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

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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

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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.

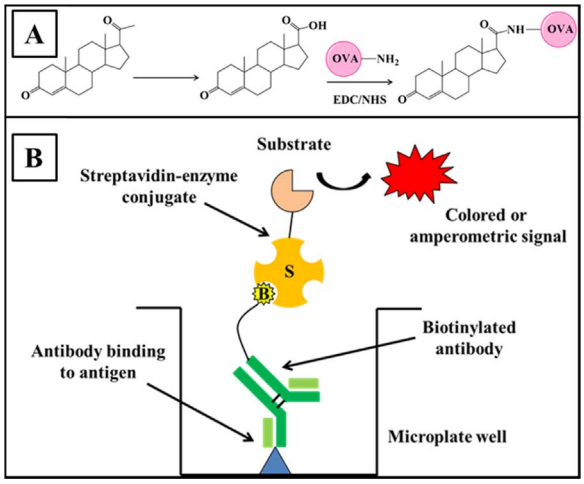

Fig. 1. (A) Synthetic route of P4 coating antigen; (B) Schematic diagram of the labeled Avidin-biotin method.

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

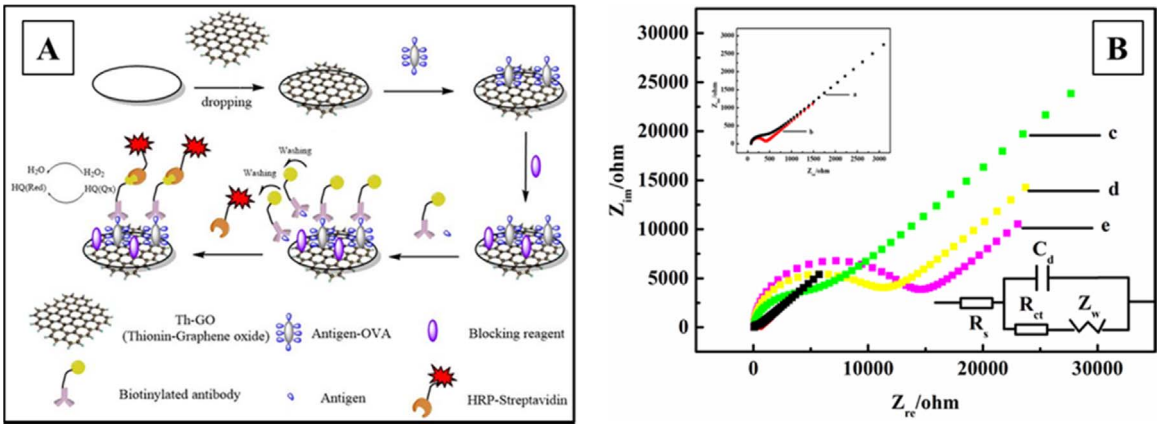

Fig. 2. (A) Schematic diagram of structured immunosensor; (B)Electrochemical impedance spectroscopy of structured immunosensor: (a) bare GCE; (b) Thi-GO/GCE; (c) Ag/Thi-GO/GCE; (d) blocking reagent/Ag/ Thi-GO/GCE; (e) biotin-Ab/blocking reagent/Ag/Thi-GO /GCE in 0.1 mol L^{-1}KCl solution containing 1 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$.

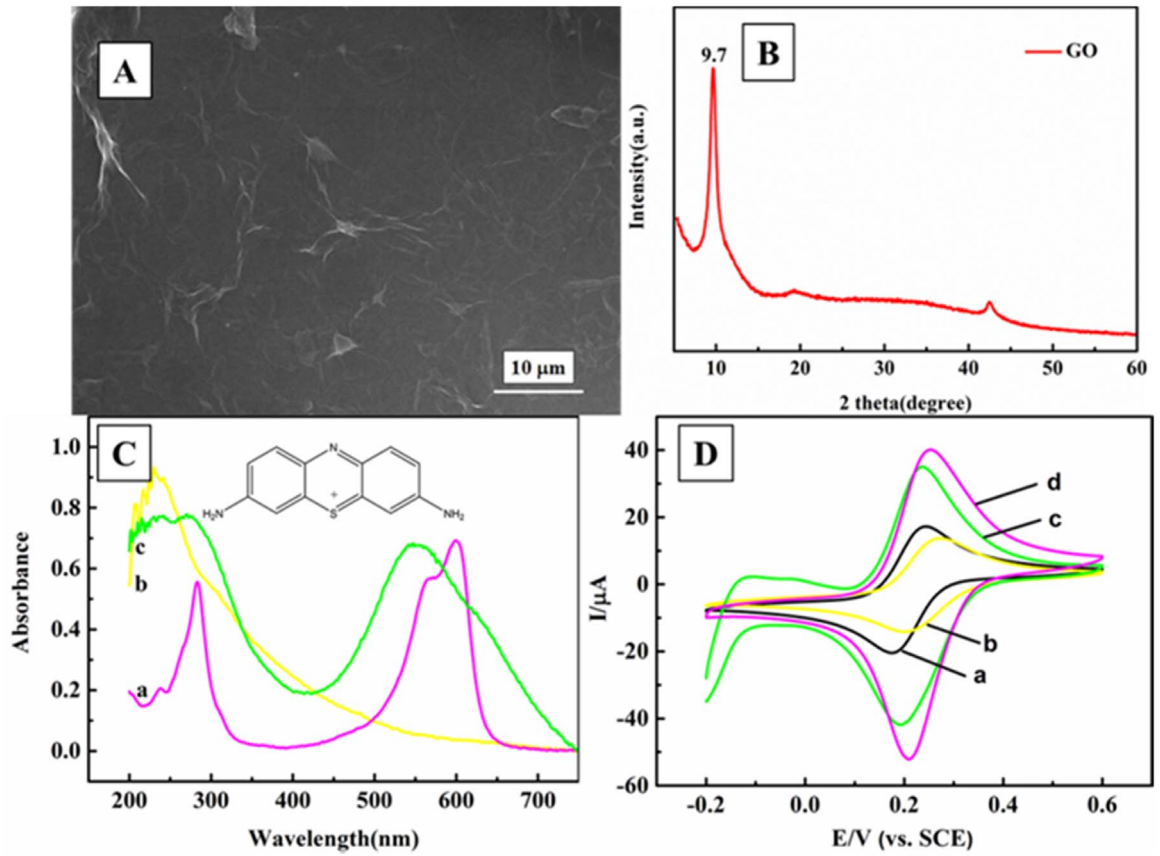

Fig. 3. SEM (A) and XRD (B) of GO; (C) The characterization by UV–vis spectra of (a) Thi, (b) GO and (c) Thi-GO; (D) Cyclic voltamograms at a scan rate of 50 mV s^{-1}. (a)bare GCE, (b) GO/GCE, (c)Thi/GCE and (d) Thi-GO/GCE in 0.1 mol L^{-1} KCl solution containing 1 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$.

1.44 mM H_2O_2. The electrochemical cathodic current change value before and after the addition of H_2O_2 was used as the signal response. Then we could further obtain the relation curve between the concentrations of P4 and the current change value.

3. Results and discussion

3.1. Characterization of Thi-GO composites

We have evaluated the obtained GO through scanning electron microscopy (SEM). As shown in Fig. 3**A**, the obtained GO has layer shape structure with most big specific surface area and wrinkled sheet-like structures, which were typically observed for graphene-based materials. Further, the X-ray diffraction (XRD) was performed and indicated that a strong diffraction peak at 9.7° which is the characteristic peak of GO (Fig. 3**B**). Compared to the graphene, the diffraction peak shifted left. All the information indicated that the GO was formed.

Thionine with positive charge could absorb onto the surface of GO through electrostatic interaction [31,32]. Also, thionine could couple with GO by means of π-π reaction. To validate the existence of Thi-GO composites, the UV–vis absorption spectrums were performed. Two main features can be seen from Fig. 3**C (a)**: (1) a characteristic absorption peak at 228 nm, which causes fromπ→π* of C=C, and (2) a shoulder at ~290–300 nm, corresponding to n→π* transition of the C=O bond [33]. Also, two

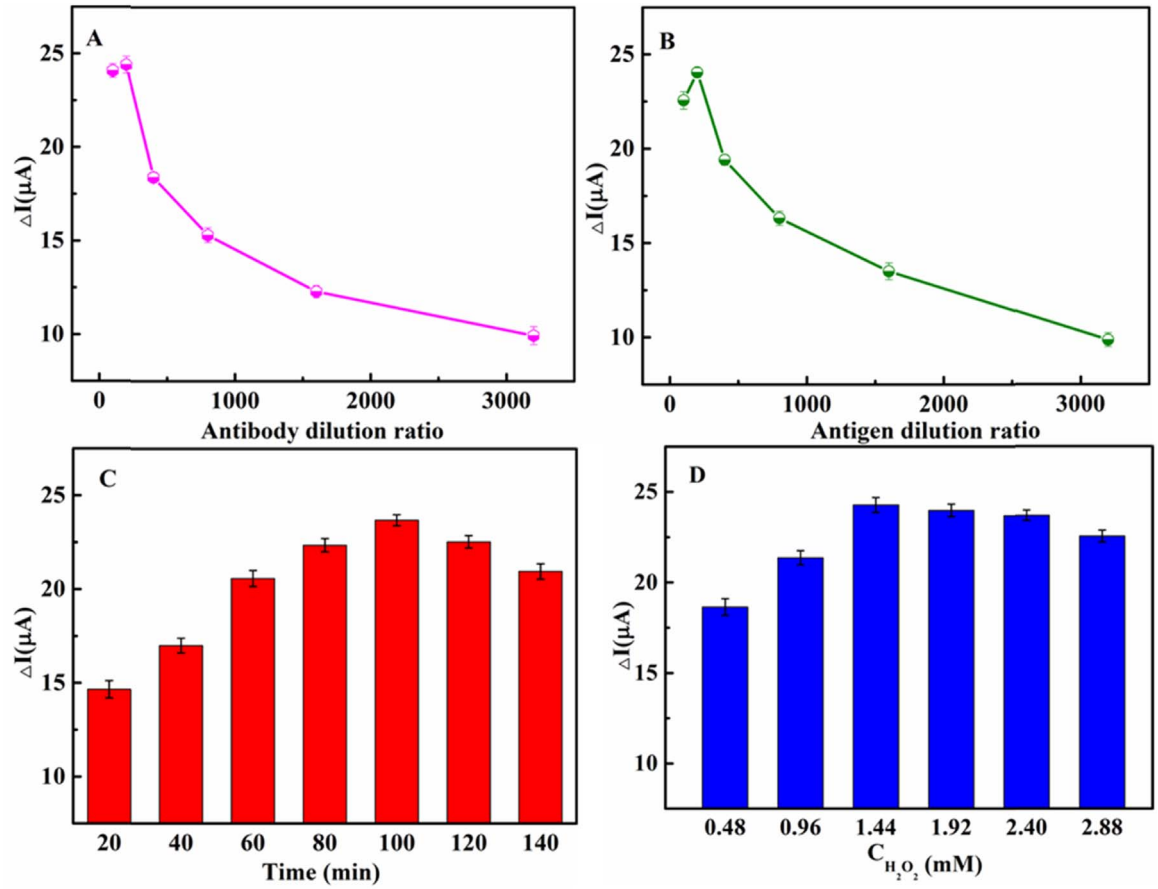

Fig. 4. Effect of several factors on the electrochemical signal of the immunosensor. (A) the dilution ratio of the antibody, (B) the dilution ratio of the P4 antigen, (C) the incubation time of the antibody and antigen, and (D) the concentration of H_2O_2.

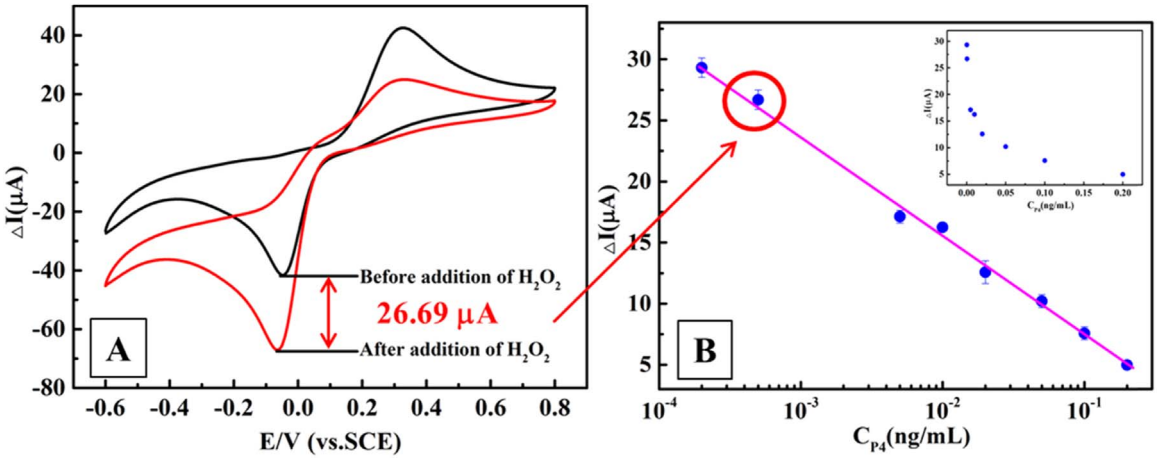

Fig. 5. The cyclic voltamograms (A) and calibration curve (B) of immunosensor; the relationship between the concentration of P4 (C_{P4}) and the current response (ΔI) in the standard solution (as shown in inset in (B)).

characteristic absorption peaks of thionine are observed. As shown in Fig. 3**C (b)**, a peak at 282 nm is attributed to π→π* of aromatic nucleus and another peak at 598 nm is due to n→π* of C=N bond. From the Fig. 3**C (c)**, it has the two characteristic absorption peaks of thionine. Therefore, the Thi-GO composites were formed.

3.2. Electrochemical behaviors of structured immunosensor

The CVs of different modified electrodes in 0.1 mol L^{-1} KCl solution containing 1 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 50 mV s^{-1} are shown in Fig. 3**D**. Compared to the bare GCE, the oxidation/reduction currents on Thi, Thi-GO composites modified GCE are all enhanced, except GO modified GCE which is due to the low electroconductivity of GO. The sequence of the values of apparent electroactive surface areas (*A*) for different electrodes is Thi-GO/GCE > Thi/GCE > GCE > GO/GCE [34]. It is clearly indicated that the Thi-GO composites modified GCE has more favorable electron transfer kinetics.

Electrochemical impedance spectroscopy (EIS) is usually applied for evaluating the quality of biosensor. It could show the immobilization of biomolecule onto the surface of sensor through impedance

Table 1
Comparison of available biosensors for analysis of P4.

Methods	Linear range ($ngmL^{-1}$)	Detection limit ($ngmL^{-1}$)	Reference
a CdSe/ZnS quantum dot-based direct binding assay	0.385–4.55	0.21	[39]
an electrochemical immunosensor	0.16–50	0.16	[20]
a quantum dot-based fluorescent immunoassay	0.3–14.5	0.1	[40]
a tyrosinase-colloidal gold-graphite-Teflon biosensor	0–40	0.43	[41]
a Thi-GO composites and BAS immunosensor	0.0002–0.2	6.3×10^{-5}	This work

change [35]. The EIS of different assembled electrodes performed with frequency range from 10 mHz to 100 kHz. Fig. 2**B** illustrated the EIS obtained from bare GCE (curve a), Thi-GO/GCE (curve b), Ag/Thi-GO/GCE (curve c), blocking reagent/Ag/ Thi-GO/GCE(curve d), and Biotin-Ab/blocking reagent/Ag/Th-GO /GCE (curve e) using $[Fe(CN)_6]^{3-/4-}$ as the redox probe. A big interfacial resistance on bare GCE was exhibited (curve a), after coated with Thi-GO composites on the electrode surface, we observed that resistance decreased obviously compared with the bare GCE (curve b). The phenomena likely because that the presence of Thi-GO composites could promote electron transfer due to their unique excellent and electronic properties. However, after next biomolecule (Ag, Ab and so on)was immobilized on the electrode, the interfacial resistance increased remarkably and gradually (curve c,d and e) due to the increase of the thickness of the interface and inherent property of protein. This was the direct evidence of successful binding of biomolecule on the electrode surface.

3.3. Optimization of analytical conditions for the immunosensor

The quantity of Ab is very important for competitive immunoassay because it can have a direct effect on the response values of the amperometric sensor. The dilution ratio of the antigen was fixed at 1:200 (in CB). Different dilution ratios of biotinylated antibody were investigated from 1:3200 to 1:100 (in PBS). As shown in Fig. 4**A**, the signal response exhibited a continuous increase until the dilution ratio reached 1:200, and the response value showed a slight decrease with the further reduced dilution ratio. Therefore, the dilution ratio of 1:200 was chosen for subsequent experiments.

Generally, a much higher amount of antigens can combine with a much higher amount of antibodies, which can bring a much higher amperometric response. However, if there were superfluous antigens, the electrode surface may be overspread with biomolecules; this could prevent the electron transfer due to the steric-hindrance between protein molecules. Thus, a suitable antigen dilution ratio should be considered and the effect of the P4-OVA antigen concentration was examined separately. As shown in Fig. 4**B**, the dilution ratio of the coating antigen was changed from 1:3200 to 1:100. The results showed that the response values increased at a lower dilution ratio and decreased at a higher dilution ration, thus a dilution ratio of 1:200 for P4-OVA antigen was selected because it was much more suitable.

Subsequently, the incubation time of the antibody and antigen reaction was studied. It takes some time for the interaction of antibody and antigen to reach equilibrium, indicating that the response values would be lower at earlier time points. As appeared in Fig. 4**C**, the signal response of the immunosensor sharply increased with the incubation time before 100 min, indicating a tendency to thoroughly capture HA antigens on the electrode surface during this time period. After 100 min, the responses decreased slowly, which may be associated with the loss of antibody activity with the increase of the incubation time. Therefore, 100 min was adopted as the optimal incubation time for the immunoassay.

The concentration of H_2O_2 can greatly affect the final response values of the sensor, since it is the substance of the HRP. Therefore, the concentration of H_2O_2 was investigated from 0.48 mM to 2.88 mM. As shown in Fig. 4**D**, the amperometric response increased from 0.48 mM to 1.44 mM, which may be due to the fact that enough H_2O_2 can enhance the catalytic ability. However, a further increase in the H_2O_2 concentration to 2.88 mM may bring a slight decrease in the catalytic current. At higher H_2O_2 concentrations, the modified microelectrode was supplied with substrate for a longer period of time to allow the reaction to proceed over a larger window of time. This, together with the possibility of fouling the electrode surface by the reaction products, results in a lower response; the concentration of H_2O_2 also attains a saturation level at 1.44 mM. Therefore, 1.44 mM was selected as the concentration of H_2O_2.

3.4. Performance of the structured immunosensor

Under the optimal conditions, the resulted immunosensor based on Thi-GO platform and BAS was applied to detect different concentrations of P4. In a typical competitive immunoassay, a standard P4 solution at a known concentration was added into the incubation solution containing a certain concentration of biotinylated antibody, and a certain volume of the mixture was immediately incubated on the immunosensor surface. Then P4 in the incubation solution competed with the immobilized P4-OVA on the immunosensor surface to combine with the limited binding sites of Ab and form an immunocomplex. As shown in Fig. 5**A**, the possible catalytic mechanism by HRP may be described as the following: Eqs. (1)–(3). [36].

Table 2
Results of the determination of P4 in spiked milk samples with immunosensor and LC-MS/MS (n =3).

Method	Spiked ($ngmL^{-1}$)	Mean Found ($\overline{X} \pm SD$) ($ng\ mL^{-1}$)	Mean Recovery (%)	CV (%)
immunosensor	0	0	–	–
	0.15	0.126 ± 0.004	84.0	3.46
	0.5	0.482 ± 0.02	96.4	4.15
	1.5	1.53 ± 0.04	102.0	2.65
	5	4.573 ± 0.08	91.46	1.74
	10	9.685 ± 0.43	96.85	4.44
	15	14.4 ± 0.26	96.0	1.8
LC-MS/MS	0	0	–	–
	0.15	0.146 ± 0.001	97.2	8.36
	0.5	0.521975 ± 0.03	104.4	5.73
	1.5	1.41 ± 0.011	94.3	0.79
	5	4.87865 ± 0.11	97.57	2.34
	10	10.1 ± 0.59	100.99	5.83
	15	13.89 ± 0.62	92.6	4.43

$$HRP_{Red} + H_2O_2 \rightarrow HRP_{Ox} + H_2O \quad (1)$$

$$HRP_{Ox} + HQ \rightarrow HRP_{Red} + BQ \quad (2)$$

$$BQ + H^+ + 2e^- \rightarrow HQ \text{ (electrode reaction)} \quad (3)$$

Where BQ (benzoquinone) is the oxidation state of HQ, and HRP_{Ox} is the oxidation states of HRP in the process of the enzymatic reaction. In the HRP catalyzing H_2O_2 + HQ system, the HRP in reduced state (HRP_{Red}) reduces H_2O_2 into H_2O and HRP_{Red} itself converts into oxidized state HRP_{Ox}. Then, the exchange electrons between HRP_{Ox} and HQ produce HRP_{Red} and benzoquinone (BQ). BQ receive electrons from the electrode, which electrochemically produces HQ. Thus, HQ recycles in the process resulting in increase of the current signal. Therefore, the behavior of different immunosensors can be evaluated by comparing the current before and after the addition of H_2O_2. Then a lower concentration of P4 resulted in a larger current change due to the formation of more immunocomplex on the sensor surface. Otherwise, a higher concentration of P4 resulted in a little current change. ΔI gradually decreased with the increase in P4 concentrations (Fig. 5**B**). Furthermore, a linear response was discovered between ΔI and the logarithm of P4 concentration in the range of 0.0002–0.2 ng mL^{-1} as ΔI (μA) =-(0.71 ± 0.45)-(8.15 ± 0.2)×lg C_{P4} (ng mL^{-1}) with a correlation coefficient of 99.6%, where ΔI was the current change and C was P4 concentration. In addition, according to the equation [37,38]: $x_L = x_{b1} + 3\,s_{b1}$, where x_L was the detection limit, x_{b1} was the mean signal of the blank measures, s_{b1} was the standard deviation of the blank measures, a value of 3 was chosen for the equation as the constant. This usually corresponds to a confidence level of about 90% in a practical sense, while the detection limit can be calculated as 6.3×10^{-5} ng mL^{-1}. Finally, we compared the analytical performance of the proposed electrode with some recent references, as shown in Table 1. It reveals that the proposed sensor has a wider linear range, lower detection limit and higher sensitivity.

In this work, after making six measurements of blank, the x_{b1}=33.07 and s_{b1}=0.15 was obtained. Thus, $x_L = x_{b1} + 3\,s_{b1}$=33.07+3×0.15=33.52 and □I(A)=−0.71−8.15×lg C_{P4}(ngmL^{-1}) , $c_L=10^{(I+0.71)/(-8.15)}=10^{(33.52+0.71)/(-8.15)}$=6.3×10^{-5}ngmL^{-1}, where □I is the $x_L$at 33.52. To conclude, the LOD of this proposed immunosensor for the detection of P4 is 6.3×10^{-5} ng mL^{-1}.

To evaluate the selectivity of the immunosensor for the detection of P4, the cross-reactivity (CR) against a range of analogues to P4, such as ethisterone, testosterone, medroxyprogesterone acetate, β-estradiol, and estriol were examined under the same experimental conditions. As shown in Table S1, among all the analogues, the CRs were lower than 0.1%, suggesting that these analogues have no capability for the detection of P4 in the real world. Although its structural analogues also belong to the class of biogenic amines, and even some compounds displayed a similar chemical structure to P4, this electrochemical immunosensor showed a high specificity for the detection of P4. The superior specificity capability of the constructed sensor is partially decided by the prepared antibody.

The reproducibility of the immunosensor was an important parameter for practical application. In this study, reproducibility was confirmed by six measurements of every concentration, which resulted in the relative standard deviations of 3.5%, 2.85% and 4.61% at P4 concentrations of 0.0005, 0.005 and 0.05 ng mL^{-1}, respectively.

The stability of the biosensor was also explored by checking current response periodically. When the biosensor was not in use, it was stored in refrigerator at 4 °C. After 2 weeks, the catalytic current of the immunosensor decreased to about 86.3% of its original value, indicating the retention of the specific binding ability of the antigens was accepted.

3.5. Detection of progesterone in spiked milk

The spiked milk samples with P4 concentrations at 0.15, 0.5, 1.5, 5, 10, and 15 ng mL^{-1}were analyzed after dilution of 100 times, respectively (n=3). Average recoveries were observed from 84.0% to 102.0% with the coefficient of variance (CV) ranging from 1.74% to 4.44% after all the spiked milk samples were analyzed by immusensor (Table 2), which indicated that the matrix effect of the sample was negligible using the sample preparation method.

To confirm the reliability of the proposed immunosensor, spiked milk samples were subjected to P4 analysis by LC–MS/MS. The results of the immunosensor were in good agreement with those of LC–MS/MS with a correlation coefficient (R^2) of 0.998 (**Fig. S1**), revealing good reliability and accuracy for this immunosensor.

4. Conclusion

In summary, by combining of Thi-GO composites and BAS strategy, we have developed an ultrasensitive electrochemical immunosensor for progesterone P4 and demonstrated its use in sensitive and selective detection ofP4 in milk samples. The fabricated Thi-GO composites electrode platform was an ideal substrate for artificial antigen immobilization with acceptable stability and excellent bioactivity. Due to the signal amplification strategy of Thi-GO composites electrode platform and biotin-avidin-system, the electrochemical response of the established immunosensor was greatly enhanced, showing a linear response range for P4 between 0.02–20 ng mL^{-1} with a low detection limit of 0.0063 ng mL^{-1} in milk samples. Meanwhile, the proposed electrochemical immunosensor also exhibited great specificity, acceptable stability and reproducibility. It was anticipated that the amplified method based on the Thi-GO composites electrode platform and the BAS label could be expanded for the construction of other immunosensors. This made it potentially attractive for the detection of other important lower molecular contaminant in food.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.04.054.

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