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Research paper

An electrochemical immunosensor based on pristine graphene for rapid determination of ractopamine

Shaopeng Qi, Bo Zhao, Bo Zhou, Xiaoqing Jiang *

Jiangsu Key Laboratory of New Power Batteries, College of Chemistry and Materials Science, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210023, PR China

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ABSTRACT

A new electrochemical immunosensor for fast determination of ractopamine (RAC) is fabricated based on pristine graphene (PG). The PG provides a microenvironment beneficial the immobilization of RAC, promotes the electron transfer, and raises the sensitivity of the immunosensor. The free RAC in solution can be effectively measured based on the competitive immunoreaction between RAC-antibody and RAC. The calibration graph shows linearity over the concentration ranges of 0.1–10 and 10–4000 ng mL^{-1}. The proposed immunosensor displays a satisfactory stability, selectivity, and reproducibility and has been applied to the quantificational detection of RAC in real pork samples.

1. Introduction

Ractopamine (RAC) is a dietary β-adrenergic agonist and was originally employed to treat respiratory diseases [1]. However, it was also illegally employed to enhance protein accretion when used as animal feed [2]. There were reports about abuse of β-agonists for growth promoting purposes in the past few years [3,4]. More seriously, the drug residues which accumulate to a certain degree in animal tissues are detrimental to the health of consumers and lead to some symptoms such as headache, vomiting, fever, dizziness, nervousness, nausea, muscular tremors, chills, and cardiac palpitations [5–7]. Therefore, β-agonists is now among the list of banned animal feed additives in China, Europe, and many other countries [8].

Up to now, various analytical methods of detecting β-agonists residues in animal tissues have been developed, including gas chromatography–mass spectrometry [9], liquid chromatography-mass spectrometry [10–13]. However, these methods inevitably count on exorbitant apparatus and involve complex operating steps or consume long time. To overcome these weaknesses, a number of optical and electrochemical sensors or assays have been proposed, using molecular-imprinted polymers [14], aptamers [15], and more often antibodies [16,17] as biosensing elements.

Among the variety of sensors reported, electrochemical immunosensor has been a research topic in recent years based on its advantages of high simplicity, reliability, accuracy, and fast response [18,19]. Notably, great concern for preparing an excellent electrochemical immunosensor has put emphasis on how to immobilize the immune-reagent efficiently and effectively. The material modified on electrode surface greatly influences the performance of immunosensor, since it has two important functions. First, it can adjust the charge transfer between electrode surface and solution. Second, it can change the active electrode surface area for immobilizing biomolecules. Recently, carbon materials have been brought into focus. Graphene as the newest member of carbon family has been increasingly applied to biosensors in recent years owing to its outstanding physical, chemical and other properties [20–23]. Very recently, a number of works on modification of glassy carbon electrode (GCE) with chemical reduced graphene oxide to fabricate biosensors or immunosensors have been published. However, few works on fabricating sensors with pristine graphene (PG) has been reported [24].

In this work, we fabricated a new type electrochemical immunosensor for fast determination of RAC by use of PG. The PG was easily prepared by exfoliating graphite and then dispersing PG sheets in some organic solvents with certain organic salts as assistants [25]. We had demonstrated that thus as-made PG sheets contained little oxygen and had good electrical conductivity. It had been used in electrochemical sensor [26]. In this work, a novel immunosensor has been fabricated with PG for the quantificational detection of RAC based on a competitive mechanism, utilizing potassium ferricyanide ($K_3[Fe(CN)_6]$) as the redox probe. This PG based immunosensor shows satisfied detection signals for the RAC in both lab and real pork samples. For all we know, this is

* Corresponding author.
E-mail address: jiangxiaoqing@njnu.edu.cn (X. Jiang).

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the first work on the determination of RAC with electrochemical immunosensor based on PG.

2. Experimental

2.1. Reagents

Graphite powder (particle size < 30 μm), dimethyl sulfoxide (DMSO), ethanol, $K_3[Fe(CN)_6]$, potassium sodium tartrate, and potassium chloride were purchased from Shanghai Chemical Reagent Co. Ltd. Bovine serum albumin (BSA) was obtained from Beijing Yuanheng Shengma Biology Technology Research Institute. RAC and RAC monoclonal antibodies (RmAb) were bought from Sigma-Aldrich and Guangzhou food-safe biological technology Co. Ltd, respectively. Mabuterol (MAB), terbutaline (TER), Zilpaterol (ZIL), and clenbuterol (CLE) were purchased from Aladdin. Phosphate buffer solution (PBS, pH 7.4, 0.1 M) was prepared by potassium dihydrogen phosphate and disodium hydrogen phosphate dodecahydrate. Pork samples were obtained from local supermarkets. All solutions were prepared with ultrapure water (resistivity of 18.25 MΩ cm).

2.2. Preparation of PG

The PG dispersion was prepared from graphite powder according to our previous work [27]. First, a mixture of graphite powder (1 g), potassium sodium tartrate (2 g), and DMSO (50 mL) was placed in an ultrasonic bath with a frequency of 40 kHz for 3 h at room temperature and then followed by 3000 rpm centrifugation for 30 min using a UNIVERSAL 320 centrifuge. The supernatant was transferred out using a pipette, followed by mixing with some inorganic salts for aggregating the dispersion. The sediment was then washed in succession with ultrapure water and ethanol. Finally, the aggregate was re-dispersed in DMSO (3.5 mg mL^{-1}) for further use.

2.3. Preparation of the modified electrode

The immunosensor was fabricated as follows. First, 5 μL of the PG dispersion (3.5 mg mL^{-1} in DMSO) was dropped onto the surface of a cleaned GCE and dried in air (labeled as PG/GCE). Second, 2 μL of a 0.2 mg mL^{-1} RAC solution (dissolved in water) was dropped onto the surface of PG/GCE to adsorb RAC on PG through π-π interaction [28] between PG and RAC. To avoid aspecific combination, the modified electrode (labeled as RAC-PG/GCE) was incubated in bull serum albumin solution (5 wt%) for 30 min at 37 °C, then followed by incubating in PBS (pH 7.4, 0.1 M) containing monoclonal antibody against ractopamine (RmAb) and RAC (antigen) for certain time. Thus prepared electrode is labeled as RmAb-RAC-PG/GCE. The amount of antibody and the time of immunoreaction were optimized to acquire the best signal responses.

2.4. The mechanism of immunoassay

The determination of RAC is based on a competitive mechanism [29] (shown in Fig. 1), which involves in the specific interaction between RmAb and RAC. The more free RAC in incubation solution, the less RmAb can bind those RAC immobilized on the electrode, then the larger the current on the electrode.

2.5. Preparation of the pork samples

The pork sample was crushed by mechanical mincing machine. The smashed pork sample (about 1 g) was precisely weighed using an electronic balance and homogenized with different amounts of RAC stock solution (0.2 mg mL^{-1}) and then ultrasonicated for 30 min to achieve sample's final RAC concentration of 100, 1000, and 4000 μg kg^{-1}. Then, 4 mL of newly prepared acetonitrile-acetone extraction solution (volume ratio 4:1) was added into spiked samples [30]. The samples were wobbled continuously for 20 min and then centrifuged for 40 min at 2000 rpm. The clear supernatant was separated and transferred into test tubes and dried by nitrogen. Finally, the solution for determination was prepared by adding 1 mL PBS buffers into the test tubes, and then stored at 4 °C.

3. Result and discussion

3.1. Characteristics and electrochemical behaviors of different modified electrodes

The morphology of PG was shown in Fig.2a. TEM image of transparent graphene flakes drop-coated onto a holey carbon grid can be observed. High quality of the PG has been evidenced by our previous work [27]. Fig.2b shows the RmAb was embedded steadily and evenly on the surface of PG. This is due to the specific binding of antibody.

The electrochemical performances of GCE, RAC-PG/GCE, and RmAb-RAC-PG/GCE are tested by cyclic voltammogram (CV) in PBS solution (pH 7.4) containing 5 mM $K_3[Fe(CN)_6]$ used as the electrochemical probe. As shown in Fig. 2c, a pair of redox peaks can be seen on the GCE (black dash line). After being coated with PG-RAC, a significant increase in the redox peak currents can be observed (red solid line), owing to the excellent electron transfer ability of PG [27]. This proves the PG can enlarge the electrochemical response to a large extent. In contrast, a noticeable reduction of the peak currents and an enlargement of the peak-to-peak potential interval between the redox peaks are clearly observed on the RmAb-modified RAC-PG/GCE (blue dash dot line). This phenomenon indicates that there should be a stabilized combination between the RmAb and RAC modified on the surface of electrode and this antigen-antibody complex served as an obstacle and therefore significantly blocked the transport of electron and the spread of the redox probe to the electrode interface.

3.2. Optimization of the experimental conditions

The experimental conditions that could affect the performance of the immunosensor in the determination of RAC, involving the time of immunoreaction and the amount of antibody, were optimized. The differential pulse voltammetry (DPV) peak currents of electrodes incubated with different incubation times in the presence of the certain concentration of antibody (20 μg mL^{-1}) were measured in PBS (pH 7.4) buffer solution containing 5 mM $K_3[Fe(CN)_6]$. As described in Fig. 3a, with increasing the incubation time the peak current of RAC-PG/GCE initially has a rapid decrease (from 0 to 10 min) and then continues to decrease slowly as the incubation time is up to 45 min. The current decrease should be owing to the specific immunoreaction between RAC and RmAb. The decrease in peak current tends to stop as the incubation time exceeds 45 min, suggesting the immunoreaction should be almost finished within 45 min. Therefore, 45 min was used as the optimal incubation time in this work.

The volume of RmAb incubation solution dropped on PG/GCE may also have a significant impact on the current response. In order to investigate this impact, different volumes of 20 μg mL^{-1} RmAb solution were used to coat the RAC-PG/GCE. The electrodes were then incubated at 37 °C for 45 min and washed warily with PBS buffer (pH 7.4). As we expected, the volume of RmAb