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Glucose oxidase and ferrocene labels immobilized at Au/TiO_2 nanocomposites with high load amount and activity for sensitive immunoelectrochemical measurement of ProGRP biomarker

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ABSTRACT

Progastrin releasing-peptide (ProGRP) is a sensitive, specific, and reliable tumor marker with small cell lung cancer (SCLC), which may indicate an early tendency of cancer metastasis, causing high mortality rate. Thus, aiming for a more convenient assay system of SCLC, a novel immunoelectrochemical measurement for sensitive detection of ProGRP was developed in this work *via* Au nanoparticle/graphene modified immunosensor with ferrocene and glucose oxidase-multifunctionalized Au/TiO_2 nanocomposites as a trace label. At first, Au nanoparticles (nano-Au) were attached on the TiO_2 nanoparticles surface by using 3-aminopropyltriethoxy silane (APTES) as linkage reagent to obtain Au/TiO_2 nanocomposites (nano-Au/TiO_2). Next, glucose oxidase (GOD) and ferrocene labeled secondary antibodies (Fc-Ab_2) were used to bind Au/TiO_2 nanocomposites with high load amount and good biological activity, and because the increased surface area and biocompatibility of nano-Au/TiO_2, the electrode can provide amplified signals. On the other hand, the nano-Au functionalized graphene sheets (GS) were used for the biosensor platform for increasing the surface area as well as improving the electronic transmission rate to capture a large amount of primary antibodies (Ab_1). Then in presence of glucose, amplified signals can be obtained by an electrochemical sandwich immunoassay protocol. Based on the proposed immunosensor, the current is linear with the concentration of ProGRP being within a concentration range from 10.0 to 500 pg/mL with a limit of detection down to 3.0 pg/mL (S/N = 3).

1. Introduction

Lung cancer, which is the leading cause of cancer mortality, has two main subtypes, small cell lung cancer (SCLC) and nonsmall cell lung cancer (NSCLC) (Han et al., 2008). Recently, progastrin releasing-peptide (ProGRP) have been reported to be a sensitive, specific, and reliable tumor marker for staging, monitoring treatment, and predicting relapse in patients with SCLC (Yamaguchi et al., 1983). Usually, the upper normal limit of ProGRP in the circulation is 50 pg/mL (Molina et al., 2004). Since the ProGRP level at the time of diagnosis was correlated with the disease extent, the observation of an increase in serum ProGRP level reflects the predictor and course of SCLC as well as an effective index in discriminating SCLC and NSCLC.

Detection antibodies immobilization is a key step in fabrication of a sensitive sandwich immunosensor. Nowadays, titanium dioxide nanoparticles (nano-TiO_2) have been widely used to immobilize proteins or enzymes on electrode surface for either mechanistic study of the proteins or fabricating electrochemical biosensors (Wang et al., 2010; Mun et al., 2010; Ji et al., 2009). In recent years, the Au nanoparticles functionalized nano-TiO_2 (nano-Au/TiO_2) have been investigated to enhance the surface-to-volume ratio, biocompatibility and stability (Milsom et al., 2007; Zhang et al., 2008). Thus, the spherical gold nanoparticles capped nano-TiO_2 composite nanoparticles were prepared and introduced for the binding of glucose oxidase (GOD) and ferrocene labeled secondary antibodies (Fc-Ab_2) in this work. Because of the large surface area and good biocompatibility of nano-Au/TiO_2, the large load amount of GOD and Fc-Ab_2 were obtained on the nano-Au/TiO_2 surface with good biological activity, which resulting in the improvement of the intensity of the signal and ultrasensitive detection.

Graphene sheets (GS), a single layer of carbon atoms with a hexagonal arrangement in a two-dimensional lattice, had attracted enormous attention since it was first reported in 2004 (Novoselov et al., 2004), because of a few intriguing attributes, such as its fast electron transportation, high thermal conductivity, excel-

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lent mechanical stiffness and good biocompatibility (Chen et al., 2008; Li and Kaner, 2008; Geim, 2009). Recent researches have made efforts to increase the graphene solubility by covalent or non-covalent functionalization method (Wu et al., 2010). Thus, a water-soluble polymers, such as polyvinylpyrrolidone (Shan et al., 2009), chitosan (Kang et al., 2009), and Nafion (Li et al., 2009) were used as disperser to prepare homogeneous GS solution. In our previous work (Liao et al., 2010), we have developed a Nafion–cysteine (Cys/Nf) composite membrane for the biomolecule immobilization. Compared with pure Nafion film, the Cys/Nf composite membrane demonstrated more stability, biocompatibility and favorable for mass and electron transfer to the electrode surface. Herein, Nafion not only acts as an effective solubilizing agent for dispersing graphene nanosheets (GS-Nf), but also as a cation exchange polymer for Cys loading to obtain a nanostructural graphene-Nafion–cysteine composite membrane with the further enhancement of the porosity, surface area and electronic transfer rate. Moreover, gold nanoparticles (nano-Au) have been self-assembled on the cysteine/Nafion-graphene (Cys/GS-Nf) composite membrane by the –SH of the embedded cysteine. Finally, the nano-Au embedded Cys/GS-Nf composite membrane was employed for the antibody molecules immobilization.

In view of the advantageous features of spherical gold nanoparticles capped nano-TiO_2 composite nanoparticles and nano-Au embedded Cys/GS-Nf composite membrane, a sensitive sandwich-type electrochemical immunosensor was constructed using ProGRP as the model analyte. Based on the ferrocene and GOD-multifunctionalized Au/TiO_2 nanocomposites, amplified response signals could be achieved by efficient catalysis of the GOD towards the electrochemical reduction of glucose, resulting in the low detection limit of ProGRP. The details of the attractive response performances of the proposed immunosensor and potential merits for protein detection are substantiated as follows.

2. Experimental

2.1. Reagents and materials

ProGRP and anti-ProGRP were purchased from Advanced Life Science Institute, Inc., Saitama, Japan. Graphene nanosheets were obtained in Pioneer Nanotechnology Co. (Nanjing, China). The reagents gold chloride ($HAuCl_4$), Nafion (5%), 3-aminopropyltrimethoxysilane (APTES, 97%), titania nanoparticles (nano-TiO_2, 5%, pH 2.0–3.0), gold chloride ($HAuCl_4$), ferrocenemonocarboxylic (Fc-COOH), bovine serum albumin (BSA, 96–99%), $NaBH_4$ and sodium citrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and used without further purification. L-Cysteine (L-Cys) was purchased from Kangda Amino Acid (Shanghai, China). Distilled water was used throughout this study. $LiClO_4$–glycine buffer, pH 7.4 (0.1 M) was used as working buffer throughout the experiment for electrochemical immune-detection. Phosphate buffered solutions (PBS) (pH 7.4) were prepared using 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.1 M KCl and kept at 4 °C before use. All other chemicals were of analytical grade and used as received without further purification. Gold colloids were produced by reducing gold chloride tetrahydrate with citric acid at 100 °C for half an hour (Frens, 1973). The mean size of the prepared Au colloids was about 16 nm, which was estimated from transmission electron microscopy (the graph not shown).

2.2. Apparatus

The cyclic voltammetric (CV) experiments were carried out on a CHI 660C electrochemistry workstation (Shanghai CH Instruments, China) connected to a personal computer. A conventional three-electrode system was used with and a saturated calomel electrode (SCE) as reference electrode, a platinum wire as auxiliary electrode and the modified glassy carbon electrode (Φ = 4 mm) as working electrode. The assembling interface was tracked by scanning electron microscopy (SEM, S-4800, Hitachi, Japan) at an acceleration voltage of 20 kV. The pH measurements were made with a pH meter (MP 230, Mettler-Toledo Switzerland) and a digital ion analyzer (Model PHS-3C, Dazhong Instruments, Shanghai, China).

2.3. Preparation of Au/TiO_2 nanospheres

The Au/TiO_2 nanocomposites were prepared as following procedure according to the literature (Morrill et al., 2009). Briefly, 5 mL methanol solution was prepared and stirred which contained 1% APTES, 5% deionized water, and 1 mM acetic acid. Then 100 μL nano-TiO_2 was added in to the resulting mixture and stirred for 4 h for the APTES covalent linking on the surface of nano-TiO_2. Next, it was washed by centrifugation with methanol and deionized water for three times until the filtrate was neutral. After that, 5.0 mL of the APTES-treated nano-TiO_2 was added drop by drop to the 5.0 mL of gold colloidal under stirring condition for 1 h and then separated by centrifugation. After discarding the supernatant, the obtained Au/TiO_2 nanocomposites were dispersed in 1 mL phosphate buffer (pH 7.4).

2.4. Preparation of Fc-Ab_2 and GOD-labeled nano-Au/TiO_2 bioconjugates

At first, ferrocene labeled secondary ProGRP antibody (Fc-Ab_2) was fabricated with the aid of EDC and NHS as coupling agents. Briefly, a proper amount of EDC and NHS was added into Fc-COOH solution for the activation of carboxyl. And then Ab_2 was added into the mixture which was allowed to stay overnight at room temperature under continuous stirring at 4 °C. Next, it was centrifuged for 15 min at 5000 rpm and washed twice with working buffer. 0.2 mL of 330 μg/mL of Fc-Ab_2 solution was added into the obtained nano-Au functionalized nano-TiO_2 nanospheres (nano-Au/TiO_2) solution for Fc-Ab_2 attachment. Then the mixture was allowed to react under gently stirring for 24 h at 4 °C, followed by centrifugation.

Subsequently, 1.0 mg GOD was dissolved in the Fc-Ab_2 labeled nano-Au/TiO_2 followed by incubation at 4 °C for 8 h under gently stirring. Herein, GOD played two roles: first, amplifying the response of the sandwich immunoreaction based on the biocatalysis towards glucose; second, it was employed to block the unspecified sites of nano-Au/TiO_2 nanospheres and prevent non-specific adsorption. At last the multi-labeled nano-Au/TiO_2 nanospheres were collected and redispersed in 1 mL PBS and then stored at 4 °C before use. The assembly process of Fc-Ab_2 and GOD multi-labeled nano-Au/TiO_2 nanospheres is shown in Fig. 1(A).

2.5. Fabrication of proposed immunosensor

A glassy carbon electrode (GCE) with 4 mm diameter was firstly polished with 0.3 and 0.05 μm alumina to obtain mirror-like surface, respectively. To remove the physically adsorbed substance, it was rinsed with deionized water and ethanol in ultrasonic bath and dried at room temperature.

Subsequently, 1 mg GS was dissolved in 1 mL 1% Nf solution by ultrasonic dispersion. Then 10 μL of the resulting suspension was coated onto a pretreated GCE surface and dried in the air. Then GS-Nf modified GCE was immersed into Cys solution (pH 2.3) for 6 h. Next, nano-Au monolayer was obtained by immersing the modified

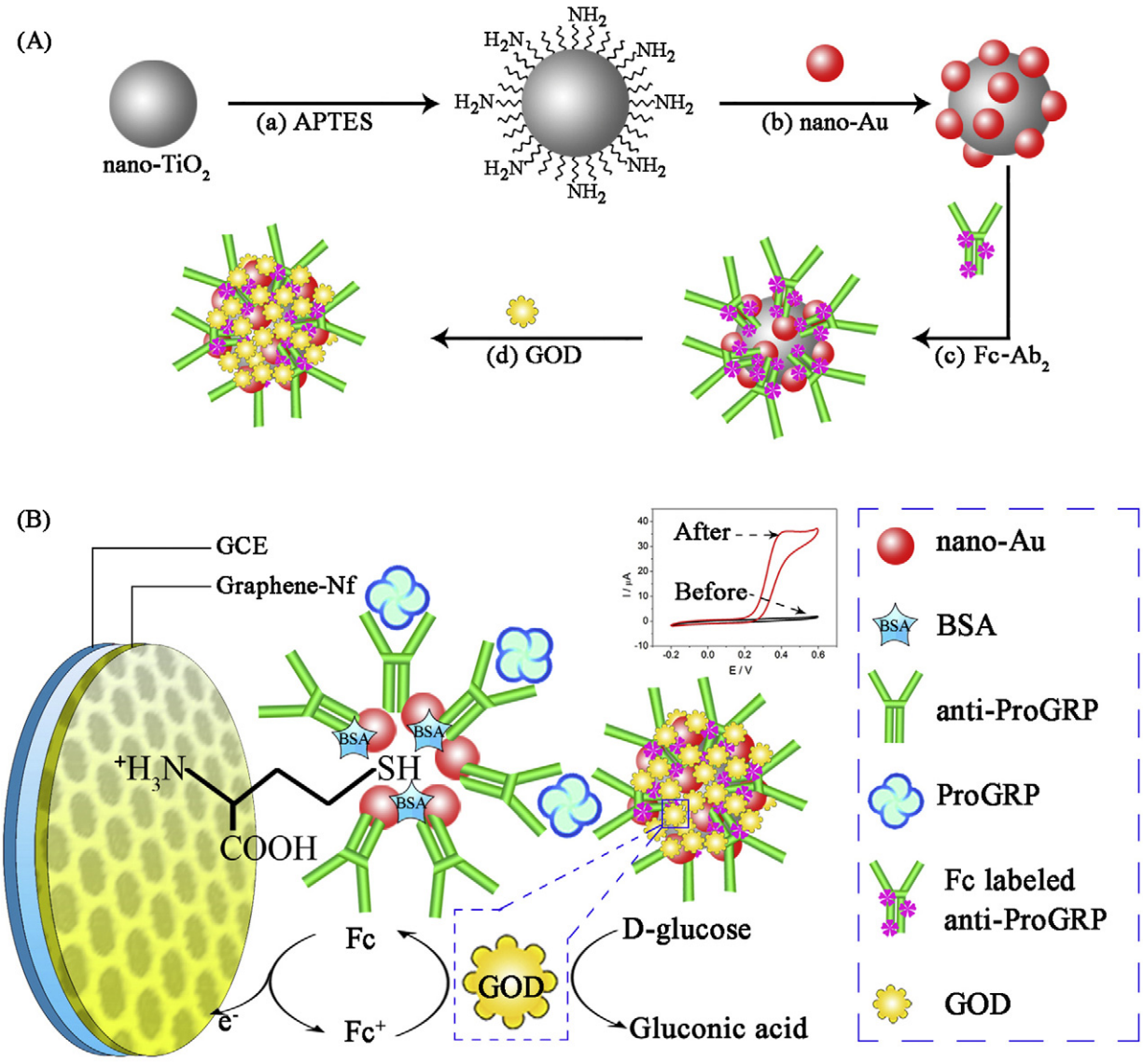

Fig. 1. The schematic diagrams of the preparation Fc-Ab_2 and GOD multilabeled nano-Au/TiO_2 nanospheres (A) and the fabrication of immunosensor and amplified voltammetric responses for ProGRP detection (B).

GCE into the prepared Au colloid for 3 h. Subsequently, primary ProGRP antibody (abbreviated as Ab_1) was attached by incubating at 4 °C for 12 h with 20 μL of 80 μg/mL anti-ProGRP in pH 7.4 PBS. The immunosensor was then washed with PBS containing 0.05% Tween-20 to remove the physical adsorbed Ab_1 and then incubated for 1 h with 20 μL of 1% BSA, followed by washing with 0.05% Tween-20 in PBS. Ultimately, the obtained immunosensor was stored at 4 °C when not in use. Fig. 1(B) may illustrate self-assembly procedure and the mechanism of the strategy in this work to fabricate the immunosensor.

2.6. Detection principle of the immunosensor

The immunosensors were produced with CV scans between −0.2 and +0.6 V *vs.* SCE at a scan rate of 50 mV/s in an unstirred electrochemical cell containing 0.1 M $LiClO_4$–glycine buffer (pH = 7.4) with an appropriate concentration of glucose at room temperature. After incubation with ProGRP standard solution, 10 μL of the prepared multilabeled nano-Au/TiO_2 nanospheres was added and left incubated to form a sandwich immunocomplex for 20 min at 37 °C.

Quantification of ProGRP protein was based on the voltammetric response which was recorded as the change of oxidation peak current of CVs before and after sandwich immunoreaction. Unless otherwise stated, all measurements were performed at room temperature (About 25 ± 2 °C).

3. Results and discussion

3.1. SEM characterizing of Cys/Nf-GS and nano-Au/Cys/Nf-GS composite membrane

The surface images of the Cys/GS-Nf and nano-Au/Cys/GS-Nf composite membrane were investigated by using scanning electronic microscope (Fig. 2). The SEM of graphene sheets dispersed by Nf (Fig. 2A) clearly shows a wrinkled paper-like structure with irregular size. Then the Au nanoparticles appear as bright dots, which occupy almost all of the surface of Cys/GS-Nf with fairly even, ordered, and close-packed distribution in Fig. 2B. The diameter of Au nanoparticles ranges from 14.2 to 17.6 nm, with a mean diameter of 16.2 nm, showing a narrow size-distribution, which confirmed the citrate reduction method could yield Au nanopar-

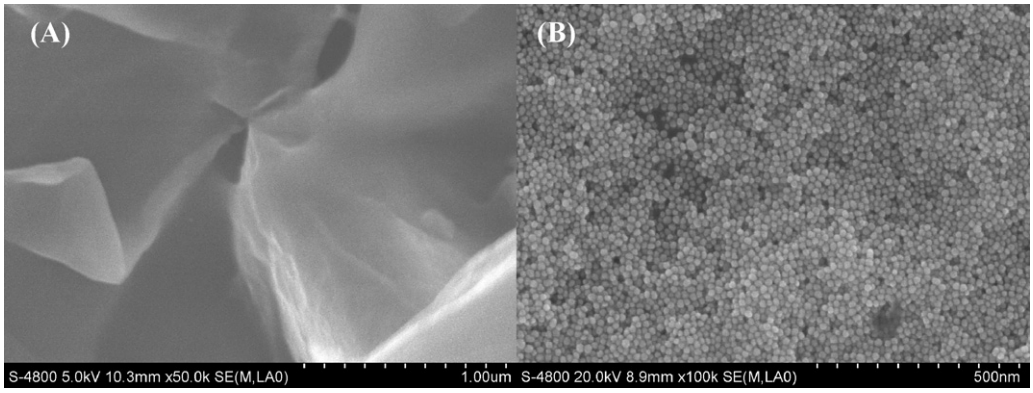

Fig. 2. SEM images of the Cys/GS-Nf (A) and nano-Au/Cys/GS-Nf (B).

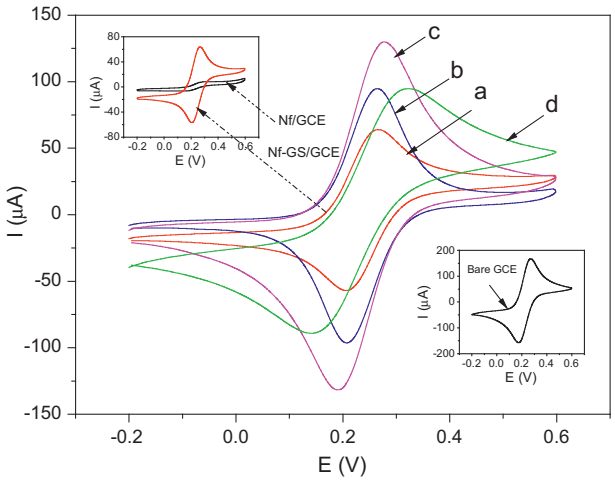

Fig. 3. CVs for bare Au GCE (insert in the right corner), GS-Nf (a), Cys/GS-Nf (b), nano-Au/Cys/GS-Nf (c), and Ab_1/nano-Au/Cys/GS-Nf (d) modified GCE in 5 mM potassium ferricyanide solution (pH 7.4) containing 0.1 M KCl at 50 mV/s. The insert in the top left corner was the comparison with the GS-Nf and pure Nf modified GCE.

ticles with desirably uniform size. Furthermore, the uniformly distributed, dense Au nanoparticles demonstrate the efficient interaction between Au nanoparticles and –SH functionalized Cys/GS-Nf nanocomposite.

3.2. Electrochemical characterization of proposed immunosensor

The cyclic voltammetric behavior of the step by step surface modification of the GCE in 5 mM potassium ferricyanide solution (pH 7.4) containing 0.1 M KCl from −0.2 to 0.6 V at a scan rate of 50 mV/s is shown in Fig. 3. The CV wave of bare GCE showed a well-defined redox wave in the right corner of Fig. 3, corresponding to the reversible redox reaction of ferricyanide ions. The curve a in Fig. 3 was the CV of GS-Nf modified GCE. Compared with the pure Nf modified GCE (the CV of Nf modified GCE was exhibited in the top left corner of Fig. 3), the voltammetric response increased obviously as the doping of GS in Nf film could greatly promote electron and mass transfer. Then the voltammetric response of Cys/GS-Nf modified electrode further increased (curve b in Fig. 3), certifying the protonated Cys (with positive charged) could be absorbed into the perfluorosulfonated cation polymer *via* the ion exchange effect. And the incorporated Cys/GS-Nf membrane was more favorable for mass and electron transfer to the electrode surface than the Nf-GS membrane. When the nano-Au was adsorbed in Cys/GS-Nf membrane (curve c in Fig. 3), a large peak current was obtained owing to nano-Au may act as a bridge of electron transfer and promote the electron transfer. After antibody and BSA were successively loaded onto the electrode surface, curve d in Fig. 3 shows that the voltammetric response decreases and significant shift of the peak position as the formation of a hydrophobic protein layer which insulates the conductive support and the interfacial electron transfer.

3.3. The comparison of different signal amplification strategies

In order to evaluate the effect of GOD and Fc-Ab_2 multilabeled nano-Au/nano-TiO_2 nanospheres bioconjugates (GOD/Fc-Ab_2/nano-Au/TiO_2) for the signal amplification, three kinds of Ab_2 functionalized probes were prepared and the results are shown in Fig. 4. The Ab_2 functionalized probes are: (a) Fc labeled secondary antibody (Fc-Ab_2), (b) GOD and Fc-Ab_2 multilabeled nano-TiO_2 nanospheres bioconjugates (GOD/Fc-Ab_2/nano-TiO_2), and (c) GOD/Fc-Ab_2/nano-Au/TiO_2. To control the reaction condition, the same batch of immunosensors was incubated with the 50, 100, and 200 pg/mL ProGRP concentration, respectively, and then variously labeled probes were utilized as multi-labeled bioconjugates. It can be found that the GOD/Fc-Ab_2/nano-Au/TiO_2 offers highest oxidation peak currents than that obtained for the Fc-Ab_2 and GOD/Fc-Ab_2/nano-TiO_2. As shown in Fig. 4(D), the experiment results confirmed the nano-Au/TiO_2 can immobilize more Fc-Ab_2 and GOD than TiO_2 nanoparticles. More importantly, the attached GOD exhibits efficient catalysis towards glucose, illustrating the good biological activity.

3.4. Optimization of main experimental conditions

The goal of this study was to control the optimal experimental conditions to obtain the excellent performance of the proposed immunosensors for 100 pg/mL ProGRP detection. Several key parameters, such as the glucose concentration, pH of the detection solution, and incubation time were optimized by CVs. The amount of glucose in the detection solution is the key factor influencing the response of the immunosensor. SFig. 1(A) shows the relation between the oxidation peak currents and the glucose concentration. It can be seen that with the augment of glucose, the oxidation peak currents increased dramatically because the electrochemical catalysis of GOD. Then the trend gradually slowed down till the glucose up to 1.8 mM indicating that the saturation concentration had reached. Therefore, the optimum concentration of glucose was 1.8 mM.

As the pH of the detection solution might influence not only the electrochemical performance of ferrocene, but also the activity of GOD and immobilized immunoproteins, the pH dependence of the voltammetric response was investigated over the pH values from 5.0 to 9.0 in the detection solution containing 1.8 mM glucose (SFig. 1(B)). It was found that the immunosensor displayed an optimum

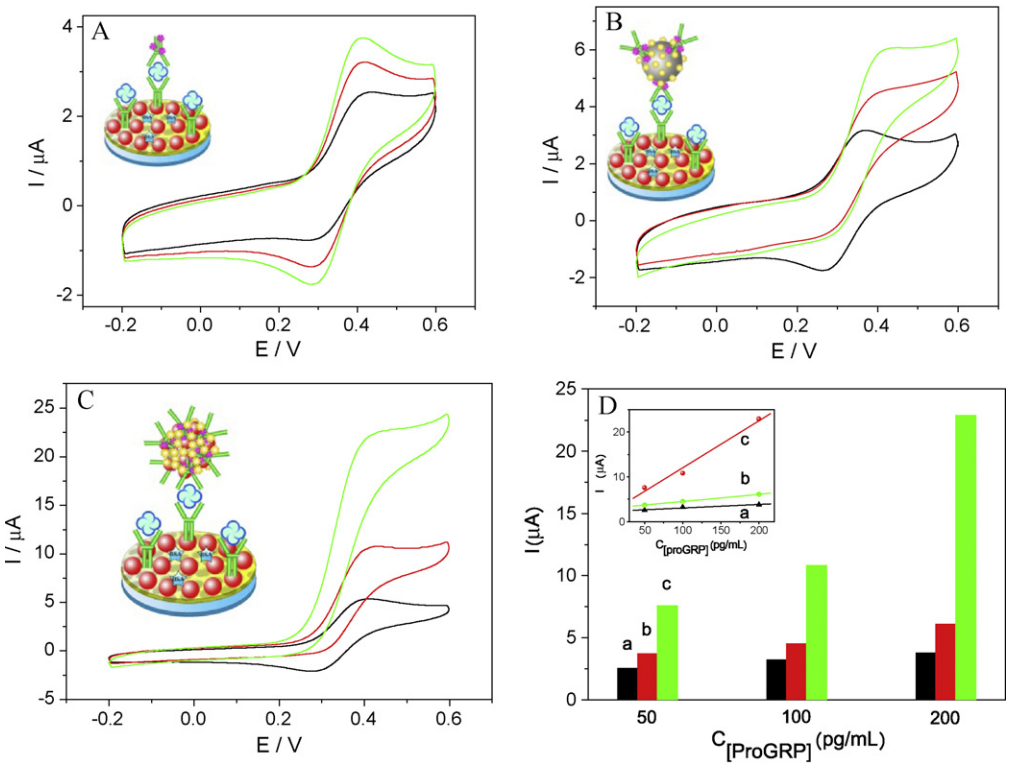

Fig. 4. The CVs after the sandwich immunoreaction of the resulted immunosensor with variously labeled probes. (A) Fc-Ab_2, (B) GOD/Fc-Ab_2/nano-TiO_2, and (C) GOD/Fc-Ab_2/nano-Au/TiO_2. All of them were detected in the presence of 1.8 mM glucose in working buffer at 50 mV/s. (D) showed the voltammetric responses *vs.* ProGRP concentration for the three labeled probes.

sensitivity of the response at pH 7.0. Thus, the optimal pH of 7.0 was chosen in the further study.

The formation of immunocomplex on electrode surface depends on the time of incubation. Thus, the voltammetric responses were recorded with the incubation time at the incubation temperature of 37 °C. The proposed immunosensors were incubated with 100 pg/mL of ProGRP with different incubation times, then 10 μL of the prepared multi-labeled nano-Au/TiO_2 nanospheres was added to form a sandwich immunocomplex for 20 min at 37 °C. Finally, the biosensors were taken into the detection solution containing 1.8 mM glucose. The results showed the maximum voltammetric response occurred at the time of 25 min (SFig. 1(C)). When the incubation time was longer than 25 min, the voltammetric response maintained a constant value, indicating a saturated binding of antibodies and antigens. Thus, the incubation time of 25 min were chosen as the optimal incubation conditions for the immunoassay.

3.5. The quantificational detection of ProGRP

The analytical performance of the prepared biosensor was explored by quantifying the standard ProGRP solution with different concentrations according to the described conditions. As expected, the voltammetric response increased with the concentration of ProGRP. The corresponding CV waves are presented in Fig. 5(A). The standard calibration curve for ProGRP detection is shown in Fig. 5(B). It can be found the linear range covered from 10.0 to 500 pg/mL with a regression equation of $y = 4.523 + 0.065x$ (pg/mL) and correlation coefficient of 0.996. The detection limit corresponding to three times the standard deviation of the blank solution was estimated as 3.0 pg/mL. In order to evaluate the non-specific fouling effect on the proposed immunosensor performances. ProGRP standard solutions were replaced by BSA standard solutions with corresponding concentrations, and the responses are displayed as control line in Fig. 5(B), which indicated no significant nonspecific adsorption of the proposed immunosensor. Furthermore, the comparison of the performances of different immunosensors which employed GOD as signal enhancer is shown in Table 1. It could be found that the proposed immunosensor exhibited satisfactory detection limit and linear range. The reasons might be that (1) the relatively large amounts of anti-ProGRP were immobilized onto nano-Au/Cys/Nf-GS multilayer, which could enhance the access chance of the antigen and antibody; (2) the employment of GOD and Fc loaded onto the Au/TiO_2 nanospheres provided strong catalytic effect and good electron transfer tunnel for reduction of glucose.

3.6. The selectivity, reproducibility, and stability of the immunosensor

To confirm that the observed voltammetric response is generated from the antibody–antigen specific interaction or nonspecific protein interaction, selectivity was investigated when the immunosensor was incubated with the samples containing the following three kinds of potential interfering substances: neuron-specific enolase (NSE), carcinoembryonic antigen (CEA, 20 ng/mL), α-fetoprotein antigen (AFP, 20 ng/mL), and BSA. The peak current responses to 10 ng/mL ProGRP standard solutions with and without interferences showed a difference of less than 5.1%, which suggests that the selectivity of the immunosensor based on the specific antigen–antibody immunoreactions was acceptable. The reproducibility of the immunosensors was evaluated by intra- and interassay coefficients of variation (CVs). The CVs of 12 immunosensors were 6.7% for a single batch and 9.2% for different batches at 10 ng/mL ProGRP. This revealed that the developed biosensor held

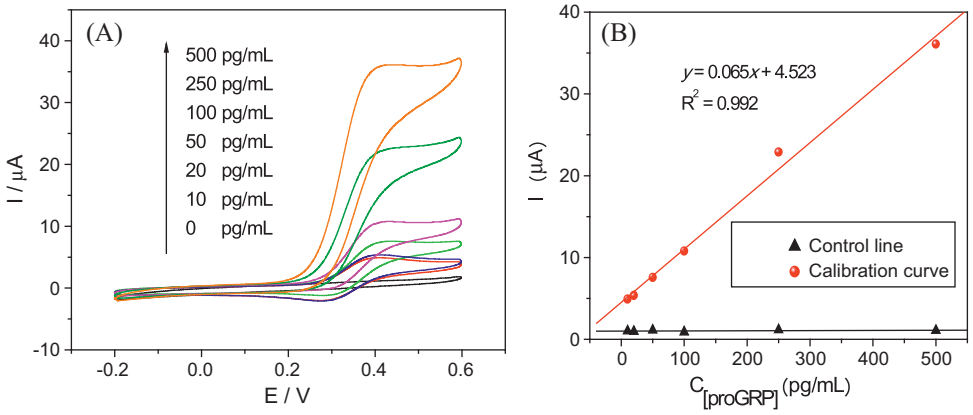

Fig. 5. The CV response of the immunosensors to different concentrations of ProGRP (A) and the calibration curve (B).

Table 1
Comparison of the performances of different immunosensors employed GOD as signal enhancer.

Detection method	Detection target	Linear range	Detection limit	Reference
CV	ProGRP	10.0–500 pg/mL	3.0 pg/mL	This work
CV	IgG	5.0×10^{-6} to 9.6×10^{-4} mol/L	5.0×10^{-7} mol/L	Tang et al. (2009)
DPV	CEA and AFP	2.5 pg/mL to 2.0 ng/mL for CEA; 2.5 pg/mL to 2.5 ng/mL for AFP	1.4 pg/mL for CEA; 2.2 pg/mL for AFP	Lai et al. (2009)
CV	DNP[a] and anti-DNP, respectively	200 nM to 2 mM for DNP; 1 μg/mL to 0.1 mg/mL for anti-DNP	–	Lee et al. (2008)

[a] Dinitrophenyl.

potentials for quantitative assay of ProGRP proteins with desirable reproducibility.

The longtime stability of the immunosensor was investigated within several weeks of storage. The immunosensors were stored at 4 °C and measured intermittently (every 3 days) in working buffer, no apparent change was found within 90 days, it yielded the RSD of 2.7%. The stored immunosensors were used to detect the 100 pg/mL ProGRP and the analytical performances did not show an obvious decline, demonstrating good bioactivity of GOD/Fc-Ab_2/nano-Au/TiO_2 bioconjugates.

3.7. Clinical application

Eleven serum samples from lung cancer patients and healthy volunteers were analyzed to evaluate performance of the proposed immunosensor in clinical analysis. The accuracy of ProGRP determination was examined by comparing the results with those from the enzyme-linked immunosorbent assay (ELISA) analysis (the results have been given in SFig. 2). The regression equation of the concentration of ProGRP obtained from the proposed system (y) and ELISA technique (x) was $y = 0.857x + 32.96$ with a coefficient of 0.992, which revealed that the proposed immunosensor is in good agreement with the conventional ELISA.

4. Conclusions

In conclusion, we have demonstrated the applicability of a sensitive voltammetric immunosensor for detection ProGRP biomarker based on nano-Au embedded Cys/GS-Nf composite as antibody immobilization matrix and Fc-Ab_2 and GOD-multifunctionalized Au/TiO_2 nanocomposites as a trace label. Due to the low background response and the improved high load amount and activity of the GOD, the sensitivity of the immunosensor for ProGRP was enhanced with a detection limit of 3.0 pg/mL, a value below the commonly accepted concentration threshold in clinical diagnosis. Besides this nano-Au/Cys/GS-Nf nanocomposite film can provide a favorable microenvironment for biomolecule and effectively keep their activities. Moreover, the large surface area, rich active sites, and together with the good electrical properties, made the Au/TiO_2 nanospheres become a promising nanomaterial for signal amplifying applications.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.02.043.

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