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Label free electrochemical aptasensor for ultrasensitive detection of ractopamine

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ABSTRACT

A label free electrochemical (EC) aptasensor for ultrasensitive detection of ractopamine (RAC) was developed. A special immobilization media consisting of gold nanoparticles/poly dimethyl diallyl ammonium chloride–graphene composite (AuNPs/PDDA-GN) was utilized to improve conductivity and performance of the biosensor. The RAC aptamer was attached on AuNPs of the composite membrane via Au–S bond. The fabrication process of the EC aptasensor was characterized by electrochemical impedance spectroscopy and cyclic voltammetry. The peak currents obtained by differential pulse voltammetry decreased linearly with the increasing of RAC concentrations and the sensor responds approximately logarithmically over a wide dynamic range of RAC concentration from 1.0×10^{-12} mol/L to 1.0×10^{-8} mol/L. The linear correlation coefficient of the developed aptasensor was 0.998, the limit of detection was 5.0×10^{-13} mol/L. The proposed EC aptasensor displayed good stability, reproducibility and robust operation in animal urine. Particularly, the generality of the fabrication approach of electrochemical aptasensor is highlighted with a further example for illegal drugs detection via the aptamer identification.

1. Introduction

β-Agonist are drugs that can promote bronchodilation, vasodilatation and increase heart rate, and used for the treatment of pulmonary disease and asthma (Kuiper et al., 1998). RAC is one of the typical β-agonists. It was used as feed additive in animal feeding due to its roles in decreasing adipose tissue deposition and increasing protein accretion in livestock (Xiong et al., 2006). However, excessive amount of RAC in animal meats may pose health risks to human being when the meats were consumed (Carry et al., 2009). The use of RAC in stock breeding has been banned by the majority of countries, such as China (MOA Regulation 176, 2002, PR China) and European Union (Commission decision, 1996/23/EC). Routine monitoring of RAC abuse had attracted great attention to the government regulatory agencies and the food industry.

Convention analytical methods, such as liquid chromatography (LC) (Koole et al., 1999; Blomgren et al., 2002), gas chromatography–mass spectrometry (Hernandez-Carrasquilla, 2000) and LC–tandem mass spectrometry (LC–MS/MS) (Nielen et al., 2008; Shao et al., 2009), have been developed to detect trace residue of RAC. These chromatography methods often suffer from complicated sample preparation procedures, which were time-consuming. Some rapid detection methods including enzyme immunoassay (Elliott et al., 1998), visual probes (Zhou et al., 2013), immuno chromatographic assay sensor (Zhang et al., 2009; Wang et al., 2015), surface-enhanced Raman scattering immunoassay (Zhu et al., 2011) and so on, have been developed for the determination of RAC. However, these new methods either require antibodies that were difficult to obtain or the sensitivity don not meet the MRL requirements. Electrochemical (EC) sensors show great potential due to their simple determination procedure, fast response time, high sensitivity, lower cost and portability (Chen et al., 2011). Recently, some EC and electrochemiluminescent (ECL) sensors have been developed for detection of RAC in various sample matrixes (Yang et al., 2014; Wei et al., 2015; Li et al., 2013). These EC or ECL sensors exhibited high sensitivity and fast analysis time. However, the selectivity of these EC or ECL methods depended on either monoclonal antibody for RAC or electrocatalytic

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oxidation of RAC. The sensitivity and selectivity were limited in complicated sample matrix due to the absence of specific recognition elements.

Aptamers are a kind of synthetic oligonucleotide sequence (Ellington and Szostak, 1990). They exhibit excellent biological recognition capability and can be exploited as analytical biosensors (Hermann, 2000). Aptamers have been synthetically evolved for a wide range of targets ranging from metal ions, small molecules, to proteins and even entire cells (Jo et al., 2011; Lee et al., 2010; Meyer et al., 2011). Compared with antibodies, aptamers possess some outstanding features, including high specificity, stability, easy modification with electrochemical active markers, optical markers, enzymes and other desired substances (Khezrian et al., 2013). It is facillius for the aptamer to transduce the recognition events into detectable chemical signals (Kang et al., 2008). In the case of small molecular compounds targets, the binding of aptamers induces conformational switch of the aptamer (Citartan et al., 2012). Such switch can then be transduced via colorimetric (Barthelmebs et al., 2011; Zheng et al., 2011), fluorescence (Sassolas et al., 2011; Yildirim et al., 2012), size (Alsager et al., 2014), and electrochemical responses (Lin et al.,2012). Among them, electrochemical aptasensors offer the lowest detection limit, as well as rapid, label-free quantification of analyte over a wide range of concentrations (Radi, 2011). Graphene has recently attracted tremendous interest because of its unique thermal, mechanical, and electrical properties (Novoselov et al., 2004). One of the most promising applications of graphene is electrochemical sensing (Zhou et al., 2009; Shan et al., 2009).

To our knowledge, the EC aptasensor for RAC based on the aptamer recognition has not been reported. Herein, we proposed an EC aptasensor for sensitive determination of RAC. A single strain DNA aptamer designed for recognition RAC was used for selective detection of RAC. The RAC aptamer was modified on the AuNPs/PDDA-GN composite film on a glass carbon electrode surface (GCE). The interaction between aptamer and RAC can be captured by the EC probe of ferricyanide and monitored by cyclic voltammetry (CV) and differential pulse stripping voltammetry (DPV). The proposed EC sensor showed excellent selectivity and could be used directly for the analysis of urine samples without any sample pre-treatment. Particularly, the EC aptasensor was applied to the determination of RAC in swine urine samples with satisfactory results.

2. Experimental

2.1. Chemicals and apparatus

All reagents were of analytical grade and used without further purification. Solutions were prepared using high pure water with a resistance of 18 MΩ cm. RAC, clenbuterol, salbuterol, dopamine, oxytetracycline, penicillin and Poly dimethyl diallyl ammonium chloride (PDDA, MW=100,000–200,000, 20 wt% in water) was purchased from Aldrich Company. The RAC aptamer was a single strain DNA which was provided by Dr. Yao who is from Jinan University, Guangzhou of China. The RAC aptamer contained functional sequence of AGTGCGGGC with a molecular weight of 4.06 KDa. The base pairs (bp) and dissociation equilibrium constant (K_d) for RAC of the aptamer were 13 bp and 1.66×10^{-6} mol/L. The ΔH and ΔG of aptamer binding with RAC were -0.9×10^{2} cal/mol and -7.88×10^{3} cal/mol. 5′ of RAC aptamer was modified with functional group of $-(CH_2)_3$–SH. $HAuCl_4\cdot4H_2O$ was bought from Sinopharm Chemical Reagent Co., Ltd. Graphene oxide (GO) dispersion (1 mg/mL) was bought from XF NANO, INC. Hydrazine hydrate (40 wt%) was purchased from Sinopharm Chemical Reagent Co., Ltd. Bovine serum albumin (BSA) was purchased from Beijing Cell chip Biotechnology Co., Ltd. Sodium citrate, $CaCl_2$, $MgCl_2$, and NaCl were purchased from Sinopharm Chemical Reagent Co., Ltd. Phosphate-buffered saline (PBS, 10 mM) with various pH values was prepared with stock standard solution of Na_2HPO_4 and NaH_2PO_4. The pH of the solution was measured with a PB-10 pH meter (Sartorius, Germany). Cyclic voltammetry (CV) and differential pulse stripping voltammetry (DPV) were carried out by a CHI 760 C electrochemical workstation (CHI Instrument Company, Shanghai, China). A three-electrode system was used in the measurements, composed of a GCE (2 mm in diameter) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and the Pt wire as the counter electrode.

2.2. Preparation of AuNPs

AuNPs were prepared by the reduction of $HAuCl_4$ with sodium citrate according to the conventional method (Hua et al., 2013). Briefly, 15 mL trisodium citrate (3.88×10^{-2} mol/L) was added into 150 mL of boiling solution of $HAuCl_4$ (1×10^{-3} mol/L) with magnetic stirring and the mixed solution was heated to boiling under stirring for another 30 min, producing a wine-red color solution. The solution was cooled to room temperature citrate-stabilized suspension of AuNPs was formed. The final AuNPs were characterized by using transmission electron microscope (TEM) and UV–vis (Fig. 2A and C). The size of AuNPs was about 13 nm and the concentration was 10 nmol/L. The stock solution of AuNPs was stored in a refrigerator at 4 °C for further use.

2.3. Preparation of PDDA-GNs

The preparation of PDDA-GNs was performed according to method from literature (Xue et al., 2011). Firstly, 1.5 mL GO dispersion, 1 mL PDDA (0.2 wt%), 1 mL hydrazine hydrate (4 wt%) and 4 mL H_2O was added into a flask, followed by ultra-sonication for 5 min to form a homogeneous dispersion. After stirring for 30 min at room temperature, the mixture was heated to 100 °C for 30 min. Finally, a black PDDA-GNs dispersion with a concentration of 0.25 mg mL^{-1} was obtained. Excessive PDDA and hydrazine hydrate was removed by centrifugation at 15000 rpm for 10 min. The PDDA-GNs precipitate was rinsed with water twice and then redispersed into 1 mL H_2O for the further use.

2.4. Fabrication of the sensor

GCE was polished with alumina slurry until a mirror-like surface was obtained. Then it was washed in absolute ethanol and ultra pure water for 5 min under ultrasonication. Finally, it was dried under a stream of nitrogen. 10 μL PDDA-GNs dispersion was dropped onto the surface of the GCE, dried under infrared lamp, and rinsed with water to remove loosely adsorbed graphene. The AuNPs/PDDA-GNs/GCE was prepared by immersing PDDA-GNs/GCE into AuNPs solution for 2 h and then reinsed with water and dried under a stream of nitrogen. The Citrate-capped AuNPs with negative surface charge can be adsorbed onto the PDDA-GNs with positive surface charge by electrostatic adsorption. The modified electrode (AuNPs/PDDA-GNs/GCE) was immersed in thoil modified RAC aptamer solution at 4 °C overnight. The resulted electrode was incubated in BSA solution (0.25%, w/w) for 1 h in order to block possible remaining active sites and avoid non-specific adsorption. The finished sensor (aptamer/AuNPs/PDDA-GNs/GCE) was stored at 4 °C in PBS (pH 7.0) when not use. The procedures used for construction of the sensor were shown in Fig. 1.

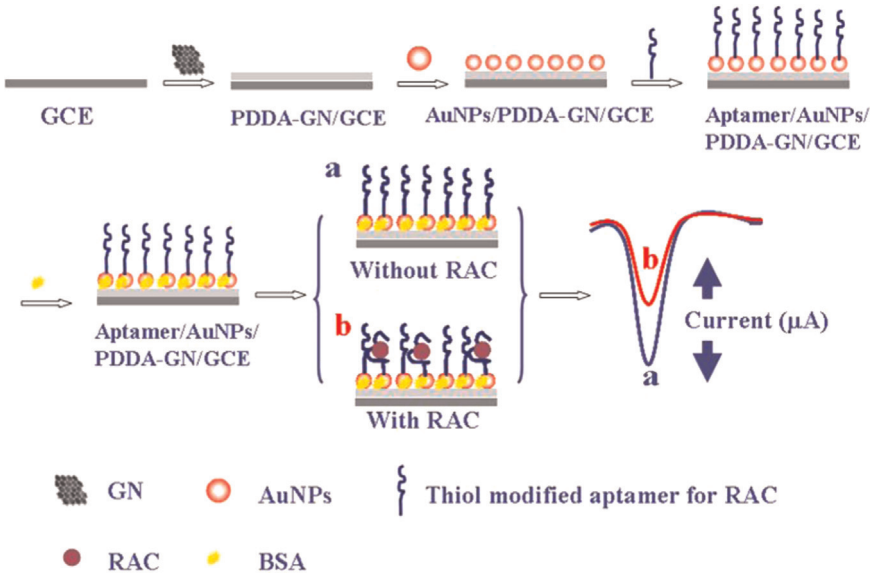

Fig. 1. Schematic illustration of the aptasensor fabrication process.

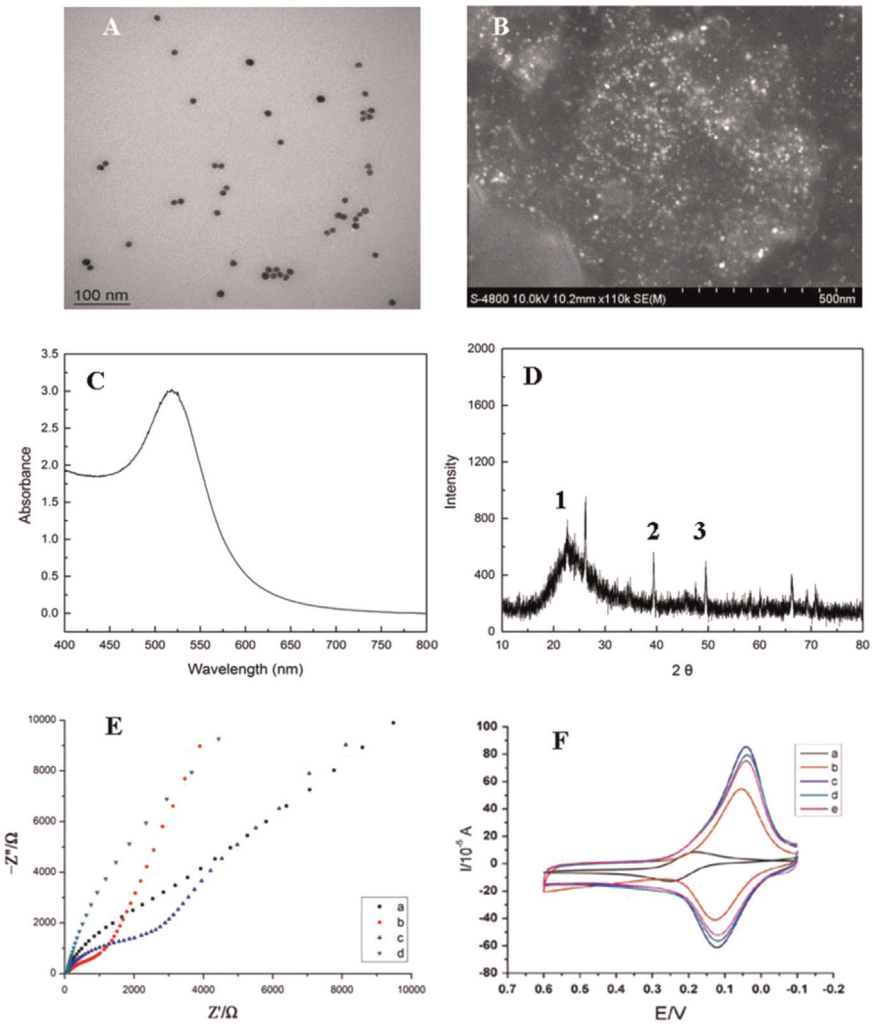

Fig. 2. (A) TEM image of AuNPs. (B) SEM image of modified electrode. (C) UV–vis spectrum of AuNPs. (D) XRD spectrum of modified electrode. (1) GN, (2) and (3) AuNPs. (E) EIS of modified GCE under different conditions (measurement in 1 mM $[Fe(CN)_6]^{3-/4-}$ + 10 mM PBS solution). (a) bare GCE, (b) AuNPs/PDDA-GNs/GCE, (c) BSA/RAC-aptamer/AuNPs/PDDA-GNs/GCE, (d) after addition of RAC (1×10^{-8} mol/L). (F) Cyclic voltammograms of fabrication process for the modified electrode (measurement in 1 mM $[Fe(CN)_6]^{3-/4-}$ + 10 mM PBS solution): (a) bare GCE, (b) PDDA-GNs/GCE, (c) AuNPs/PDDA-GNs/GCE, (d) aptamer-RAC /AuNPs/PDDA-GNs/GCE (e) BSA/aptamer-RAC /AuNPs/PDDA-GNs/GCE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Determination of RAC by using the developed EC aptasensor

Determination of RAC in PBS buffer (pH=7.0) was performed as following: The modified electrode was incubated in PBS solution for 35 min, then gently rinsed with water and immersed in 1 mM $[Fe(CN)_6]^{3-/4-}$ +10 mM PBS solution containing RAC at different concentrations for DPV analysis. The DPV scanned from −100 mV to 500 mV with a pulse amplitude of 50 mV and a pulse width of 200 ms.

For determination of RAC in urine, the urine sample was diluted 2 times with pH 7.0 PBS buffer, and the analysis was carried in the same procedure as in PBS buffer.

2.6. Validation of the developed EC aptasensor

The validity of EC aptasensor was verified by analyzing swine urine samples spiked with RAC at different levels and real contaminated urine samples. The results were further validated and compared with those from UPLC–MS/MS.

3. Results and discussion

3.1. Principle of RAC detection using EC aptasensor

Fig. 1 presented the detailed fabrication and detection process of the proposed EC aptasensor for RAC. The GCE surface was first modified by AuNPs/PDDA-GN composite. This led to increase the effective surface area of electrode and provided an enhanced loading surface for the subsequent immobilization of aptamer. The thiol modified aptamer was attached to the surface of AuNPs via the aid of coordinate binding. Here, the RAC aptamer modified GCE could produce a well-defined DPV signal depicted as “a” in Fig. 1. When RAC as target was introduced, the aptamer selectively bond with it and resulted in the hinder of the electron transition from the electrode surface and thus, a decrease in peak current of EC aptasensor was observed (depicted as “b” in Fig. 1).

3.2. Characterization of EC aptasensor

X-ray diffraction (XRD) and scanning electron microscope (SEM) were employed to characterize the prepared EC aptasensor. Fig. 2B showed the SEM image of the developed EC aptasensor, clearly illustrated morphology on surface of modified electrode. It was indicated that the GN and AuNPs were modified successfully on GCE electrode. As shown in Fig. 2D, XRD spectrum of GN on EC aptasensor exhibited a character peak at $2\theta=23.7^\circ$ (Wang et al., 2008). The XRD character peaks of AuNPs were shown at $2\theta=39.2^\circ$ and 49.4°.

In order to further understand the characterization of the modified electrode, the electrochemical behaviors of GCE under different conditions were examined by electrochemical impedance spectroscopy (EIS) and CV. As shown in Fig. 2E, bare GCE and AuNPs/PDDA-GNs/GCE had low resistance (curves a and b). Resistance increased dramatically with the modification of RAC aptamer and BSA (curve c). Further, the semi circle domain increased (curve d) after the addition of RAC (1×10^{-8} mol/L), this indicated RAC greatly hindered the electron transfer. The corresponding CV curves in $[Fe(CN)_6]^{3-/4-}$ were shown in Fig. 2F. Curve a indicated that the redox-label $[Fe(CN)_6]^{3-/4-}$ revealed a reversible CV at the bare GCE. After modified with PDDA-GNs, the peak current increased greatly, indicating that the introduction of the graphene played a role in the increase of the electro active surface area and provided the conducting bridges for the electron-transfer of $[Fe(CN)_6]^{3-/4-}$ (curve b). When AuNPs were adsorbed onto the GNs/GCE, the peak currents was increased (curve c), which attributed to the good electrical conductivity of AuNPs that could significantly enhance the transduction of the electron. After the aptamers for RAC was immobilized on the electrode surface, the peak currents decreased obviously (curve d), the electrode showed remarkable blocking of the redox probes from effective charge transfer at the interface owing to their non-electroactive property that obstructs electron transfer. The peak currents also decreased (curve e) after BSA was used to block non-specific sites.

The CV was also used to investigate electrochemical mechanism by the relationship between the peak current and the scan rate. As shown in Fig. S1, the peak current increased wit the increasing of the potential scan rate in 1 mM $[Fe(CN)_6]^{3-/4-}$ in the range of 10–200 mV s^{-1}. From the inset graph in Fig. S1, the linear relationship with good correlation coefficients was observed between the peak currents and the square root of scan rate, the linear regression equations were as following: $I_{pa}(A)=-3.02\times10^{-5}+1.02\times10^{-5}\,v^{1/2}\,(mV\,s^{-1})^{1/2}$, $R^2=0.998$; $I_{pc}(A)=2.09\times10^{-5}-6.55\times10^{-6}\,v^{1/2}\,(mV\,s^{-1})^{1/2}$, $R^2=0.996$, suggesting the electrode reaction was a diffusion controlled process.

3.3. Influence of pH on the current response of sensor

The pH values of the solution (10 mM PBS) had a great effect on aptamer binding RAC and electrochemical behavior of the EC aptasensor. To better understand the origin of such effect, systematic CV spectrum of the aptamer binding RAC (1.0×10^{-8} mol/L) at different pH values were recorded (Fig. S2). As shown in Fig. S2, it was seen that the current responses change increased from pH 6.0–8.0 to reach the maximum and decreased at pH 9.0. This phenomenon shown the different pH values led to the different EC responses of the developed EC aptasensor. We presumed the mechanism of EC behaviour as followed: RAC aptamer adsorbed H^+ or OH^- at different pH values, which lead to the different electrochemical responses. In this experiment, the RAC aptamer adsorb lots of OH^- when the pH higher than 7 and have negative charge on the surface. Electrostatic repulsion played an important role between RAC aptamer and $[Fe(CN)_6]^{3-/4-}$, which made the decrease of peak currents. Considering the response and the activity of aptamer binding RAC, the pH 7.0 phosphate buffered solution was chose for further experiments.

3.4. Influence of incubation time

The influence of incubation time on the signal current response was investigated. The developed EC aptasensor was incubated in RAC standard solution at a constant concentration of 1.0×10^{-8} mol/L for different time intervals. The result was shown in Fig. S3. It was obvious that the absolute current response decreased with increasing incubation time and reached a platform at 35 min. The results indicated that the saturated formation of aptamer and RAC complex on the modified electrode. Therefore, the incubation time of 35 min was adopted in this work.

3.5. Detection of RAC with the sensor

To further demonstrated the assay for the detection of RAC, quantitative analysis was investigated by DPV under the optimal experimental conditions. The sensor was incubated in a series concentration of RAC in pH 7.0 PBS solution for 35 min, then detected in 1 mM $[Fe(CN)_6]^{3-/4-}$ +10 mM PBS solution by DPV. Fig. 3 showed the values of peak current decreased with the increase concentrations of RAC. This was attributed to the fact that the formation of aptamer–RAC complex hindered the diffusion of $[Fe(CN)_6]^{3-/4-}$ towards the electrode surface. The linear relationship between the cathodic peak current of the sensor and the RAC concentration was observed in range from

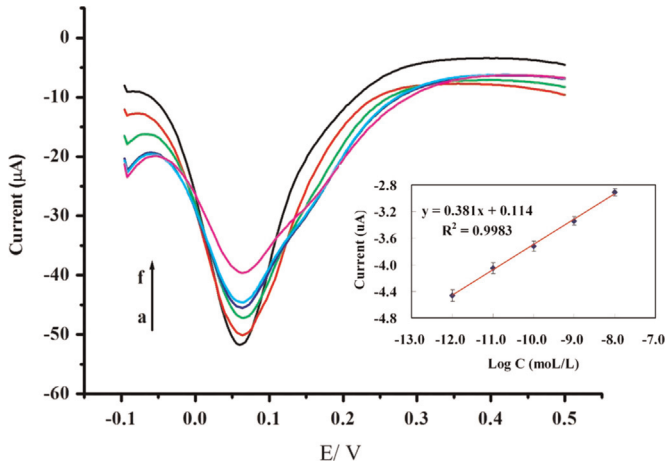

Fig. 3. DPV of the sensor incubated with different concentrations of RAC (measurement in 1 mM $[Fe(CN)_6]^{3-/4-}$ + 10 mM PBS solution, a–f: 0, 1×10^{-12}, 1×10^{-11}, 1×10^{-10}, 1×10^{-9} and 1×10^{-8} mol/L). The inset shows the linear relationship between the peak currents and RAC.

Table 1
Comparison between the present work and other reported EC methods for the determination of RAC.

Methods	LODs (mol/L)	Dynamic range (mol/L)	Ref.
EC sensor	7.4×10^{-8}	1.0×10^{-10}–1.0×10^{-7}	Zhang et al. (2014)
EC sensor	1.5×10^{-8}	5.0×10^{-8}–4.5×10^{-7}	Zhang et al. (2012)
EC sensor	5.0×10^{-10}	1.0×10^{-9}–2.7×10^{-6}	Bai et al. (2014)
EC sensor	5.0×10^{-12}	3.0×10^{-11}–3.0×10^{-8}	Wang et al. (2013)
EC sensor	1.0×10^{-10}	1.0×10^{-10}–1.0×10^{-6}	Zhou et al. (2014)
EC sensor	6.0×10^{-8}	8.5×10^{-8}–8.0×10^{-6}	Yang et al. (2014)
ECL sensor	2.5×10^{-11}	7.4×10^{-11}–8.9×10^{-9}	Li et al. (2013)
EC aptasensor	1.0×10^{-12}	1.0×10^{-12}–1.0×10^{-8}	This method

1.0×10^{-12} mol/L to 1.0×10^{-8} mol/L with a linear correlation coefficient of 0.998. The detection limit of RAC was estimated as low as 5.0×10^{-13} mol/L (defined as $S/N=3$).

A lot of EC sensor methods for RAC have been reported and the representative method was shown in Table 1. Comparing with the published electrochemical methods in Table 1, advantages of the developed EC aptasensor based on RAC aptamer was apparent: (1) It could be seen that the detection limit of our aptasensor, is obviously better than other sensors. (2) The four orders of magnitude dynamic range in lower concentration level obtained in this study was wider than most reported RAC EC sensors and rivaled by only a few, with most only operating within 2–3 orders of magnitude of their level of detection. (3) The superior performance of the aptasensor over the reported sensor highlighted the benefits that can be attributed to the novel fabrication strategy of the aptasensor.

3.6. Selectivity of the sensor

The specificity of the sensor plays an important role in analyzing biological samples in situ without separation, and some common potentially interfering substances were investigated to test the selectivity of the EC aptasensor. The aptasensor was incubated in 1.0×10^{-8} mol/L RAC and some potential interference species (1.0×10^{-8} mol/L), including clenbuterol (CLEN), salbuterol (SAL), oxytetracycline (OTC), penicillin (PEN), dopamine (DPA), Ca^{2+}, Mg^{2+} and Na^{+}, respectively. As shown in Fig. S4, it was found that when the same concentration of these potential interferences was added, no obvious signal change of EC aptasensor was obtained even with DPA, which is a kind of naturally compound in animal body. The results were demonstrated that the EC aptasensor was specific to RAC.

3.7. Stability and reproducibility of the sensor

Stability is one of the most important properties required for a biosensor. For stability test, the sensor was used to detect the same RAC concentration (1.0×10^{-8} mol/L) after keeping it in 4 °C for two weeks. The peak current did not have an obvious change (lower than 7.4%), which showed that the sensor had good stability after long-time stored.

To investigate the reproducibility for the sensor, 4 different sensors were examined by 8 independent determinations in the measurement of RAC at concentration of 1.0×10^{-8} mol/L, respectively. For the same sensor, RSD < 2.9%. For the different sensor, RSD < 4.7%. The results demonstrated that the sensor had a good reproducibility.

3.8. Real sample analysis

For the aim of evaluating the applicability of the sensing system, the sensor was used for determining the recoveries of four different spiked concentrations of RAC in blank swine urine samples. For the analysis of spiked samples, the swine urine was diluted 2 times with pH 7.0 buffer and added standard solutions of RAC. As shown in Table 2, the recovery was in the range of 92.6–103.0%, the relative standard deviation (RSD) was varied from 3.7% to 5.5%, which indicated that the developed assay might be applied for the determination of RAC in swine urine samples.

In order to further valid the developed method, 12 swine urine samples were tested using the developed EC aptasensor, and the results were compared with LC–MS/MS (Nielen et al., 2008). The results indicated that 4 urine samples were determined as positive. As listed in Table 3, the results of the proposed EC aptasensor method were in good agreement with the reference LC–MS/MS method. With good performance, this newly developed method could be used in analysis of ultra trace amount of RAC in swine urine sample matrixes.

4. Conclusions

In conclusion, an effective and label free EC aptasensor for the detection of RAC using aptamer-bound AuNPs and graphene composite film was developed. High sensitivity, a wide dynamic range, superb selectivity and good performance in real urine samples were achieved. Under the optimal conditions, good linearity was achieved in the range of 1.0×10^{-12}–1.0×10^{-8} mol/L, the limits detection of RAC was 5.0×10^{-13} mol/L. The proposed method could be successfully applied to the determination of RAC in spiked swine urine and real swine urine samples with high recoveries from 90.1% to 104.0% and good agreement with UPLC–MS/MS results, the repeatability was satisfactory with RSD < 5.5%. Particularly, the fabrication strategy of the developed electrochemical aptasensor is a general approach to detect small

Table 2
Recoveries of RAC in swine urine with detection of the developed EC aptasensor ($n=6$).

Sample	Added (mol/L)	Found (mol/L)	Recovery (%)	RSD (%)
1	1.0×10^{-8}	1.03×10^{-8}	103.0	3.7
2	1.0×10^{-9}	9.72×10^{-8}	97.2	4.3
3	1.0×10^{-10}	9.57×10^{-9}	95.7	5.5
4	1.0×10^{-11}	9.26×10^{-10}	92.6	5.1

Table 3
Comparison of the analysis results for RAC in really contaminated samples by the developed EC aptasensor and reference LC–MS/MS ($n=5$).

Sample	This method		LC–MS/MS	
	Concentration (ng/mL)	RSD (%)	Concentration (ng/mL)	RSD (%)
Urine 1	1.04 ± 0.049	4.7	0.92 ± 0.058	6.3
Urine 2	14.6 ± 0.74	5.1	15.9 ± 0.60	3.8
Urine 3	6.14 ± 0.36	5.8	6.95 ± 0.032	4.6
Urine 4	0.26 ± 0.020	7.7	0.22 ± 0.015	6.9

molecules of illegal drugs based on the conformational change of aptamers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.09.050.

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