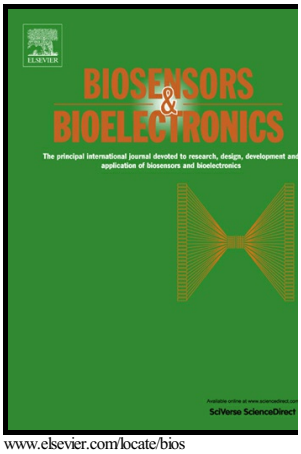

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Electrochemical aptasensor for the detection of adenosine by using PdCu@MWCNTs-supported bienzymes as labels

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Abstract: A highly sensitive electrochemical adenosine aptasensor was fabricated by covalently immobilizing 3′-NH_2-terminated capture probe (SSDNA1) and thionine (TH) on Au-GS modified glassy carbon electrode. 3′-SH-terminated adenosine aptamer (SSDNA2) was adsorbed onto palladium/copper alloyed supported on MWCNTs (PdCu@MWCNTs)-conjugated multiple bienzymes, glucose oxidase (GOx), and horseradish peroxidase (HRP) (SSDNA2/PdCu@MWCNTs/HRP/GO_X). Then, it was immobilized onto the electrode surface through the hybridization between the adenosine aptamer and the capture probe. The signal was amplified based on the gradual electrocatalytic reduction of GOx-generated hydrogen peroxide by the multiple HRP through the mediating ability of the loaded multiple TH. However, the peak current of TH decreased in the presence of adenosine because the interaction between adenosine and its aptamer made SSDNA2/PdCu@MWCNTs/HRP/GO_X release from the modified electrode. Various experimental parameters have been optimized for the detection of adenosine and tests for selectivity, reproducibility and stability have also been performed. Under the optimal condition, the proposed aptasensor displayed a wide linear range (10~400 nM) with the low detection limit (2.5 nM), which has been applied in human serum samples with satisfactory results. Thus, the combination of Au-GS as a sensor platform and PdCu@MWCNTs/HRP/GO_X as labels can be a promising amplification strategy for highly sensitive adenosine detection.

***Keywords*:** adenosine; aptamer; electrochemical biosensor; PdCu@MWCNTs.

1. Introduction

Similar to antigens and antibodies, aptamers can combine many types of targets such as small molecules, proteins, drugs and even viruses (Wu et al., 2015a). Due to their outstanding advantages such as the simplicity of synthesis, ease of labeling, excellent stability, wide applicability, and high sensitivity, aptamers are considered to be ideal candidates as molecular recognition elements in biosensors. Various aptasensors based on fluorescence (Guo et al., 2015; Wu et al., 2015b), colorimetry (Wei et al., 2007; Luo et al., 2015), electrochemiluminescence (Fang et al., 2008; Liu et al., 2014), electrochemistry (Zuo et al., 2007; Ding et al., 2010) and other assays (Yao et al., 2015) have been developed. Among them, electrochemical aptasensors have attracted a great deal of attention recently, which are proved to be powerful analytical tools owing to the integrated advantages of electrochemistry and aptamer with high specificity and affinity.

For sandwich-type methods, using functional nanomaterials and enzymes as labels for signal amplification, have also been developed to achieve ultrasensitive detection, due to the superiority of low detection limits and high sensitivity (Wang et al., 2011). Various nanomaterials such as carbon nanotubes (Akter et al., 2012; Yu et al., 2006), silica nanoparticles (Lin et al., 2011), carbon spheres (Du et al., 2010), etc. have been used as nanocarriers for loading enzymes such as only one kind of enzymes–horseradish peroxidase (HRP) or mimic bi-enzyme system of Pt–AuNPs and HRP (Zhao et al., 2011; Bai et al., 2011). However, PdCu@MWCNTs-simultaneously supported bienzymes, glucose oxidase (GOx) and HRP as the label (PdCu@MWCNTs/HRP/GO_X) to detect adenosine have not been reported yet. Alloying of noble metals with non-noble metals (Fe, Co, Ni, Cu etc.) is one of the effective approaches to enhance the catalytic activity and the selectivity (Yang et al., 2011). In recent years, multi-walled carbon nanotubes (MWCNTs) have attracted considerable attention due to their unique structure and properties, including large surface areas, high electrical conductivity, good thermal and chemical stability, as well as rich surface chemical functionalities (Tan et al., 2015). Therefore, the integration of MWCNTs with PdCu alloy might potentially provide excellent

opportunity for signal amplification in sensor. PdCu@MWCNTs not only immobilize more bienzymes and aptamers but also exhibit superior electrocatalytic activity. In this work, PdCu@MWCNTs-simultaneously supported bienzymes, glucose oxidase (GOx) and HRP were used as the label for multiple signal amplification.

Adenosine is an endogenous nucleoside modulator released from almost all the cells. It takes part in biological activities such as the extension of the blood vessels, increment of the blood flow of the arteries, anti-arrhythmia and improvement of the oxygen supply of cardiac muscle. Moreover, it is also an essential intermediate in the synthesis of ATP, adenine, adenylic acid and vidarabine (Huang et al., 2011; Wang et al., 2010). Therefore, the determination of adenosine is very important.

Here, the mixture of thionine (TH) and Au-GS was used to immobilize 3′-NH_2-terminated capture probe (SSDNA1). PdCu@MWCNTs was synthesized and incubated with bienzymes (glucose oxidase and horseradish peroxidase) and 3′-SH-terminated adenosine aptamer (SSDNA2) to fabricate the electrochemical aptasensor, aiming to improve the analytical performances of the aptasensor. Then, the adenosine aptamer was immobilized onto the electrode surface through its hybridization with the capture probe. In the absence of adenosine, the signal of TH was amplified greatly by the gradual HRP-electrocatalyzed reduction of hydrogen peroxide, which was generated by the enzyme GOx. In the presence of adenosine, TH peak current decreased due to the relaease of aptamer from the capture probe and the formation of a complex between SSDNA2/PdCu@MWCNTs/HRP/GO_X and adenosine. Therefore, sensitive quantitative detection of adenosine was achieved by measuring the differences of TH current response by using square wave voltammetry (SWV) technique. The preparation, characterization, optimal conditions, and analysis of real samples for the detection of adenosine were discussed in detail. The proposed aptasensor was used to detect adenosine in human serum samples with satisfactory results.

2. Experimental

2.1. Materials and reagents

Adenosine, horseradish peroxidase (HRP) and glucose oxidase (GOx) were

purchased from Aladdin Chemical Shanghai Co., Ltd. Thionine (TH) was obtained from Sinopharm Chemical Reagent Ltd Co. (Beijing, China). The sequences of 3′-NH_2-terminated capture probe (SSDNA1: 5′-TCTCTTGGACCC-$(CH_2)_6$-NH_2-3′) and 3′- SH -terminated adenosine aptamer (SSDNA2: 3′-SH-AGAGAACCTGGGGGAGTATTGCGGAGGAAGGT-5′) were synthesized by Shanghai Sangon Biotech. Co., Ltd. (China).

2.2. Apparatus

All electrochemical measurements were performed on a CHI 760D electrochemical workstation (Shanghai CH Instruments Co., China). Transmission electron microscope (TEM) images were obtained from a Hitachi H-800 microscope (Japan). Electrochemical impedance spectroscopy (EIS) was performed in $[Fe(CN)_6]^{3-/4-}$ working solution in the frequency range of 0.1~10^5 Hz. A conventional three-electrode system was used for all electrochemical measurements: the modified glassy carbon electrode (GCE, 4 mm in diameter) as working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire electrode as the counter electrode.

2.3. Synthesis of PdCu@MWCNTs

PdCu@MWCNTs was prepared according to the reported method (Yang et al., 2011). In brief, an aqueous solution of $PdCl_2$ (3.3 mg/mL, 3.8 mL), 20 mg of $CuSO_4 \cdot 5H_2O$, and 50 mg of glutamate were mixed together in 40 mL of ethylene glycol. The pH of the system was adjusted to 11 by the dropwise addition of the 8 wt% KOH/EG solution under stirring. During the process, the green solution turned into dark blue. Subsequently, 30 mg of pristine MWCNTs were added, and the solution was ultrasonicated and stirred for 2 h to obtain a homogeneous suspension. Upon completion, the suspension was transferred into a 50 mL of Teflon-lined stainless-steel autoclave. The autoclave was sealed, heated at 160^{o}C for 6 h, and air-cooled to room temperature. Finally, the product was collected by filtration and washed several times with doubly distilled water. The obtained product was dried at 40^{o}C under vacuum for 8 h.

2.4. Preparation of SSDNA2/PdCu@MWCNTs/HRP/GO_X conjugate

Figure 1A showed the procedure to prepare the SSDNA2/PdCu@MWCNTs/HRP/GO_X conjugate. SSDNA2 (5 μM), HRP (1 mg/mL) and GO_X (1 mg/mL) were added to the as-prepared PdCu@MWCNTs (1 mg/mL) solution and stirred for 24 h at 4°C. By this step, amino groups in GOx and HRP can be bonded strongly to Pd of PdCu@MWCNTs and SSDNA2 can be covalently bonded to PdCu@MWCNTs via S-Pd bonds.

2.5. Modification of electrodes

Fig. 1B showed the fabrication procedure of the aptasensor. A glassy carbon electrode was polished to a mirror-like surface by using 1.0, 0.3 and 0.05 μm alumina powder sequentially and then thoroughly cleaned before use. First, 6.0 μL of Au-GS solution (2.0 mg/mL) was added onto the electrode and then dried. The electrode was thoroughly rinsed with PBS later. Then, 6 μL of 0.5 mg/mL thionine was dropped on the electrode surface. After that 6 μL of 5 μM SSDNA1 which was dispersed in Tris-HCl was coated on the electrode surface and incubated for 2 h at 4 ^{o}C. Finally, the electrode was incubated for another 4 h in SSDNA2/PdCu@MWCNTs/HRP/GO_X solution. After washing, the electrode was ready for measurement. To measure the adenosine, 6 μL of adenosine solution in different concentration was dropped onto the electrode surface and incubated for 45 min. The sensing was found by monitoring the change of TH peak current, with the use of square wave voltammetry (SWV) technique in pH 8.0 PBS containing 1.0 mM glucose.

Fig.1C shows the possible mechanism that is described as follows (Jeong et al., 2013):

β-Glucose+ $O_2 \xrightarrow{GOx} H_2O_2$+glucolactone (1)

$H_2O_2 + HRP_R \rightarrow H_2O + HRP_O$ (2)

$HRP_O + TH \rightarrow HRP_R + TH^+$ (3)

Firstly, TH signal is greatly enhanced in the absence of adenosine due to the gradual electrocatalytic reduction of GOx-generated hydrogen peroxide by the multiple HRP labels through the mediating ability of the loaded multiple thionine (TH). Secondly, because the aptamer-target complex is more stable than the

aptamer-DNA duplex, the aptamer prefers to form the aptamer-adenosine complex rather than the aptamer-DNA duplex, which causes the separation of the aptamer probe from the electrode surface in the presence of adenosine. In this way, HRP and GO_X are released from the surface of the electrode along with the aptamer, and hence, the electronic signal of TH decreases. The decreased signal of TH can reflect the concentration of the adenosine.

3. Results and discussion

3.1. Characterization of PdCu@MWCNTs

As shown in Fig. S1 (Supporting Information), PdCu alloyed nanoparticles were formed on MWCNTs to obtain PdCu@MWCNTs, which was observed in agreement with the literature (Yang et al., 2011).

3.2. Characterization of the aptasensor

EIS has been proven to be one of the most powerful tools for probing the features of a modified electrode surface (Wang et al., 2013; Peng et al., 2012). The semicircle diameter equals the charge transfer resistance R_{ct}. The impedance spectra of different modified electrodes were recorded in 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol/L KCl aqueous solution and the corresponding results are shown in Fig. 2A. When Au-GS and thionine was modified onto the electrode, the resistance decreased (curve b) a little compared with bare GCE (curve a). The reason was that Au-GS and thionine were excellent electrically conducting materials, which made electron transfer easier. After incubation with SSDNA1, the semicircle increased remarkably (curve c) because SSDNA1 was immobilized on the electrode successfully and blocked the electron transfer. Then a larger semicircle diameter in curve d is seen after incubation with SSDNA2/GO_X/HRP/PdCu@MWCNTs, confirming the high resistance of the electrode interface as the prepared aptasensors was blocked with non-conductive substances due to hybridization of SSDNA2 with SSDNA1. Finally, R_{ct} decreased again (curve e) after adenosine was immobilized on the electrode. The reason was that the formation of aptamer-adenosine would result in the dissociation of aptamer from the electrode surface.

The stepwise construction process of the aptasensor was also characterized by

the cyclic voltammetry (CV) in 5.0 mmol/L $K_3[Fe(CN)_6]$ and 0.1 mol/L KNO_3 aqueous solution, as shown in Fig. 2B. Compared with the bare electrode (curve a), the current was enhanced after Au-GS and thionine were immobilized on it (curve b). Subsequently, an obvious decrease in the signal was obtained (curve c) when capture probe SSDNA1 was conjugated to the electrode surface, which was because that SSDNA1 obstructed the electron transport. After incubated with SSDNA2/GO_X/HRP/PdCu@MWCNTs, a decrease of the current was detected (curve d). The reason was that half-duplex DNA structure is formed on the surface of the electrode through the hybridization between the 12-base capture probe and the 32-base adenosine aptamer. The signal (curve e) increased again because more non-electroactive aptamers left from the electrode surface due to the formation of aptamer-adenosine. CV results were consistent with the observation of EIS experiments, which further confirmed the successful construction of the aptasensor.

3.3. Optimization of experimental conditions

To verify the signal amplification effect, PdCu@MWCNTs, PdCu@MWCNTs/GO_X, PdCu@MWCNTs/HRP/GO_X as the label was coated onto the electrodes surface to prepare the corresponding modified electrode, respectively. The SWV responses were shown as Figure S2 (Supporting Information). It can be seen that the current of PdCu@MWCNTs/HRP/GO_X (curve a) as the label is higher than that of PdCu@MWCNTs/GO_X (curve b) and PdCu@MWCNTs (curve c), indicating the best signal amplification effect of PdCu@MWCNTs/HRP/GO_X. Therefore, PdCu@MWCNTs-simultaneously supported bienzymes, glucose oxidase (GOx) and HRP as the label to amplify the response signal of TH was selected maybe due to synergistic effect of PdCu alloy, GOx and HRP.

In order to obtain the best performance for detecting adenosine, experimental conditions were optimized including the effects of pH and the concentration of Au-GS. As shown in Fig. 3A, it was found that the peak current increased from pH 6.0 to 8.0 and it reached the maximum at pH 8.0. After that, when the pH increased from 8.0 to 8.5, the peak current decreased instead. Thus, pH 8.0 PBS was selected for the test throughout this study. With the increasing concentration of Au-GS, the peak current

for detection of 150 nM adenosine increased. However, when the concentration of Au-GS was more than 2.0 mg/mL, the peak current gradually decreased (Fig. 3B). Therefore, 2.0 mg/mL Au-GS was selected for the preparation of the aptasensor.

Under the optimized conditions, the performance of the proposed aptasensor was evaluated for the detection of adenosine. The square wave voltammetric (SWV) peak currents increased with the increase of adenosine concentrations. The linear equation in the ranges of 10 to 40 nM adenosine is ΔI=0.392+0.370c (r=0.997), while for higher concentration ranges (40~400 nM) the relationship between peak current and adenosine concentration is ΔI=13.8+0.0268c (r=0.998). Based on S/N=3, the detection limit of 2.5 nM was obtained. The proposed aptasensor had lower detection limit than most of previously reported aptasensors as well as a wider linear range than some of them, as clearly shown in Table 1. The reason was mainly attributed to the superior electrocatalytic activity and the large amount of immobilized labels of PdCu@MWCNTs.

3.4. Selectivity, reproducibility and stability

To assess the selectivity of the aptasensor, the SWV responses were measured for interfering substances such as uridine, guanosine and cytidine which belonged to the nucleosides family. It can be seen from Fig. 4A that the current response decreased significantly after dropping 200 nM adenosine onto the modified electrode surface. However, there was no remarkable decrease in the signal in the presence of 1 μM non-target molecules such as cytidine, uridine and guanosine (Fig. 4B-D). The data was also displayed in the histogram (Fig. 4E). The results demonstrated that the developed method had sufficient selectivity to detect the interaction between the adenosine and the aptamer immobilized on the electrode surface.

To evaluate the reproducibility of the aptasensor, a series of four electrodes were prepared for the detection of 200 nM adenosine. The relative standard deviation (RSD) of the measurements for the four electrodes was 2.2 %, which suggested that the reproducibility of the proposed aptasensor was quite good.

In addition, the aptasensor was stored at 4 ˚C. After 8 days, the aptasensor retained 96% of its original response. After 10 days, the responses of the aptasensor

maintained 86% of its initial responses. The above results indicated that the aptasensor possessed good bioactivity and acceptable stability.

3.5. Serum sample analysis

To demonstrate the practical application of the aptasensor, the amount of adenosine in serum sample including normal and pregnant people. Meanwhile, high performance liquid chromatography (HPLC) was used as a reference method to validate the proposed method. The detailed process of sample pretreatment using HPLC method according to the reported literature (Zhang et al., 2008). The adenosine concentration in human serum sample was measured 5 times by using two methods, respectively. The results are shown in Table S1. The relative error between the two methods was in the range from -2.2 % to 2.8 %. Based on *F*-test, the precisions of this method were not remarkably different from those obtained by HPLC method because *F* calculated is less than theoretical value. These data revealed a good agreement between the two analytical methods.

The accuracy was also studied through a recovery experiment. 20.00 nM or 100.0 nM of the standard adenosine solution was added to human serum sample. The recovery, referring to the average recovery, was calculated and the results are also shown in Table 2. The recovery was in the range from 95.1% to 103%. All these presented sufficient precision and high accuracy. There would be promising future for the proposed platform in biological fluids and pharmaceutical product application to detect adenosine.

4. Conclusions

Using adenosine as a model, a sensitive and selective aptasensor was developed based on immobilization of SSDNA1 as capture probe onto Au-GS/thionine and aptamer probe SSDNA2 adsorbed onto PdCu@MWCNTs-conjugated multiple bienzymes (HRP and GOx). The highly sensitive detection was achieved by monitoring the change of TH peak current with the use of square wave voltammetry (SWV) technique. Without adenosine, the amplification of TH peak current was caused by multiple HRP-catalyzed TH-mediated electroreduction of H_2O_2, which was

locally generated by GOx. In the presence of adenosine, HRP and GO_X were released from the electrode surface along with the aptamer, and hence, the electronic signal of TH decreased. This developed method showed high sensitivity, good selectivity and reproducibility, and acceptable stability, having the potential for the sensitive determination of aptamer-based other small molecules.

Acknowledgements

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Figure captions:

Fig. 1 Fabrication steps of the aptasensor.

Fig. 2 Electrochemical impedance spectroscopy (A) and cyclic voltammograms (B) for each immobilized step. The bare GCE (a), Au-GS/TH/GCE (b), SSDNA1/Au-GS/TH/GCE (c), SSDNA2/GO_X/HRP/PdCu@MWCNTs/SSDNA1/Au-GS/TH/GCE (d), 200 nM Adenosine/SSDNA2/GO_X/HRP/PdCu@MWCNTs/SSDNA1/Au-GS/TH/GCE (e)

Fig. 3 Effect of pH (A) and the concentration of Au-GS (B) on the response of the aptasensor to 150 nM adenosine.

Fig. 4 SWV responses of the modified electrode after dropping of 200 nM adenosine (A), 1μM uridine (B), 1μM guanosine (C) and 1μM cytidine (D) in PBS (pH 8.0). Histogram of the current change of aptasensor after reaction with adenosine and other nucleoside family (E).

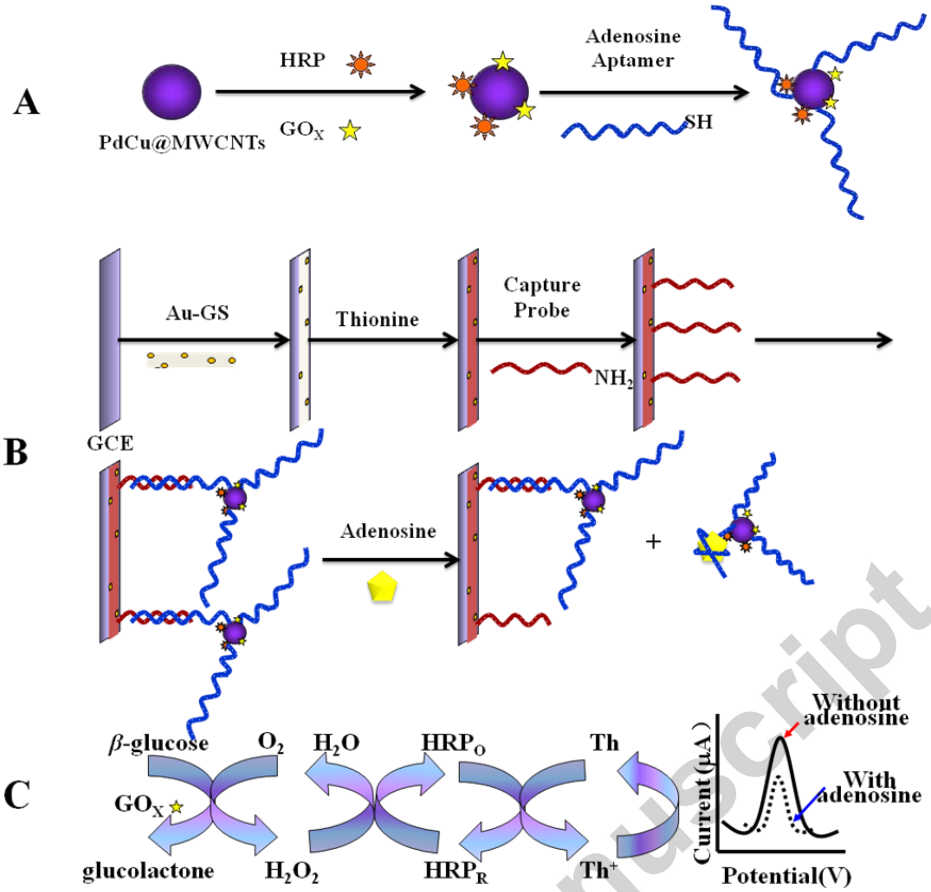

Fig. 1

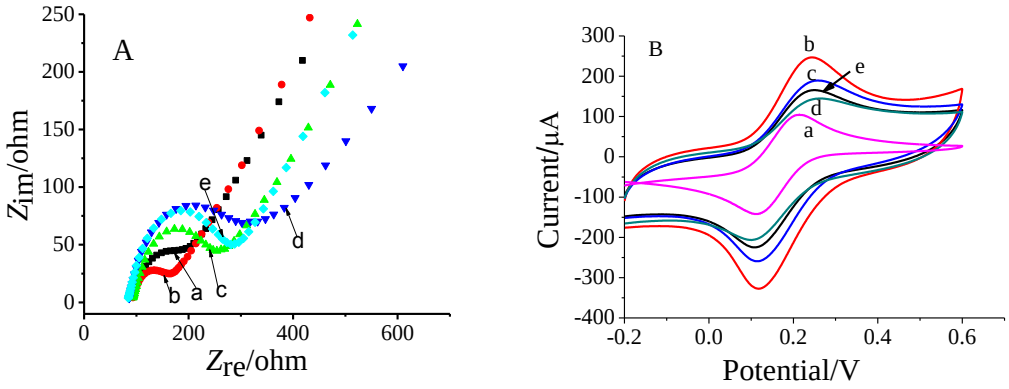

Fig. 2

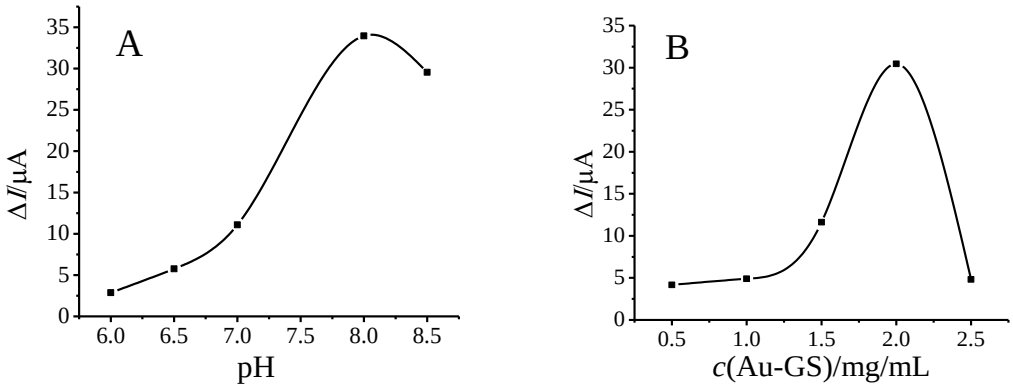

Fig. 3

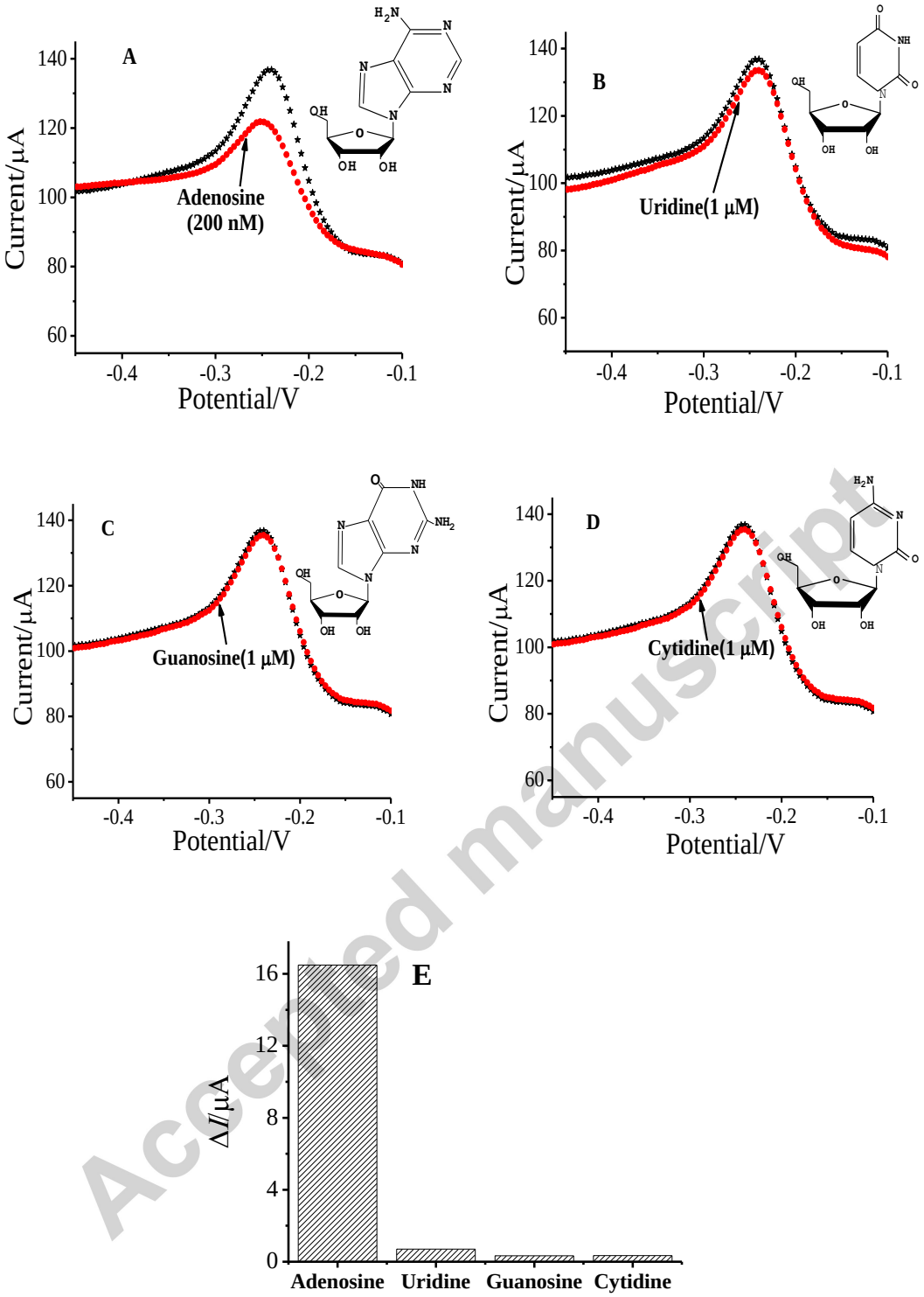

Fig. 4

Tables captions:

Table 1 Analytical parameters for the detection of adenosine with different aptasensors.

Table 2 Results for the determination of adenosine in human serum sample.

Table 1

Materials	Electrochemical technique	Detection limit (nM)	Detection range (nM)	References
$Ru(bpy)_3^{2+}$-AuNPs	ECL	5.0	10~100	Wang et al., 2010
Methylene blue	DPV	10.0	20~2000	Wang et al., 2009
DNA-loading AuNPs	CV	0.18	0.5~4	Zhang et al., 2008
Au/PCS-MBA/MCE	EIS	10.0	$10\sim10^5$	Du et al., 2008
Au/DNA(S1)/MCH/ DNA(S2)/DNA3-Au NPs	CV	0.02	0.02~3.0	Deng et al., 2009
Au/DNA/DNA duplex	DPV	10.0	10~1000	Yan et al., 2011
oPADs	Digital Multimeter	1.18×10^4	-----	Liu et al., 2012
$Ru(bpy)_3^{3+}$	CV	2×10^4	2×10^4~3×10^6	Kim et al., 2009
NPGE	DPV	100	$100\sim3\times10^6$	Kashefi-Kheyrabadi et al., 2013
This study	SWV	2.5	10~400	

Table 2

Serum sample	Content (nM)	Mean (nM, *n*=5)	Added (nM)	Recovery Value (nM)	Recovery (%, *n*=5)
Normal woman	29.55	29.58	20.00	48.12	95.1
	31.03		20.00	49.22	
	30.10		20.00	48.76	
	28.89		20.00	49.33	
	28.34		20.00	47.58	
Pregnant woman	127.6	127.9	100.0	230.5	103
	129.9		100.0	232.7	
	126.6		100.0	229.4	
	128.8		100.0	231.5	
	126.7		100.0	228.3	

Highlights

1. A sensitive aptasensor for detection of adenosine is developed.

2. Enhanced sensitivity is achieved by using PdCu@MWCNTs-conjugated bienzymes (GOx and HRP).
3. The aptasensor has low detection limit, good stability, selectivity and reproducibility.