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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