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Highly Sensitive Detection of Peptide Hormone Prolactin Using Gold Nanoparticles-Graphene Nanocomposite Modified Electrode

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A novel amperometric immunosensor for detection of prolactin has been developed using the glassy carbon electrodes modified with graphene and electrochemically deposited gold nanoparticles. The hybrid nanocomposites not only offer a appropriate microenvironment for immobilization of primary antibodies and maintain their biological activity, but also accelerate electron transfer between the immobilized proteins and electrode substrates. In the presence of prolactin, a sandwich-type immune complex was formed on the sensing interface. Finally, the streptavidin-alkaline phosphatase was bound to the biotinylated secondary antibodies, catalyzing the hydrolysis of the substrate α-naphthyl phosphate. Under optimal conditions, the immunosensor exhibited a linear response to prolactin from 100 pg mL^{-1} to 50 ng mL^{-1}, with a detection limit of 38.9 pg mL^{-1} (S/N=3). In addition, good specificity and reproducibility were achieved for the electrochemical immunosensor, which could be used for the detection of prolactin in real samples. Thus, the proposed strategy might provide a valuable platform for detection of prolactin for clinical immunoassay in the future.

Keywords: prolactin, electrochemical immunosensor, graphene, gold nanoparticles

1. INTRODUCTION

Prolactin (PRL), as a lactogenic substance presented in extracts of the pituitary gland of cow, was definitely identified in human blood in 1970 [1]. Prolactin constitutes part of luteotrophic complex and is necessary for maintenance and secretory activity of corpus luteum in mammals [2]. Recent

researches suggest that prolactin levels may be of some significance in distinguishing epileptic seizures from psychogenic hysteria presenting in an epileptiform manner [3]. When hyperprolactinemia is confirmed, the determination of prolactin in women serum is needed in the case of menstrual disturbances, galactorrhea, hypogonadism or infertility among other diseases [4].Therefore, it is imperative to detect prolactin concentration for diagnosis of disease.

Various assays have been developed for the determination of prolactin. Such as enzyme immunoassay (EIA) [5, 6], bioluminescent immunoassay [7], and chemiluminescence immunoassay [8]. Although these methods have been widely applied and accepted, most of them are expensive, time consuming and requiring highly trained personnel [9]. Thus, it is a necessary to develop a simple and new method with good specificity and high sensitivity for the detection of prolactin in clinical serum sample. Recently, electrochemical biosensors coupled with nanomaterials have attracted considerable attention because of fast response, high sensitivity, versatile functionality and low-cost instrumentation [10-13].

Graphene, a one-atom thick and two-dimensional closely packed honeycomb lattice, has been regarded as a new and effective electrode substrate for fabricating biosensor in view of its excellent biocompatibility, large surface area, and more importantly, its capability for accelerating the electron transfer between electroactive species and electrode substrates [14-17]. However, graphene application is limited because of the aggregation of graphene through van der waals interaction and π-π stacking interaction in aqueous solution [18]. To improve the homogeneity and dispersion of the graphene, chitosan has been used to stabilize graphene by non-covalently functionalized graphene on account of π-π stacking interaction and van der waals interaction [19-22]. Up to now, graphene-based hybrid materials have become a hot research topic in material science[23,24]. The hybridization can be an effective strategy to improve the functionality of materials [25]. And the integration of nanomaterials on graphene potentially paves a new way to enhance their electronic, chemical and electrochemical properties [15]. On the other hand, Numerous studies has shown that the unique properties of gold nanoparticles (AuNPs) can offer a appropriate microenvironment for biomolecules immobilization while maintaining their biological activity, and accelerate electron transfer between the immobilized proteins and electrodes [26-29]. Therefore, through non-covalently π-π stacking interaction, the integration of gold nanoparticles on graphene not only potentially takes advantage of the synergetic effects from AuNPs, but also enhances the performances of graphene in terms of electronic properties [30-32].

In this paper, a sandwich-type electrochemical immunosensor was fabricated using graphene modified electrode with electrochemically deposited AuNPs for detection of prolactin. The nanocomposites could provide an effective antibody immobilization matrix and make the immobilized immunocomponents possess bioactivityy and high stabilit. After an immuno-reaction, streptavidin-alkaline phosphatase was bound to the secondary antibodies via biotin-streptavidin chemistry. In addition, the sensitivity was enhanced by highly catalytic activity of alkaline phosphatase toward enzyme substrate α-naphthyl phosphate. Therefore, the fabricated biosensor strategy might be a promising approach for detection of other biomolecules in the field of clinical biomedical applications.

2. EXPERIMENTAL

2.1. Materials and reagents

Prolactin antigen, monoclonal prolactin antibodies (Ab1) and biotin labeled monoclonal prolactin antibodies (biotinylated Ab2) were obtained from Bioscience (Tianjin, China). Graphene (500 mg, Purity: >99.8%) was acquired from Pioneer Nanotechnology Co. Ltd. (Nanjing, China). Chloroauric acid ($HAuCl_4$), bovine serum albumin (BSA, 96-99%), streptavidin-alkaline phosphatase (ST-AP), α-naphthyl phosphate (α-NP) and chitosan (CS) were achieved from Sigma-Aldrich (MO, USA). All other reagents were of analytical grade. Deionized water purified through a Millipore system (≥18 MΩ, Milli-Q, Millipore) was employed in all experiments. 0.01 M phosphate-buffered saline (PBS) containing 136.7 mM NaCl, 2.7 mM KCl, 87 mM Na_2HPO_4 and 14 mM KH_2PO_4 (pH 7.4) was used as washing buffer. Diethanolamine (DEA) buffer contained 0.1 M DEA, 1 M $MgCl_2$ and 100 mM KCl (pH 9.6).

2.2. Apparatus

All electrochemical measurements were performed on a CHI 660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China). A conventional three-electrode system was used for all electrochemical measurement: glassy carbon electrode (GCE, 3 mm in diameter) as a working electrode, Ag/AgCl electrode as a reference electrode and platinum wire as an auxiliary electrode. The morphologies of graphene and AuNPs-graphene were characterized by scanning electron microscopy (SEM) system (FEI Nova-400, USA).

2.3. Preparation of graphene solution

Graphene solution was prepared as following steps. Firstly, chitosan solution (0.5 wt%) was prepared by dissolving 1g of chitosan powder into 200 mL of 1% (v/v) acetic acid solution with 4 h ultrasonication to form a homogeneous solution. The pH of chitosan solution was adjusted to 4.0-5.0 using 1.0 M NaOH. Then, 1 mg of graphene was dispersed in 1 mL of chitosan solution, followed by 2 h sonication to obtain black homogeneous solution.

2.4. Fabrication of the electrochemical immunosensor

Prior to the surface modification, the glassy carbon electrode was polished with 0.05 μm alumina slurries on soft lapping pads, and washed thoroughly with deionized water. Then the electrode was sonicated with 1:1 nitric acid and acetone, and deionized water for 3 min, respectively. The modified glassy carbon electrode was prepared by dropped about 10 μL of the prepared graphene/chitosan homogeneous suspension onto the surface of the electrode and dried for 2 h at room temperature. Then the modified electrode was immersed into 3 mM $HAuCl_4$ solution, and electrochemical deposition was conducted by cyclic voltammetry at a potential range from 0.2 to 0.6 V

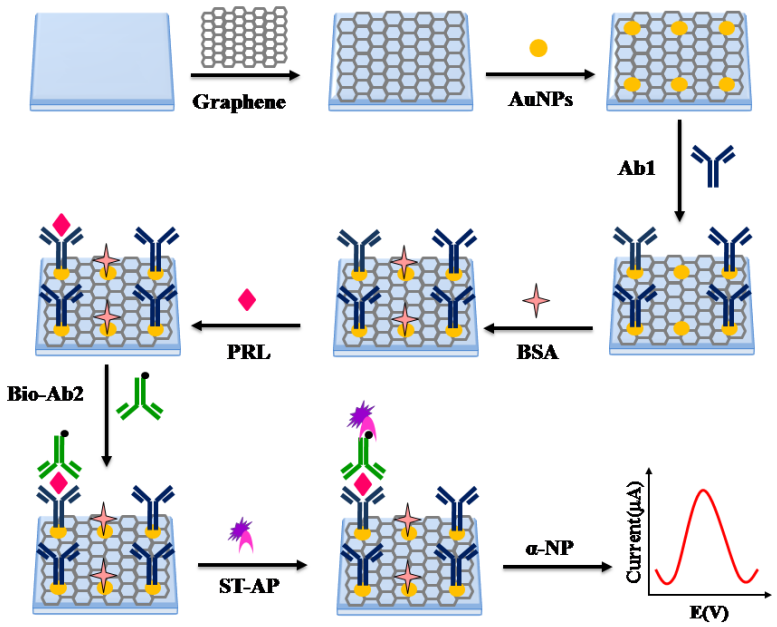
for 100 s. Finally, about 10 μL of Ab1 solution (67 μg mL^{-1}) in phosphate buffer was dropped onto the surface of the AuNPs/graphene/chitosan modified electrode, followed by overnight incubation at 4 ℃. The obtained electrode was incubated in 0.5 % (wt) BSA for 30 min at room temperature. After thoroughly washed with phosphate buffer (pH 7.0, 0.01 M), the electrochemical immunosensor was successfully fabricated and stored at 4 ℃for use.

2.5. Electrochemical measurements

For detection of prolactin, the immunosensors were incubated with different concentrations of prolactin solution and Ab2 solution for 40 min at 37 ℃, respectively. Then the electrode was washed carefully with PBS buffer, and 10 μL of 2.0 μg mL^{-1} ST-AP solution was dropped onto the fabricated electrode surface and incubated for 30 min at 37 ℃. Finally, after rinsed with PBS buffer, the different pulse voltammetry (DPV) measurements were carried out from -0.1 to 0.5 V with a pulse amplitude of 70 mV and width of 0.05 s in 5.0 mL diethanolamine solution containing 1.2 mg mL^{-1} α-NP.

3. RESULTS AND DISCUSSION

3.1. Design of electrochemical immunosensor



Scheme 1. Schematic representation of the sandwich immunosensor based on AuNPs-graphene for detection of PRL.

The principle of the electrochemical immunosensor was depicted in Scheme 1. Firstly, the electrochemical immunosensor was prepared by modifying the glassy carbon electrode with functionalized gold nanoparticles/graphene/chitosan nanocomposite film. The nanocomposites could not only provide a propriate microenvironment for immobilization of primary antibodies and maintain

their biological activity, but also offer a biocompatible film with large surface area. Secondly, The primary antibody was absorbed on the surface of AuNPs-graphene modified glassy carbon electrode. In the presence of PRL, a sandwich-type immune complex was formed on the sensing interface. Finally, the ST-AP was bound to the immunosensor surface via biotin-streptavidin interaction to produce enzymatic electrochemical signal readout for quantitative detection of PRL. The established method shows an acceptable precision and reproducibility, and has been successfully applied in direct detection of PRL in clinical serum specimens.

3.2.Characterization of graphene and AuNPs-graphene

The surface morphologies of the graphene and AuNPs-graphene was characterized by Scanning electron microscopy. As shown in Fig. 1A, in the presence of chitosan, graphene was stably immobilized on the glassy carbon electrode surface, exhibiting typical crumpled and wrinkled sheet structure. After AuNPs were electrochemically deposited on the surface of graphene, a mass of spherically shaped gold nanoparticles, size ranging from subnanometer to ~20 nm, were formed on the surface of the graphene (Fig. 1B), suggesting that gold nanoparticles were effectively deposited on the modified GCE with graphene.

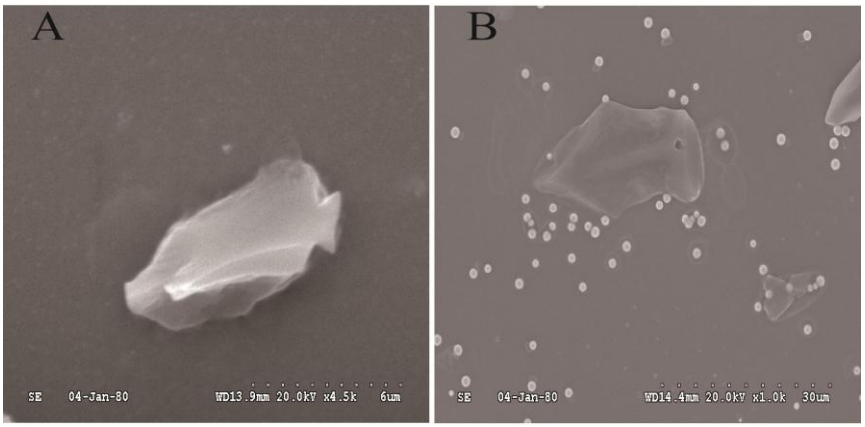


Figure 1. SEM images of graphene (A) and AuNPs-graphene (B).

3.3. Electrochemical characterization of the sensing interface

Cyclic voltammetry (CV), providing detailed information on change of the surface property of modified surface for each modification process, was a powerful tool to characterize the features of modified electrode surface. As shown in Fig. 2A (curve a), the bare GCE showed a couple of well-defined and stable redox peak in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl. With the addition of graphene, evident increases of both cathodic and anodic peak currents were observed mostly due to the high conductivity of the one-atom thick graphene (curve b). AuNPs subsequent electrochemically deposited on the surface of graphene led to further increase in the redox current of $[Fe(CN)_6]^{3-/4-}$ duo to the extraordinary conductivity of AuNPs (curve c). However, after the Ab1

(curve d) and BSA (curve e) blocking solution were immobilized on the modified electrode surface, significant decrease in redox current were observed, which suggested that biomacromolecules had been successfully immobilized on the surface and the proteins inhibited the electron transfer between the redox probe and the electrode. Finally, the introduction with PRL antigen and Ab2 (curve f, curve g) on the surface of the Ab1 modified electrode led to redox current further decrease, suggesting that the formation of antigen-antibody complex on the surface of the immunosensor blocked the current transmission. The cyclic voltammetry results were quite in a good agreement with the those obtained from square wave voltammetry (SWV) measurements (Fig. 2B). Both results of CV and SWV demonstrated that the developed biosensor worked indeed as described in the principle scheme.

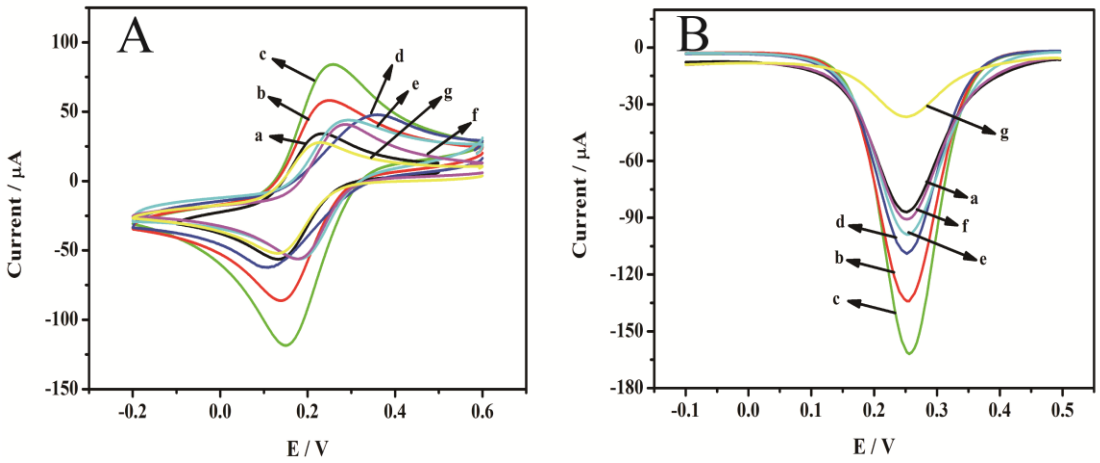


Figure 2. Cyclic voltammetry (A) and square wave voltammetry (B) of (a) bare, (b) graphene, (c) AuNPs-graphene, (d) AuNPs-graphene/Ab1, (e) AuNPs-graphene/Ab1/BSA, (f) AuNPs-graphene/Ab1/BSA/PRL, (g) AuNPs-graphene/Ab1/BSA/PRL/Ab2 modified GCE in the present of 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 mol L^{-1} KCL at 50 mV s^{-1}.

3.4. Optimization of experimental conditions

To achieve the excellent assay performance, some experimental conditions, such as the α-NP concentration, ST-AP concentration and the incubation time of antigen and antibody, were optimized. The concentration of α-NP played a important role in the measuring system. The DPV peak current of the biosensor increased with the increasing concentration of α-NP from 0.6 to 1.2 mg mL^{-1}, and then tended to a stable value at higher concentrations. Hence, the optimal concentration of α-NP was 1.2 mg mL^{-1} (Fig. 3A). The concentration of ST-AP obviously affecting the peak current of the biosensor (Figure. 3B). With the increasing concentration of ST-AP, the DPV response increased quickly up to 2.0 μg mL^{-1} and then reach a maximum value due to the saturated binding. Thus, 2.0 μg mL^{-1} was selected for the optimal concentration for electrochemical detection.

The incubation time of antigen and antibody was also the performance of the electrochemical analysis affecting the in the measuring system. In the case of incubation time, the signal responses of biosensor was found to gradually increase with the increasing incubation time and then tend towards a

plateau value after 40 min, which was denotative of a gradual increase in antibody binding till saturation on the immunosensor surface. Therefore, 40 min of incubation time was chosen as the optimal time (Fig. 3C).

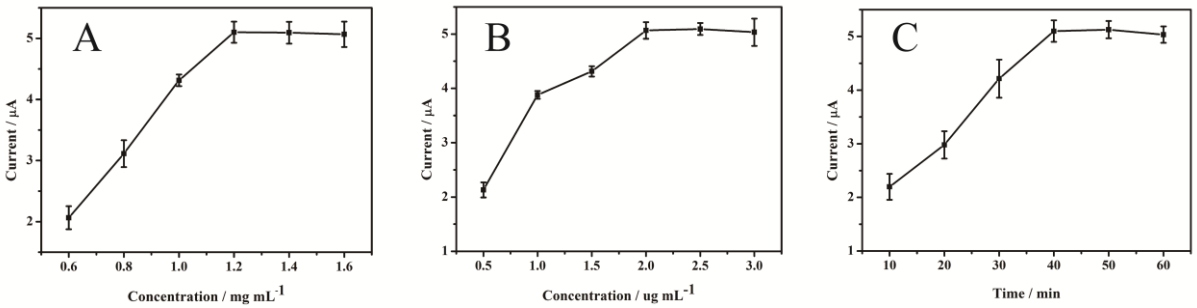


Figure 3. Dependences of DPV peak currents on α-NP concentration (A), ST-AP concentration (B) and incubation time of Ab2-Ag interactive (C), when one parameter changes while the others are under their optimal conditions. The error bars represent the standard deviation calculated from three different assays.

3.5. Analytical Performance of Electrochemical Biosensor

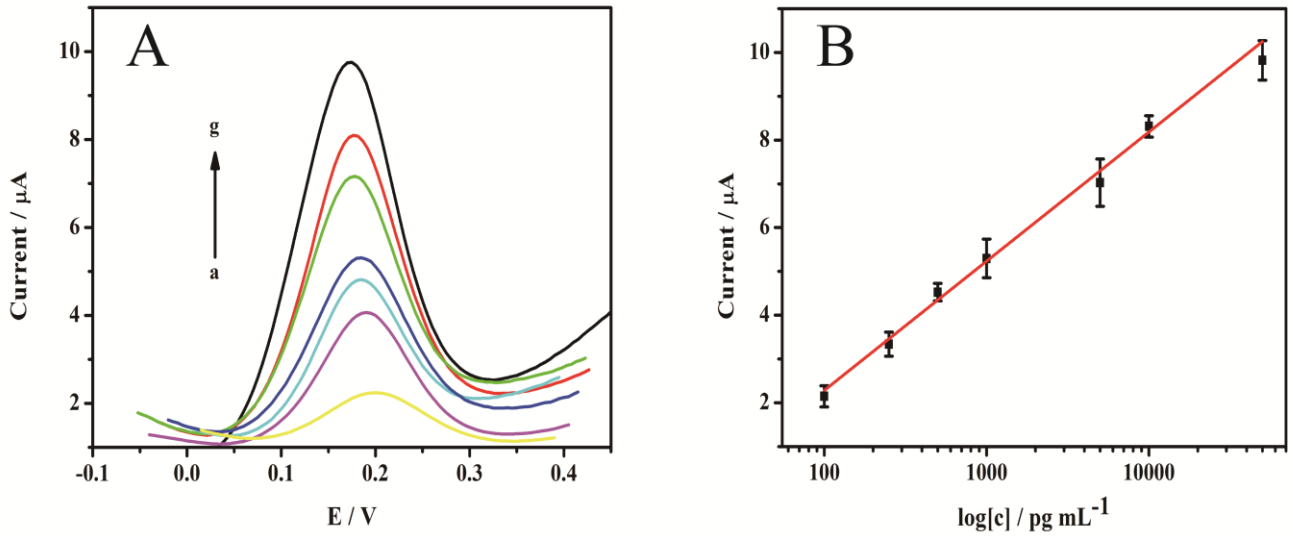


Figure 4. Typical DPV of immunosensor response to target PRL from a to g (100, 250, 500, 1000, 5000, 10 000 and 50 000 pg mL^{-1}) (A). Calibration curve of the immunosensor for PRL determination plotted on a semi-log scale (B).

To clarify the analytical performance of the established electrochemical immunosensor, various experiments were conducted by adding different concentrations of PRL to the immunosensor. Under the optimal experimental conditions, the DPV signal of the immunosensor increased with the increase of PRL concentrations, and showed a good linear relationship with the logarithm of PRL concentration from 0.1 to 50 ng mL^{-1} (Fig. 4A). The resulting calibration equation was I (μA)= -3.61 +

2.95 log c (c is the concentration of prolactin (pg mL^{-1})) with a correlation coefficient of 0.9971 (Fig. 4B). The limit of detection was calculated to be 38.9 pg mL^{-1} at a signal-to-noise ratio of 3. In order to highlight the merits of the designed immunosensor, the analytical properties were summarized in Table 1. It proved that the established immunosensor showed a much lower detection limit. Therefore, highly sensitive electrochemical immunoassay for detection of peptide hormone prolactin was achieved based on gold nanoparticles-graphene nanocomposite modified electrode.

Table 1. Comparison of various assayed method for detection of prolactin.

Analytical assay	Linear range (ng mL^{-1})	Detection limit (ng mL^{-1})	Reference
Electrochemical immunosensor	10-2000	7	[4]
Electrochemical immunosensor	0.5-180	0.1	[33]
GenWay PRL ELISA	0.35-200	0.35	[34]
Abcam, PRL human ELISA Ki	5-100	0.25	[34]
ELISA	8-1000	0.5	[35]
Chemiluminescence immunoassay	0.1-20	0.1	[36]
Electrochemical immunosensor	0.1-50	0.038	This work

3.6. Specificity and reproducibility of the immunosensor

Specificity of the immunosensor was a significant criterion for analyzing biological sample. Therefore, different proteins such as BSA, angiotension Ⅱ (Ang Ⅱ) and tumor necrosis factor-α (TNF-α) with the concentration of 100 ng mL-1 were used as interference to evaluate the specificity of the immunosensor. As shown in Fig. 5, the signal responses of BSA, Ang Ⅱ and TNF-α were similar to that of the blank (without PRL). On the contrary, the peak currents to PRL increased obviously. The error bars represented average standard errors for three measurements. These experimental results showed that the designed biosensor could effectively discriminate different proteins.

Reproducibility of immunosensor was also estimated by measuring the prolactin concentration at 100 pg mL^{-1} and 1 ng mL^{-1} with five times at the same modified GCE independently. The relative standard deviations of the intra-assay was about 3.7% and 6.9%, respectively. These results displayed that the designed immunosensor possessed an acceptable reproducibility.

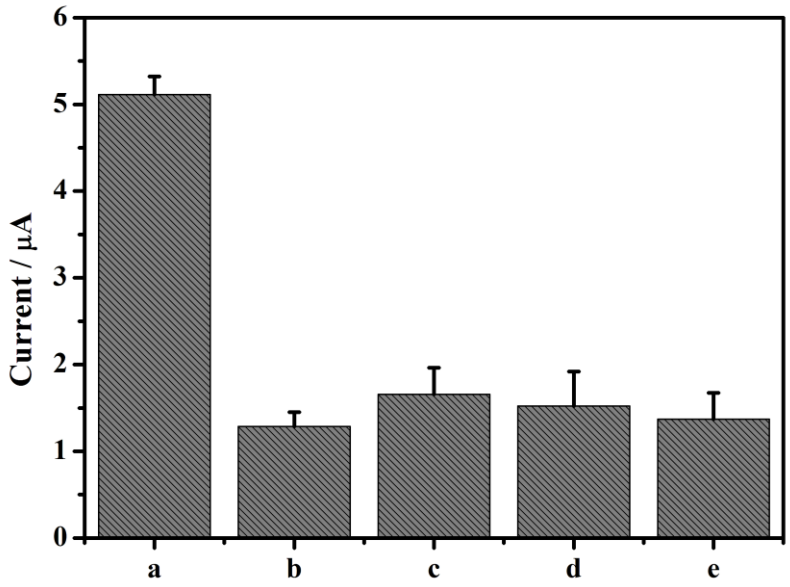


Figure 5. Responses of the immunosensor to prolactin (a), BSA (b), Ang Ⅱ, TNF-α (e) and blank (f). The prolactin concentration was 1ng mL^{-1}, other proteins were all 100 ng mL^{-1}. The error bars represent the standard deviations in three different measurements for each concentration.

3.7. Application of the immunosensor in human serum

To further evaluate the potential application of the designed electrochemical immunosensor in real sample analysis, we performed the PRL assay using serum samples, which were gained from the First Affiliated Hospital of Chongqing Medical University. Various amounts (100, 1000 and 10 000 pg mL^{-1}) of PRL were spiked into serum samples, and analyzed by the developed method. The results were listed in Table 2. The recovery values were ranged from 98.2% to 100.7% respectively, which showed that the analysis capability of immunosensor in complex mixtures was almost unaffected, and the established immunosensor could be potentially applied for the clinical determination of PRL in real biological samples.

Table 2. Recovery studies of prolactin from human serum samples.

Sample	Add/pg mL^{-1}	Found/pg mL^{-1}	Recovery/%
1	100	98.23	98.2
2	1000	1002.38	100.2
3	10000	10006.86	100.7

4. CONCLUSIONS

In conclusion, a novel electrochemical immunosensor has been successfully fabricated for the determination of prolactin by electrochemically deposited AuNPs on the surface of graphene modified electrode. The AuNPs-graphene modified GCE not only enhanced the amount of protein immobilized

on the surface of the modified GCE, but also facilitated electron transfer. The immunosensor showed a wide linear range and low detection limit for the determination of prolactin with high reproducibility and specificity. This biosensing method provides a promising potential approach for detection of prolactin in clinical applications, and might be easily extended for detection of other proteins in clinical laboratory.

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