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Triple signal amplification using gold nanoparticles, bienzyme and platinum nanoparticles functionalized graphene as enhancers for simultaneous multiple electrochemical immunoassay

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ABSTRACT

Here we demonstrated an ultrasensitive electrochemical immunoassay employing graphene, platinum nanoparticles (PtNPs), glucose oxidase (GOD) and horseradish peroxidase (HRP) as enhancers to simultaneously detect carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP). This immunosensor is based on the observation that multiple-labeled antibodies (thionine-labeled anti-CEA and ferrocene-labeled anti-AFP) recognition event yielded a distinct voltammetric peak through "sandwich" immunoreaction, whose position and size reflected the identity and level of the corresponding antigen. Greatly enhanced sensitivity for cancer markers is based on a triple signal amplification strategy. Experimental results revealed that the immunoassay enabled simultaneous determination of CEA and AFP in a single run with wide working ranges of 0.01–100 ng mL^{-1}. The detection limits reached 1.64 pg mL^{-1} for CEA and 1.33 pg mL^{-1} for AFP. No obvious cross-talk was observed during the experiment. In addition, through the analysis of clinical serum samples, the proposed method received a good correlation with ELISA as a reference. The signal amplification strategy could be easily modified and extended to detect other multiple targets.

1. Introduction

Cancer is one of the deadliest diseases for human beings. The determination of tumor markers is great important in the early diagnosis and treatment of cancers (Kulasingam and Diamandis, 2008; Ferrari, 2005). While in clinical application, as most cancers have more than one marker associated with their incidence, the determination of a single tumor marker often limits diagnostic value (Mujagić et al., 2004; Kingsmore, 2006; Freedland, 2011). Therefore, the development of simple, reliable, powerful monitoring strategies for simultaneous determination of multiple tumor markers is particularly important in clinical laboratories.

Recently the immunoassay for simultaneous determination of two or more tumor makers has been attracted much attention among the community (Chen et al., 2013; Wu et al., 2008; Chen et al., 2012; Lin and Ju, 2005). Among various measurement techniques, electrochemical immunoassay could be selected as an ideal strategy because of its portability, low cost and high sensitivity (Chikkaveeraiah et al., 2012; Li et al., 2012a). To date, the methodology to realize simultaneous multianalytes determination in electrochemistry is mainly based on spatial resolution (Lai et al., 2011; Leng et al., 2010) and multiple labels (Tang et al., 2011; Feng et al., 2012). The former mode usually requires specialized multi-working-electrode (sputter-deposited or screen-printed electrodes) or complex cutting tools to divide chips (Indium Tin Oxides) for capturing antibodies or antigens. For example, Lin et al. reported a label-free immunosensor based on modified mesoporous silica for simultaneous determination of tumor makers (Lin et al., 2011). Kong et al. introduced a branched electrode platform for simultaneous tumor makers detection based on different redox substrates (Kong et al., 2013). However, these electrodes are throwaway materials which make it be high cost and the detection sensitivity of these arrays is limited due to the nonenzymatic detection. In the latter mode, multiple-labeled antibodies are selectively bound onto the electrode surface through "sandwich" immunoreaction and the recognition event thus yield a distinct voltammetric peak, whose position and size reflect the identity and concentration of the corresponding antigens. This mode could achieve simultaneous multianalyte determination in a single run with convenient operation. Therefore, a simultaneous immunoassay for carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) based on multiple labels was carried out in this work.

Another key issue for a successful immunosensor is the signal amplification and noise reduction. New techniques using nanomaterials such as graphene (Lin et al., 2012; Liu et al., 2013a),

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magnetic particles (Peng et al., 2011), nanometallic materials (Yang et al., 2006, 2011), polymer membrane (Liu and Ma, 2013b) and enzyme (Hwang and Kim, 2005; Giannetto et al., 2011; Shang et al., 2013) are being developed to increase the sensitivity for cancer markers detection. For example, gold nanoparticles coated carbon nanotubes were used carriers to immobilize redox probe labeled antibodies for simultaneous determination of three liver cancer makers (Li et al., 2012b); Horseradish peroxidase (HRP) functionalized platinum hollow nanospheres were employed as probes for detecting CEA and AFP (Song et al., 2010); quantum dots (QDs) coated silica nanoparticles were used as labels for simultaneous detection of dual proteins by measuring the metallic component of QDs (Qian et al., 2011). However, there are two major drawbacks for these signal amplification techniques: (1) a nitrogen atmosphere for deoxygenation is necessary for removing the interference of dissolved oxygen in electrochemical assay based on HRP labels, one of the most popular labels in ELISA, which limits its clinical application; (2) harsh detection conditions including nanocrystals dissolution, high potential accumulation, and deoxygenation were required for QDs-based detection, which is not convenient for practical application.

Herein, we designed an ultrasensitive electrochemical immunosensor employing AuNPs, bienzyme and platinum nanoparticles (PtNPs) functionalized graphene as enhancers to simultaneously detect CEA and AFP. To prepare the probes, thionine (THI) labeled anti-CEA and ferrocene (Fc) labeled anti-AFP were initially conjugated on PtNPs functionalized graphene nanocomposites, respectively, and the synthesized nanocomposites were then used as carriers for HRP and GOD. With the sandwich immunoassay format, the electrochemical signals were simultaneously obtained because of the presence of different electron mediators. Because the carried GOD and HRP catalyzed the oxidation of glucose and hydrogen peroxide, the electrochemical responses were enhanced. The peak currents and positions reflected the concentration and type of the corresponding antigens. Such a system well combines the simplicity of a single electrode platform and the signal amplification without deoxygenation. This assay approach could be modified and extended to the detection of other multiple targets.

2. Experimental

2.1. Reagents and materials

Mouse monoclonal anti-CEA and anti-AFP, CEA and AFP was purchased from Linc-Bio Company (Shanghai, China). Human immunoglobulin G (IgG) was purchased from Chengwen Biological Company (Beijing, China). Graphene oxide was obtained from JCNANO (Nanjing, China). Chloroplatinic acid ($H_2PtCl_6 \cdot 6H_2O$), thionine acetate (THI), sodium borohydride ($NaBH_4$), Ferrocenecarboxylic acid (Fc), Glucose, ascorbic acid (AA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Alfa Aesar. Urea acid (UA) and albumin from bovine serum (BSA) were obtained from Beijing Chemical Reagents Company (Beijing, China). poly(ethylene imine) (PEI) was from Sigma-Aldrich. Clinical serum samples were available from Capital Normal University School Hospital. All the reagents were analytical grade and used as received.

2.2. Apparatus

Images of the nanomaterials were taken via a Hitachi (H7650, 80 kV) transmission electron microscope. X-ray photoelectron spectroscopy (XPS) analysis was carried on an Escalab 250 X-ray Photoelectron Spectroscope (Thermofisher, American) using an Al (mono) Kα radiation. All electrochemical experiments were measured on a CHI1140 electrochemical workstation (Shanghai, China). A three-electrode electrochemical cell was composed of a modified glass carbon electrode (GCE, Ø=4 mm) as the working electrode, a platinum wire as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode.

2.3. Preparation of reduction graphene oxide (rGO)

To prepare rGO, 10 mL stable dispersion of graphene oxide (1 mg mL^{-1}) was mixed with 1 mL PEI (3%) and heated under reflux at 135 °C for 3 h. The obtained black dispersion was washed three times and collected by centrifugation. The final product was re-dispersed in 10 mL ultrapure water for further use.

2.4. Preparation of PtNPs functionalized graphene nanocomposites (PGN)

The PGN were synthesized using $NaBH_4$ reduction method. Briefly, 1 mL H_2PtCl_6 (1%) solution was added into 5 mL rGO dispersion and vigorously stirred for 5 min to make the negatively charged $PtCl_6^{2-}$ ions adsorbed on the rCO surface. Then, 2.5 mL $NaBH_4$ solution was dropped into the mixture with stirring for 30 min. After several centrifugations and washings with Tris–HCl (pH=9.0), the PGN was obtained and re-dispersed into ultrapure water prior to use.

2.5. Preparation of redox probe labeled antibodies, GOD and HRP functionalized PGN nanocomposites (PGN-$Ab_{1/2}$)

Firstly, EDC and NHS were used as coupling agents to modify the antibodies with redox probe by the formation of an amide link between the carboxyl of Fc and the amino of anti-AEP or between the amino of THI and the carboxyl of anti-CEA. The obtained redox probe labeled antibodies were centrifuged at 4 °C and washed several times with PBS (pH 7.3).

Secondly, the modified antibodies were added into the PGN colloid followed by incubation at 4 °C for overnight. After centrifugation, the obtained PGN-$Ab_{1/2}$ nanocomposites were totally washed three times with PBS (pH 7.3) to remove the uncombined redox probes.

Finally, 1 mg GOD and 1 mg HRP were dissolved into the PGN-$Ab_{1/2}$ (1 mg mL^{-1}) to block the unspecified sites and prevent non-specific adsorption. After several centrifugations and washing, the synthesized PGN-$Ab_{1/2}$ carrying GOD and HRP were dispersed in PBS and store at 4 °C.

2.6. Preparation of multiple electrochemical immunoassay

The GCE (Ø=4 mm) was polished repeatedly using alumina powder and then thoroughly cleaned before use. After that, the electrode was immersed in 20 mL 1% $HAuCl_4$ aqueous solution and a constant potential of −0.2 V was applied for electrochemical deposition to obtain AuNPs. Then the GCE/AuNPs was soaked into the mixed solution containing 200 ng mL^{-1} anti-CEA and anti-AFP for 12 h at 4 °C, and BSA (1%) was employed to block possible remaining active sites and avoid the non-specific adsorption. After every step, the modified electrode was thoroughly cleaned with PBS. The as-prepared GCE/AuNPs/anntibodies were stored at 4 °C prior to use.

2.7. Electrochemical detection of CEA and AFP

All electrochemical measurements were performed at room temperature in 0.1 M pH 6.5 PBS solutions. The square wave voltammetry (SWV) scan was taken from −0.6 V to 0.6 V with a frequency of 15 Hz and a pulse amplitude of 25 mV (vs SCE). When