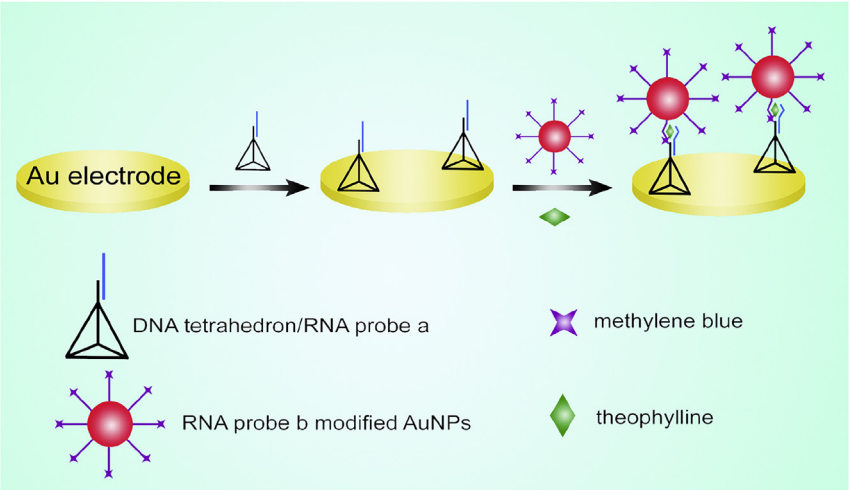


**Scheme 1.** Illustration of the AuNPs-based electrochemical aptasensor for theophylline detection.

may significantly increase the intensity of RNA density on the electrode surface. Thus, the repellant between phosphate skeleton of nucleic acids contributes to even larger semicircle portion (curve d). Since RNA probe b is labeled with MB as the electrochemical probe, SWV of MB is monitored. As shown in Fig. 3, only after the theophylline-mediated loading of RNA probe b modified AuNPs, obvious SWV peak can be observed, which is the evidence of the recognition event.

### 3.5. Amplification by AuNPs

In this contribution, AuNPs are used as the amplification element. To check the efficiency, we have compared the recognitions between DNA tetrahedron/RNA probe a with RNA probe b and RNA probe b modified AuNPs. We have chosen different concentrations of theophylline for aptamer recognition and the final SWV peaks are recorded. Since AuNPs have high specific surface area, the number of MB is significantly increased and much larger electrochemical signal is generated. Experimental results confirm that RNA probe b modified AuNPs may help enlarge the output signals greatly in the presence of theophylline with different concentrations (Fig. S3).

### 3.6. Quantitative detection of theophylline

The dependence of electrochemical signal and theophylline concentration is then investigated. The SWV curves are summarized in Fig. 4a. With the increase of theophylline concentration from 0.1 to 500 μM, the SWV peak increases correspondingly. Calibration plot in Fig. 4b describes the relationship between the two parameters in detail and a linear range is observed between 0.1 and 80 μM. The fitting equation is $y = 0.499 + 0.065\,x$ ($n = 3$, $R^2 = 0.988$), in which $y$ is the peak current (μA) and $x$ is the concentration of theophylline (μM). LOD is calculated to be 0.07 μM (signal-to-noise ratio of 3), which is quite low. Analytical performances of the proposed aptasensor are compared with those of other reported electrochemical methods in Table 2, which verify that this method represents a significant advance in theophylline assay.

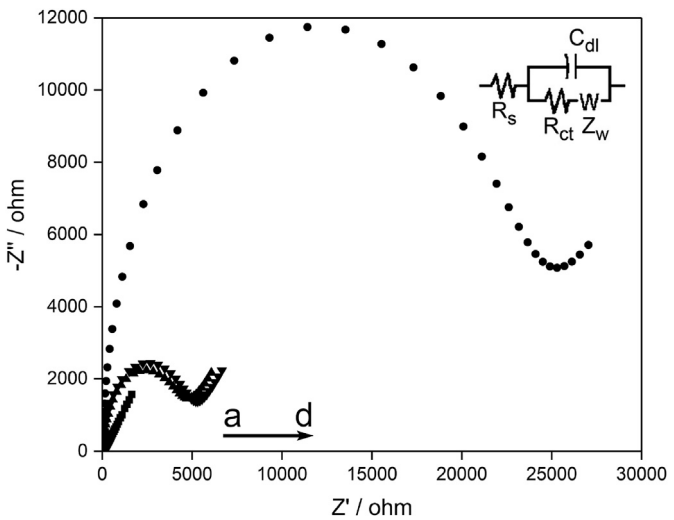


**Fig. 2.** Nyquist diagrams of impedance spectra for (a) bare electrode, (b) DNA tetrahedron/RNA probe a modified electrode, (c) after theophylline treatment, (d) after further incubation with RNA probe b modified AuNPs. Inset is the electrical equivalent circuit.

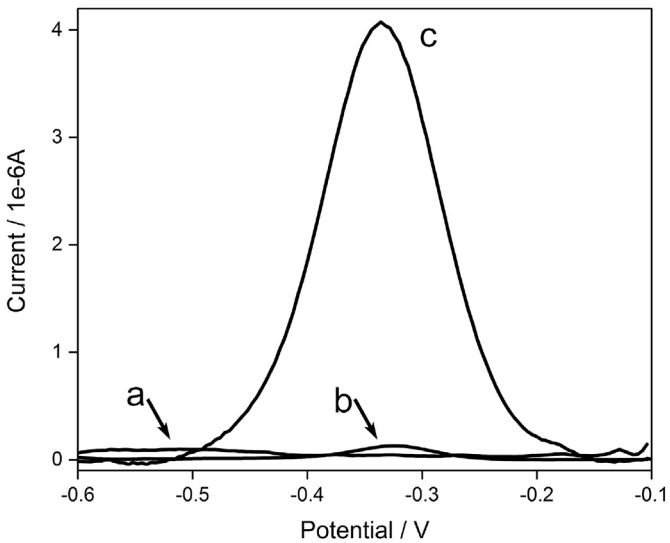


**Fig. 3.** Square wave voltammograms of (a) DNA tetrahedron/RNA probe a modified electrode, after (b) incubation with RNA probe b modified AuNPs in the (b) absence and (c) presence of theophylline (50 μM).

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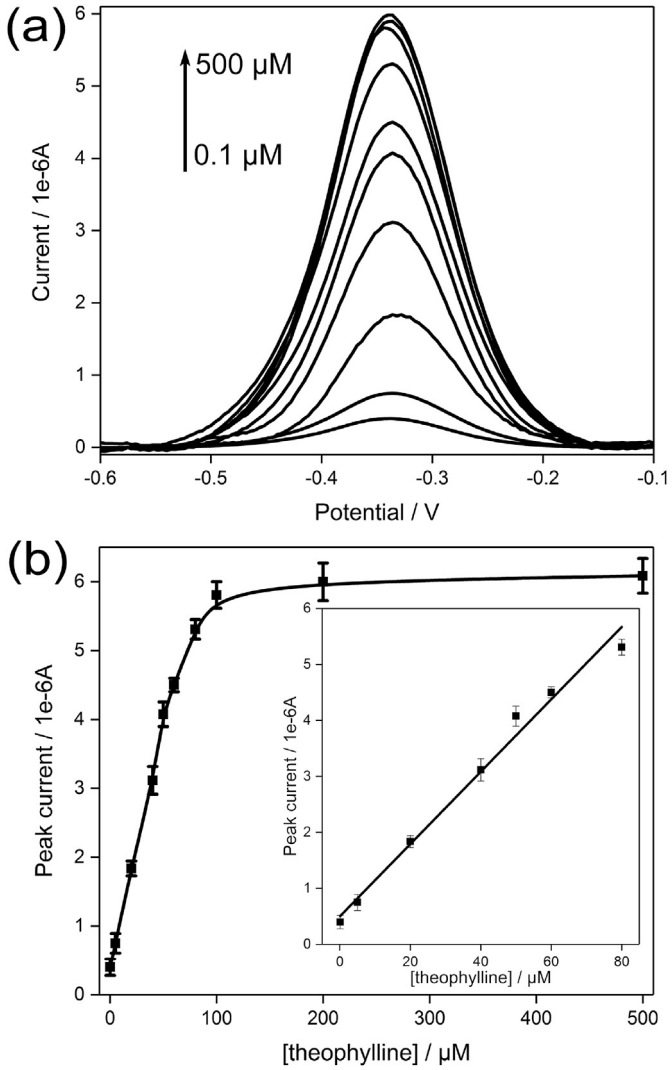


**Fig. 4.** (a) Square wave voltammograms for the detection theophylline with the concentrations of 0.1, 5, 20, 40, 50, 60, 80, 100, 200, 500 μM. (b) The calibration curve of peak current versus the concentration of theophylline. Inset shows the linear range.

We have also checked the stability and reproducibility of this method. Tetrahedral nanostructure modified gold electrodes have been stored in 10 mM Tris-HCl buffer solution (pH 8.0) with 10 mM TCEP and 0.1% (v/v) DEPC in 4 °C for 1 month. The electrodes are then used for the detection of theophylline with the concentration of 50 μM. The obtained peaks are over 93% of original values. In addition, we have modified five gold electrodes in the same manner, which are then used for the determination of theophylline (50 μM). Relative standard deviation (RSD) of the obtained peak current is calculated, which is less than 5%, demonstrating excellent electrode-to-electrode reproducibility.

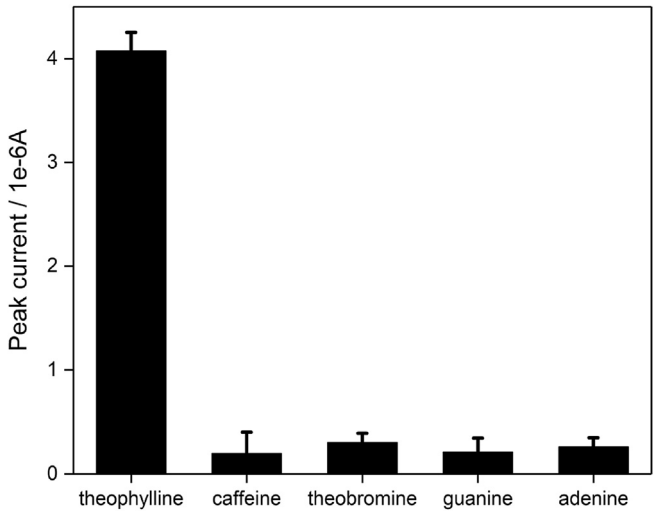


**Fig. 5.** Peak current of square wave voltammograms for the detection of theophylline, caffeine, theobromine, guanine and adenine with the concentration of 50 μM.

**Table 3**
Electrochemical detection of theophylline in real samples.

| Serum sample | Spiked (μM) | Detected (μM) | Recovery (%) | RSD (%) |
|---|---|---|---|---|
| 1 | 20 | 20.8 | 104.0 | 4.6 |
| | 50 | 51.3 | 102.6 | 5.0 |
| 2 | 20 | 20.6 | 103.0 | 3.8 |
| | 50 | 50.9 | 101.8 | 3.7 |
| 3 | 20 | 21.1 | 105.5 | 5.2 |
| | 50 | 50.5 | 101.0 | 4.1 |

### 3.7. Interference investigation and real sample analysis

To demonstrate the high selectivity and practicability of the proposed electrochemical method, different interferents and real samples are challenged. We firstly compare the SWV peak currents in the presence of theophylline, caffeine, theobromine, guanine and adenine, respectively. As depicted in Fig. 5, all interferents create much lower signal compared with that of theophylline, demonstrating the excellent selectivity. Moreover, we have spiked different levels of theophylline in serum samples, which are then tested by the proposed method. The detailed information are shown in Table 3. The detected concentrations are similar to added values and the recoveries are from 101.0% to 105.5%. All RSD values

**Table 2**
Electrochemical analysis of theophylline with the proposed aptasensor and other assays.

| Techniques | Materials | Detection range (μM) | LOD (μM) | Ref. |
|---|---|---|---|---|
| CV | screen-printed electrode | 50 to 290 | 10 | [34] |
| CV | $Ag^+$ and abasic site-incorporated duplex DNA | 0 to 50 | 3.2 | [35] |
| SWV | para amino benzene sulfonic acid | 2.5 to 300 | 2.266 | [13] |
| DPV | RNA aptamer and MB | 2 to 100 | 2 | [12] |
| DPV | RNA aptamer and ferrocene | 0.2 to 10 | 0.2 | [36] |
| DPV | CTAB | 0.5 to 1000 | 0.11 | [37] |
| DPV | $MnO_2$ nanosheets/ionic liquid functionalized graphene | 1 to 220 | 0.1 | [38] |
| DPV | poly(Alizarin Violet 3B), multi-walled carbon nanotubes and graphene | 0.5 to 120 | 0.02 | [39] |
| SWV | RNA split aptamers and AuNPs | 0.1 to 80 | 0.07 | this work |

Abbreviates: CV, cyclic voltammetry; DPV, differential pulse voltammetry.

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are less than 6%. These results verify that this method may have practical utility in biological samples.

## 4. Conclusions

In summary, this work demonstrates a highly sensitive electrochemical aptasensor for the detection of theophylline based on AuNPs-mediated signal amplification. Split RNA aptamers are modified on the surface of electrode and AuNPs, separately. After the introduction of target theophylline, a sandwich structure forms and localizes AuNPs with a huge amount of electrochemical species on the electrode interface. Therefore, highly sensitive and selective analysis of theophylline is achieved. LOD is as low as 0.07 μM and the method could successfully distinguish theophylline from other interferents with similar chemical structures. Moreover, it also performs well in serum samples. Therefore, the proposed aptasensor may have wide practical applications in the future.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2017.10.039.

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