Talanta 170 (2017) 502–508

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a progesterone immunosensor based on thionine-graphene oxide composites platforms: Improvement by biotin-streptavidin-amplified system

Xiu-Xiu Dong[a,b,1], Li-Peng Yuan[c,1], Yi-Xin Liu[a], Min-Fu Wu[a], Bo Liu[c], Yuan-Ming Sun[a], Yu-Dong Shen[a], Zhen-Lin Xu[a,*]

[a] Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou 510642, China
[b] State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China
[c] Guangdong Agriculture Industry Business Polytechnic College, Guangzhou 510507, China

ARTICLE INFO

Keywords:
Progesterone
Biotinylated antibody
Immunosensor
Thionine-graphene oxide composites
Milk samples

ABSTRACT

Progesterone (P4) is a kind of hormone that can cause neuropathic disturbances in humans when the concentration overpasses a certain degree. In this work, an electrochemical immunosensor capable of detecting P4 sensitively and selectively was developed. Thionine-graphene oxide (Thi-GO) composites with excellent biocompatibility were synthesized and coated to a clear glassy carbon electrode. P4 coating antigen (P4-OVA) was immobilized to the electrode, then sample as well as biotinylated antibody (biotin-P4 Ab) were added. The free P4 can compete with P4-OVA for binding to biotin-P4 Ab. After the further addition of streptavidin-HRP, H_2O_2 was introduced to develop electrical signal for quantitative determination of P4. After careful optimization of assay conditions, the proposed immunosensor showed a linear range from 0.02 to 20ngmL^{-1} for P4 in milk samples. The averaged recoveries from spiked samples ranged from 84.0% to 102.0%, which correlated well with standard HPLC-MS/MS. The biosensor also showed good specificity, reproducibility and stability, indicating its potential application in monitoring of P4 in a simple and low cost manner.

1. Introduction

Endocrine disrupting compounds (EDCs) have great potential harms for environment and human, which has been widely noted. EDCs usually combined to the estrogen receptor [1,2] or androgen receptor [3,4] of human which caused intracorporal hormone disturbance, disordering the normal physiological activities and triggering a series of diseases. Currently, according to the publication, the researches of progesterone (P4)are less than estrogen and androgen. However, the International Agency for Research on Cancer (IARC) carried out substantial animal experiments and partial testing in humans to conclude that P4has potential carcinogenicity [5]. It has been reported that progestational hormone including P4 could decrease the reproductive performance of aquatic organism [6–9].

Numerous analytical methods have been developed to detect EDCs, including P4. The most common techniques involved high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) [10,11], gas chromatography-mass spectrometry (GC-MS) [12,13], and laser diode thermal desorption-mass spectrometry [14,15]. Although instrumental methods offer high sensitivity and specificity, the associated high-costs and time-consuming labor requirements have inhibited the application of these methods. Therefore, the need for rapid, simple and high-throughput analysis of pesticides in a low cost manner is rapidly growing. As an alternative, antibody based immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) [16,17], have been proven to be rapid, sensitive, and low-cost screening tools for agricultural and environmental contaminants. In recent years, biosensors had become attractive analytical tools which offering fast and reduced time analysis methods, adequate selectivity and sensitivity, and low cost. Several electrochemical biosensors [18–20] had been reported for the determination of P4. Since the assignable perniciousness and low concentration level of P4 have been focused, the trace detection should be developed which would realize by means of nanomaterials to enhance signal.

Graphene oxide (GO), a nontoxic, two-dimensional carbon-based material originating from acid exfoliation of graphite, offers a new class of solution-dispersible polyaromatic platform for performing chemistry [21,22]. Compared to graphene, GO undergoes a complex interplay of

* Corresponding author.
E-mail address: jallent@163.com (Z.-L. Xu).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.talanta.2017.04.054
Received 15 February 2017; Received in revised form 10 April 2017; Accepted 21 April 2017
Available online 23 April 2017

ionic and nonionic interactions with different molecules in solution which is due to its covalently bound epoxide (1,2-ether) and hydroxyl functional groups [23,24]. As a negatively charged doping agent, the oxygen-containing functional groups on GO surfaces provide negative electricity which has great potential in the fields of biosensing [25,26].

In this work, we report a simple ion-exchange strategy for electrostatic complexation of GO with a thionine to form a complex. We found that the Thi-GO composites exhibits enhanced properties for biosensing. Moreover, since biotin-avidin-system (BAS) is a high effective technique that realizes signal amplification, the biotinylated antibody of P4was prepared and coupled with streptavidin-HRP to enhance the response signal. Therefore, a new P4immunosensor based on Thi-GO composites platforms and BAS was finally developed. It was further applied to determine P4 in milk samples.

2. Materials and methods

2.1. Reagents

Progesterone (P4),ethisterone,testosterone, medroxyprogesterone acetate, β-estradiol, biotin-hydroxysuccinimideand estriol were obtained from Aladdin Chemical Technology Co., Ltd.(Shanghai, China). Flake graphite was purchased from Shenzhen Nano Port Co., Ltd. Thionine was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Streptavidin-HRP was purchased from Thermo Fisher Scientific Co. Ltd.(Shanghai, China). P4 antibody and P4 coating antigen (P4-OVA) were synthesized in our lab. All other chemicals were of analytical grade and used as received without further purification.

In the immunoreaction, the phosphate buffer saline (PBS, 0.01 M, pH 7.4) was used by dissolving 8.5 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 2.9 g $Na_2HPO_4{\cdot}12H_2O$ in 1 L water, while carbonate buffer (CB, 0.1 M, pH 9.6) was prepared by dissolving 0.375 g Na_2CO_3, and 0.7325 g $NaHCO_3$ in 250 mL water. In the electrochemical reaction, the 1/15 M PBS (pH 6.8) solution was prepared by mixing 11.94 g $Na_2HPO_4{\cdot}12H_2O$, 4.53 g KH_2PO_4, and 7.45 g KCl in 1 L water.

2.2. Apparatus

All electrochemical measurements were performed on a CHI660D electrochemical work station (CH Instruments, Shanghai Chenhua Instrument Corporation, China) with a glassy carbon electrodes (GCE, 4 mm diameter) as work electrode, a Pt auxiliary electrode and a saturated calomel electrode as reference electrode. Scanning electron microscopy (SEM) images was carried out on a field emission scanning electron microscope of JSM-6700F. X-ray diffraction (XRD) was used Bruker D8 advance X-ray powder diffractometer (Bruker, Germany). The UV–vis spectra were performed using UV2550 Spectrometer (Shimadzu, Japan).

2.3. Preparation of Thi-GO composites

Firstly,GO were oxidized using improved Hummers' method [27]with moderate modification: graphite flakes (0.6 g) and $KMnO_4$(3.6 g) were added into three-necked flask, acid solution was poured into the three-necked flask after mixing with H_2SO_4(72 mL):H_3PO_4(8 mL)=9:1. The reaction started at 50 °C by stirring for 12 h and cooled to room temperature. The obtained solution was spilled into flask with ice. While the ice becomes water, the 30% H_2O_2 was added till no bubble producing. After washing with hydrochloric acid and distilled water till the pH > 5, cry-odesiccation, and sonication for 30 min, the GO was obtained.

Secondly, GO (5 $mgmL^{-1}$) and thionine (2 $mmolL^{-1}$) were mixed with equal volumes and then stirring for 20 min under shade environment at room temperature. The solution was centrifuged at 4000×*g* to remove the spare thionine. The Thi-GO composites was obtained after the above-mentioned precipitate drying at 50 °C.Then the Thi-GO composites was re-dissolved in 0.05 wt% chitosan solution.

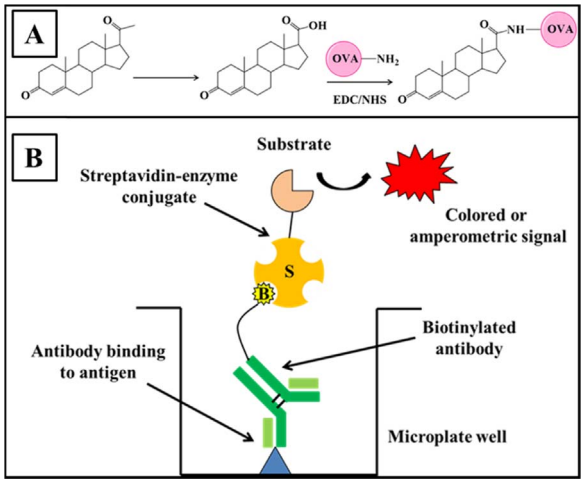

Fig. 1. (A) Synthetic route of P4 coating antigen; (B) Schematic diagram of the labeled Avidin-biotin method.

2.4. Preparation of P4coating antigen and biotinylated antibody

P4coating antigen (P4-OVA) was synthesized using the active ester method [28]. As shown in Fig. 1**A**, 13 mg (0.04 mmol) progesterone carboxylic acid mixed with 0.1 mmol EDC/NHS (1:1) and stirring at 4 °C overnight to active it. Then, the activated progesterone carboxylic acid was added into 10 $mgmL^{-1}$OVA (1 mL) under stirring. The conjugate mixture was stirred at 4 °C for 12 h and then dialyzed against 0.01 M PBS (pH 7.4).

Biotin-avidin-system (BAS) is a high effective technique that realizes signal amplification. As shown in Fig. 1**B**, the labeled avidin-biotin method (LAB) was used in this work. Streptavidin-HRP coupled with biotinylated antibody and then the HRP catalyzed in the electrochemical reaction. So we could detect the concentration of P4. The preparation of biotinylated antibody (biotin-P4 Ab) referred to the previous reports [29,30]. 1 $mgmL^{-1}$biotin-hydroxysuccinimide(dissolved in dimethyl sulfoxide, DMSO) mixed with 2 $mgmL^{-1}$ P4 antibody at volume ratio of 1:10. The mixed solution was stirred at 25 °C for 4 h and then under stirring at 4 °C for 12 h. After dialyzing against 0.01 M PBS (pH 7.4) for 3 d, the production was obtained.

2.5. Assembly of the immunosensor

Prior to modification, the bare GCE were polished successively with 0.3 and 0.05 μm alumina slurry, thoroughly washed ultrasonically in ethanol and distilled water. Then the electrode was rinsed with distilled water, and dried in the air. The immunosensor was assembled as shown in Fig. 2.

Firstly, 8 μL of Thi-GO solution was dropped onto the surface of GCE. Secondly, 8 μL of P4-OVA conjugate solution was dispersed on the surface of modified GCE and incubated at 4 °C overnight, followed by removing the un-adsorbed conjugate by washing with 0.01 M PBS. Subsequently, 10 μL of the blocking agent was dropped on the electrode surface and incubated at 37 °C for 1 h to block possible remaining active sites and avoid the nonspecific binding. Finally, the immunosensor was thoroughly washed with 0.01 M PBS and stored at 4 °C before use. Different concentrations of P4 and biotin-P4 Ab were diluted in 0.01 M PBS, and 8 μL of the mixture were immediately spotted onto the modified immunosensor surface for 2 h at 37 °C. Streptavidin-HRP was added to cause the amperometric signal change through catalyzing the reaction. The immunoreaction was based on the typical competitive procedure; P4-OVA can compete with free P4 to combine with biotin-P4 Ab, thus, washing the surface of modified electrode with PBS to remove the unbound biotin-P4 Ab, drying with nitrogen and then taking the electrochemical measurement. As for the electrochemical detection, the modified immunosensor was immersed in 1/15 M PBS solution containing 1 mM HQ and certain volumes of