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A novel electrochemical method to detect theophylline utilizing silver ions captured within abasic site-incorporated duplex DNA

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ABSTRACT

We herein describe a novel and label-free electrochemical system to detect theophylline. The system was constructed by immobilizing duplex DNA containing an abasic site opposite cytosine on the gold electrode surface. In the absence of theophylline in a sample, silver ions freely bind to the empty abasic site in the duplex DNA leading to the highly elevated electrochemical signal by the redox reaction of silver ions. On the other hand, when theophylline is present, it binds to the abasic site by pseudo base pairing with the opposite cytosine nucleobase, which consequently prevents silver ions from binding to the abasic site. As a result, redox reaction of silver ions would be greatly reduced resulting in the accordingly decreased electrochemical signal. By employing this electrochemical strategy, theophylline was reliably detected at a concentration as low as 3.2 μM with the high selectivity over structurally similar substances such as caffeine and theobromine. Finally, the diagnostic capability of this method was also successfully verified by reliably detecting theophylline present in a real human serum sample with an excellent recovery ratio within 100 ± 6%.

1. Introduction

Theophylline is one of the most commonly used bronchodilators and respiratory stimulators for the treatment of acute and chronic obstructive pulmonary disease (Barnes, 2003; Kawai and Kato, 2000). The therapeutic window for theophylline in serum is narrow with an effective concentration range of 60–100 μM (Hendeles and Weinberger, 1983). Higher concentration of theophylline over the range could cause lethal or neurological damages (Ferapontova and Gothelf, 2009b). Therefore, accurate and careful monitoring of the theophylline concentration in serum is highly demanded to prevent its toxic side effect. Currently, detection of serum theophylline has been carried out in many clinical laboratories by employing routine methods such as gas or liquid chromatography, ultraviolet spectrometry, and immunoassay (Mounie et al., 1990). However, these methods suffer from several limitations including the need for technical expertize as well as the long analysis time required for complex and multi-step procedures. In addition, selectivity of these methods is relatively low over structurally similar molecules such as caffeine or theobromine, which could cause overestimated results.

In order to overcome these limitations, an RNA aptamer-based sensing system has emerged as an alternative approach for the detection of theophylline (Jenison et al., 1994). Taking advantages of RNA aptamer exhibiting high binding affinity and selectivity toward theophylline, various signaling strategies have been developed by employing colorimetric (Pernites et al., 2011), fluorescent (Rankin et al., 2006; Stojanovic and Kolpashchikov, 2004), and electrochemical (Ferapontova and Gothelf, 2009a; Ferapontova et al., 2008) signaling methods. However, RNA molecules are basically not considered as a good sensing component because RNA synthesis is more difficult and expensive than DNA and also susceptible to the cleavage by ubiquitous ribonuclease and chemical reagents, which consequently impedes the widespread use of RNA aptamer-based assay.

Recently, Teramae et al. discovered a new class of DNA duplex aptamer to recognize theophylline where abasic (AP) site is incorporated serving as an active and strong binding receptor for theophylline (Nishizawa et al., 2010; Sankaran et al., 2006; Sato et al., 2009, 2012). This DNA duplex aptamer shows high binding affinity toward theophylline only when cytosine is positioned opposite AP site and discriminates theophylline very selectively from structurally similar substances such as caffeine and theobromine (Sankaran et al., 2006). Utilizing the unique feature, they developed a new strategy to detect theophylline, which relies on the DNA duplex probe modified with fluorescent analog of adenine, 2-aminopurine, near the AP site. Theophylline bound into the AP site changes the microenvironment of fluorescent 2-aminopurine, which consequently results in the significant reduction of fluorescence signal from 2-aminopurin (Pang et al.,

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2012). Although interesting, the need for the modification of DNA probe with 2-aminupurine might make the assay a little complicated and expensive (Li et al., 2009).

In an alternative approach aimed at eliminating the above limitations, Park et al. (2014) recently developed a modification-free method to detect theophylline by utilizing fluorescent silver nanocluster (AgNCs) as a signaling unit. In this method, the formation of AgNCs within AP site opposite cytosine moiety is controlled by competitive binding of theophylline. The strategy is quite convenient yielding high sensitivity and selectivity toward target theophylline. However, the fluorescent signal of the AgNCs might be significantly reduced by several substances present in a blood serum sample, which inevitably hinders the widespread use of the method by essentially requiring the pretreatment step to remove undesirable substances from blood serum. Therefore, there is still a significant challenge for development of novel strategy to detect theophylline in a highly stable, efficient, and cost effective manner.

In order to meet these demands, we designed a new electrochemical method to detect theophylline that could be much cheaper and more convenient than the previously reported fluorescence-based methods (Park et al., 2014; Rankin et al., 2006; Stojanovic and Kolpashchikov, 2004), which enables more widespread application even in facility-limited environments (Burke and Gorodetsky, 2012; Won et al., 2011). In this strategy, the surface of electrode is modified with the DNA duplex aptamer possessing AP-site opposite cytosine nucleobase. The electrochemical sensor is operated by generating a signal through the redox reaction of silver ions captured into the AP site (Shao et al., 2009), which is regulated by the competitive binding of theophylline into the identical AP site. This is the first report to detect theophylline in a very convenient electrochemical manner overcoming the limitations associated with the previous methods.

2. Materials and methods

2.1. Materials

All synthetic oligonucleotides were synthesized and purified by high-performance liquid chromatography (HPLC) from Integrated DNA Technologies (IDT, Coralville, USA) (Table S1). The AP site-incorporated DNAs were modified with thiolate (–SH) functional group at 5′ end. Silver nitrate, theophylline, caffeine, theobromine, creatinine, D-glucose, human serum, sodium hydrogen phosphate, sodium dihydrogen phosphate, and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and used without further purification (Park et al., 2013). Aqueous solutions were prepared using ultrapure DNase/RNase-free distilled water (Carlsbad, CA, USA).

2.2. Preparation of gold electrode platform

The coating of titanium (20 nm) on Si wafer (100) was followed by 200 nm gold (99.999%) thin layer formation by an e-beam evaporator. The gold-coated electrode surface was immersed into piranha solution (H_2SO_4:H_2O_2=4:1) for 15 min (Baek et al., 2013). The electrode surface was then thoroughly washed with PBS (50 mM sodium phosphate, 75 mM sodium chloride, pH 7.2) and dipped into the 200 μL of aqueous solution containing 1 μM AP site-incorporated DNA (Table S1) for 4 h. After washing the electrode with PBS, 1 mM mercaptohexanol solution was applied onto the electrode surface for 30 min to block bare surfaces uncovered by the AP site-incorporated DNA. Finally, the modified electrode with a surface coverage of 12.3×10^{12} molecules/cm^2 were washed with PBS and water, and dried under N_2 atmosphere. The amount of DNA immobilized on the electrode was determined to be 12.3×10^{12} molecules/cm^2 based on the Beer Lambert Law by measuring the absorbance difference of the DNA solution at 260 nm before and after immobilization process.

2.3. Theophylline detection procedure

200 μL of 1 μM complementary single-stranded DNA having different bases opposite the AP site (Table S1) was allowed to hybridize with the AP site-incorporated DNA (AP-middle) on the gold electrode surface for 1 h at 60 °C. The solution was then cooled down to 25 °C and the electrode was washed with PBS. A solution containing theophylline at different concentrations or theobromine, caffeine, D-glucose, and creatinine at 250 μM was applied to the gold electrode and incubated for 45 min at 15 °C. After washing the electrode surface with PBS and water, the electrode was immersed into the solution containing 10 μM $AgNO_3$, incubated for 45 min and again completely washed with PBS and water. Detection procedure described above is summarized in Fig. S1 for a better understanding.

2.4. Electrochemical measurement

The gold electrode was immersed into 1 mL of phosphate buffer (20 mM) containing 100 mM sodium acetate for 10 min before electrochemical measurement. The gold matrix electrode was used as a working electrode with Ag/AgCl reference electrode and platinum counter electrode. Cyclic voltammetry (CV) was performed using a reference 600 electrochemical analyzer (GAMRY, Warminster, PA, USA) connected to a computer for data analysis from 0.5 V to −0.5 V using 10–200 mV s^{-1} scan rates, and the CV under 100 mV s^{-1} was used to detect and quantify target theophylline.

2.5. Preparation and analysis of Human serum samples

A human serum sample diluted 20 times with the phosphate buffer (20 mM phosphate, 100 mM sodium acetate, pH 7.4) containing various concentrations of theophylline was directly applied to the electrode. The sample was analyzed by following the above-mentioned detection procedure. To determine the amount of theophylline, the calibration curve was first constructed by a set of standards containing a known amount of theophylline in human serum. The electrochemical peak current from the unknown samples was measured and interpolated to determine the concentration of theophylline present in human serum based on the calibration curve.

3. Results and discussion

3.1. The overall detection procedure

The overall procedure for electrochemical system to detect theophylline is illustrated in Fig. 1. The AP site within duplex DNA immobilized on the surface of electrode serves as a binding pocket for theophylline through pseudo base pairing with the opposite cytosine that is stabilized by nucleobases adjacent to the AP site (Li et al., 2009; Pang et al., 2012). At the same time, the cytosine nucleobase positioned opposite the AP site also captures silver ions through its strong interaction (Ma et al., 2011; Park and Park, 2014) when the AP site is not occupied by target theophylline (Fig. 1). The silver ion captured within the AP site then produces electrochemical signal through its redox reaction on the electrode surface. On the other hand, when theophylline is paired with cytosine base in the AP site, it prevents silver ions from binding to

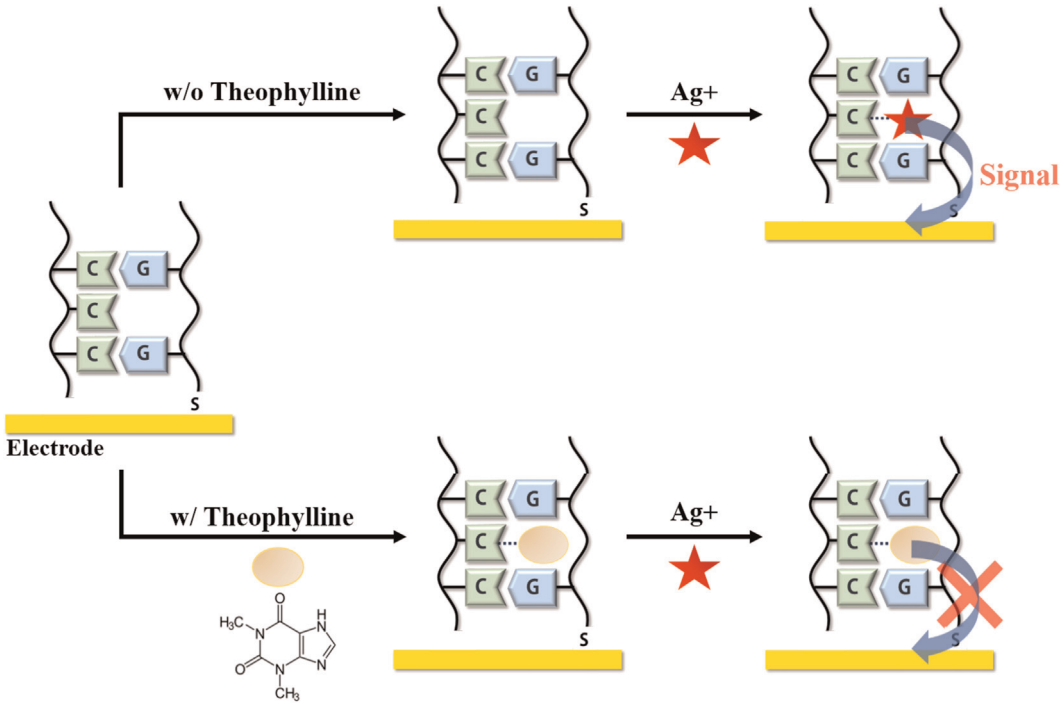

Fig. 1. Schematic illustration of electrochemical system to detect theophylline based on the redox reaction of silver ions captured into AP site-incorporated duplex DNA.

the AP site. As a result, the electrochemical signal produced through redox reaction of silver ions captured into the AP site-incorporated duplex DNA would be greatly reduced, which can be used to identify theophylline level in a sample.

3.2. Investigation of the theophylline detection system

To explore the feasibility of this strategy, we first examined the electrochemical signal produced by silver ions captured into the AP site-incorporated duplex DNA. A high electrochemical signal is produced on the electrode surface in the absence of theophylline as a result of the strong interaction of silver ions with cytosine moieties placed opposite the AP site in duplex DNA. As envisioned, the peak is significantly reduced when theophylline is present in a sample (Fig. 2). On the other hand, no significant peak current is observed regardless of the absence and presence of theophylline when the full-matched duplex DNA without AP site is immobilized on the electrode surface, which clearly confirms that the AP site is essentially required to capture silver ions and generate the electrochemical signal (Fig. 2).

Next, to investigate the electrochemical signaling mechanism of our sensing system, we measured peak current of cyclic voltammogram at varying scan rates. The result shows that the oxidation peak current quite linearly increase as a function of scan rate, indicating the current completely and exclusively relies on the surface-confined signal produced by the silver ion captured within AP site-incorporated duplex DNA immobilized on the electrode surface (Fig. S2) (Kwon et al., 2008). We also investigated the effect of immersion time of silver nitrate on the current signal. The result demonstrates that the electrochemical signal by silver ions first increases with increasing immersion time but seems to be saturated over 45 min. Based on this result, 45 min immersion time is chosen to produce electrochemical signal in further experiments (Fig. S3). In addition, we investigated how long our sensing system stably exhibits the different current signal by the presence of target, theophylline, during the treatment with $AgNO_3$ solution. As presented in Fig. S4 (a), the peak current of silver ions increases with increasing treatment time with $AgNO_3$ solution in

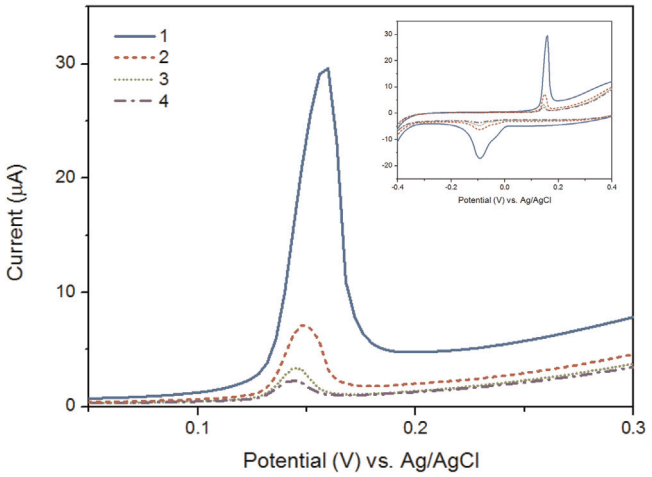

Fig. 2. Oxidation peak signal of cyclic voltammetry generated by silver ions within AP site-incorporated duplex DNA or full-matched duplex DNA in the absence and presence of theophylline (AP site-incorporated duplex DNA in the absence (1) and presence (2) of theophylline and full-matched duplex DNA in absence (3) and presence (4) of theophylline). Inset: cyclic voltammogram containing oxidation peak at +0.15 V and reduction peak at −0.09 V.

both cases with and without theophylline and the apparent signal difference is observed after 20 min. As the treatment time further increases, the signal difference more increases and reaches maximum value around 70 min. Based on this result, our sensing system is supposed to stably produce the different current signal by the presence of theophylline during the wide treatment time with $AgNO_3$ ranging from 20 min to 100 min. We further examined electrochemical signals generated by silver ions entrapped into AP site located at varying positions within duplex DNA. As presented in Fig. S5, all the three duplex DNA probes produce current signal with very similar intensities indicating that the position of AP site within duplex DNA is not significant in this strategy.

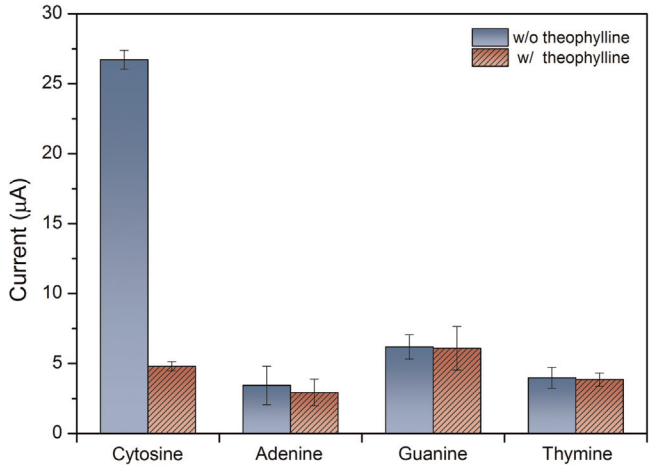

Fig. 3. Oxidation peak current produced by silver ions within duplex DNA probes incorporating AP site opposite four different nucleobases (Cytosine, Adenine, Guanine, and Thymine) in the absence and presence of 250 μM theophylline.

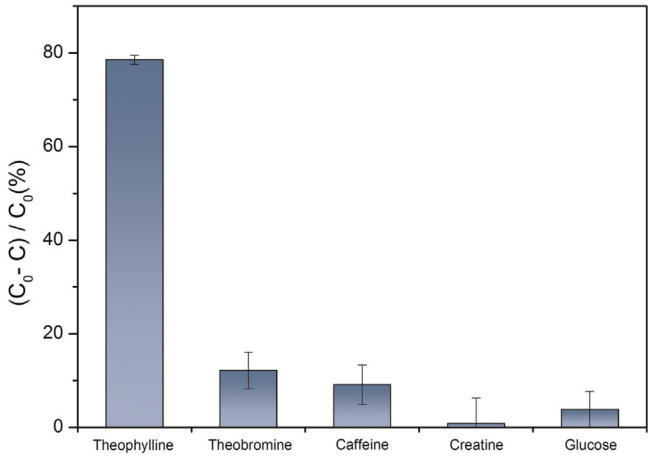

Fig. 4. Selectivity of the new electrochemical assay. The concentrations of theophylline, theobromine, caffeine, creatinine, and D-glucose are 250 μM. The degree of signal reduction was defined as $(C_0-C)/C_0$ (%), where C_0 and C are the oxidation peak current in the absence and presence of theophylline and other reagents, respectively.

3.3. Influence of nucleobase opposite AP site

The effect of the type of nucleobases opposite AP site in duplex DNA was then investigated on the electrochemical signal produced by silver ions. For this purpose, oxidation peak currents produced by silver ions within duplex DNA probes incorporating AP site opposite four different nucleobases (cytosine, adenine, guanine, and thymine) were measured in the absence and presence of theophylline. The four duplex DNA probes possess identical sequence except for a single base opposite the AP site. As shown in Fig. 3, intense electrochemical signal is obtained only from the duplex DNA having cytosine paired with AP site, which is quite consistent with the results of previous study using isothermal titration calorimetric method (Shukla and Sastry, 2009). Importantly, the presence of theophylline significantly reduces the electrochemical signal from the duplex DNA having cytosine opposite the AP site (Fig. 3). On the other hand, the other three duplex DNA probes having adenine, guanine, or thymine as a base opposite AP site do not produce significant electrochemical signal and furthermore the signals are negligibly influenced by the presence of theophylline. All these results confirm that cytosine base at the position opposite the AP site is essentially required within the duplex DNA probe to construct this strategy.

3.4. Selectivity and sensitivity

In order to evaluate the selectivity of this sensing system, structurally similar methylxanthine derivatives and other components present in blood serum were investigated for their abilities to reduce the electrochemical signal, which were then compared with that of theophylline. As presented in Fig. 4, only theophylline results in significant reduction of electrochemical signal. In contrast, there is no significant signal reduction induced by the presence of methylxanthine derivatives such as caffeine and theobromine, which are structurally different from theophylline only by a single moiety attached to the xanthine ring. The electrochemical signal is also negligibly reduced by creatinine and D-glucose, which are generally present in blood serum. These results demonstrate that our sensing system possesses high selectivity only toward target theophylline.

The detection sensitivity of our system was also determined by measuring the oxidation peak current as a function of the concentrations of theophylline (Fig. 5(a)). The result shows that electrochemical current peak decreases with increasing concentrations of theophylline up to *ca.* 100 μM, but appears to be saturated over 100 μM. Excellent linear relationship ($R^2=0.99$) is observed in a range from 0 to 50 μM and the limit of detection (LOD) (3σ/slope) is determined to be *ca.* 3.2 μM which is comparable to those of previous studies (Fig. 5(b)) (Ferapontova and Gothelf, 2009b; Rankin et al., 2006; Stojanovic and Kolpashchikov, 2004). As the concentration of theophylline decreases, the slight peak shift to positive potential is observed (Fig. 5(a)). This phenomenon is frequently observed in a system where signaling molecules are bound to DNA and ascribed to the decreased electron transfer rate of silver ions (Carter et al., 1989; Liu et al., 2002). More specifically, as the concentration of theophylline decreases, silver ions are more captured within AP site incorporated duplex DNA, which results in a closer contact with neighboring silver ions. This increases the electron transfer path by diversifying electron transfer routes, thereby leading to the reduction of electron transfer rate (Campos and Ferapontova, 2014). As a result, the peak potential is shifted to the positive direction as the concentration of theophylline decreases.

3.5. Detection of theophylline in serum sample

To verify the practical utility of the system, we finally employed our system to detect theophylline present in human blood serum containing various biological components which may interfere the detection reliability of the strategy. As presented in Fig. S6, electrochemical signal is reduced with increasing theophylline in 5% human serum, showing almost similar pattern with the case using artificial sample containing only theophylline. The excellent linear relationship ($R^2=0.95$) is also observed in the same range of 0–50 μM (Fig. S6). For unknown samples, our method exhibits high reproducibility and precision with a coefficient of variation (CV) less than 7% and a recovery ratio within 100 ± 6% (Table 1). These results are within an acceptable range when compared with previous study, ensuring the reliability and reproducibility of our strategy (Park et al., 2014).

4. Conclusions

In the study described above, we successfully developed a novel, label-free strategy to electrochemically detect theophylline by utilizing AP site-incorporated duplex DNA serving as both binding pocket for silver ions and recognition site for theophylline. The strategy of our sensor basically relies on the electrochemical

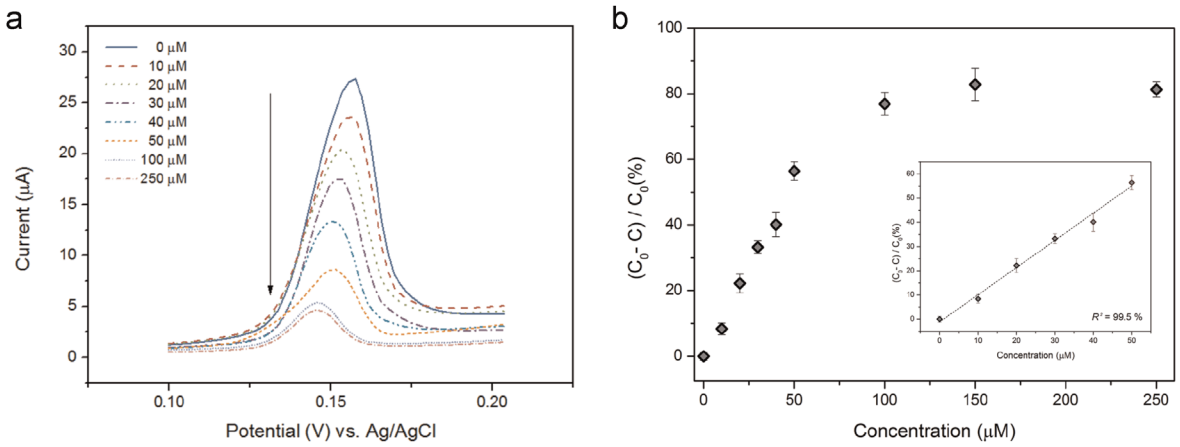

Fig. 5. Quantitative analysis of theophylline based on the redox reaction of silver ion captured into AP site-incorporated duplex DNA. (a) Cyclic voltammogram for various concentrations of theophylline. (b) The degree of signal reduction as a function of theophylline concentration; inset, linear range from 0 to 50 μM.

Table 1
Determination of theophylline in diluted human serum.

Added theophylline (μM)	Measured theophylline (μM)[a]	SD[b]	CV[c] (%)	Recovery[d] (%)
15	14.2	0.89	6.26	94.7
45	45.3	0.62	1.36	101.0

[a] Mean of three measurements.
[b] Standard deviation of three measurements.
[c] Coefficient of variation = SD/mean × 100.
[d] Measured value/added value × 100.

signal from silver ions captured into cytosine moiety placed opposite the AP site, which is greatly influenced by the competitive binding of theophylline into the same AP site. Our method shows great clinical utility in the practical application to detect theophylline from real serum samples yielding high selectivity and sensitivity. Importantly, our method employs relatively small electrochemical device and inexpensive signaling substance compared with the conventional fluorescence-based methods, which enables the realization of very convenient and cost-effective miniaturized system to detect theophylline. Finally, this is the first approach to utilize the redox reaction of silver ions captured into the AP site for the accurate determination of theophylline. The basic signaling strategy developed in this study would serve as a new platform to construct novel electrochemical systems to detect other biological molecules.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.056.

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