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Dual signal amplification of horseradish peroxidase functionalized nanocomposite as trace label for the electrochemical detection of carcinoembryonic antigen

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ABSTRACT

In this study, a novel tracer, horseradish peroxidase (HRP) functionalized nanocomposites, was designed to label the signal antibodies for the detection of carcinoembryonic antigen (CEA) using an electrochemical immunosensor. To construct high-performance immunosensor, gold nanoparticles (AuNPs) dotted thionine functionalized carbon nanotubes (Thi-CNTs), which could increase the surface area, was used to immobilize the primary antibody (Ab_1). The preparation of the HRP functionalized nanocomposites and labeling of signal antibody were performed by one pot assembly of HRP and signal antibody on AuNPs onto polyaniline (PAN) functionalized carbon nanotubes (AuNPs-PAN@CNTs). The high content HRP on the surface of the AuNPs-PAN@CNTs amplified the detectable signal for sandwich-type immunoassay. Electrochemical impedance spectra and cyclic voltammetry are used to investigate the assemble process of the immunosensor. Furthermore, differential pulse voltammetry was utilized to monitor the response signal of immunosensor. Under the optimal conditions, the immunosensor displayed an excellent linear response for the detection of CEA ranging from 0.02 to 80.0 ng mL^{-1} with a detection limit of 0.008 ng mL^{-1} (at 3 σ). In addition, the immuosensor was applied to serum samples, and the results obtained were agreement with the reference values.

1. Introduction

As is well known, the cells or the organ can release specific proteins into the circulation system during tumors developing. The levels of these proteins in serum are associated with the stages of tumors. They can be used as tumor markers for screening and clinical diagnosis of cancer. Therefore, reliable and sensitive tests methods of tumor markers are very important in early clinical diagnosis [1,2]. Immunoassay, a promising approach for selective and sensitive analysis, has recently gained increasing attention in the quantitative detection of tumor markers and screening of cancers. Compared with other immunological methods including fluorescence, chemiluminescence, surface-plasmon resonance, or quartz crystal microbalance, electrochemical methods have attracted considerable interest due to their high sensitivity, low cost and good selectivity. So far, different electrochemical immunosensors, particularly amperometric immunosensors, have been developed for the determination of tumor markers [3–7]. Usually, enzyme-labeled second antibodies were utilized for improving sensitive of immunoassays due to the amplification of the measured product. However, enzyme molecules and signal antibody are labeled using usually a ratio of 1:1 in the traditional immunoassays for sterical reasons, resulting in the detectable signal is limited [8,9].

In order to achieve highly sensitive immunosensors, various signal amplification strategies have been developed. One of the most popular strategies is using functionalized nanomaterials as tracers to load a large amount of enzymes [10]. To date, nanomaterials including carbon nanotube [11,12], carbon nanosphere [13] and gold nanoparticles [14,15] have been used as nanocarriers for loading multiple enzymes to amplify the signal intensity of immunosensors. For example, Fu and his coworkers have reported HRP-encapsulated silica nanoparticles as trace labels for the electrochemical detection of IgG [16]; Zhang and his coworkers have reported HRP functionalized AuNPs-graphene nanolabels for the electrochemical detection of CEA [17]; Ju and his coworkers used glucose oxidase-functionalized nanocomposites to label the signal antibodies for ultrasensitive multiplexed measurement of tumor markers [18].

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Recently, polymeric nanocomposites (PNCs), which has good stability and abundant sites to bind biomolecules [19,20], have been attracted considerable attention in sensing field. Among PNCs, the nanocomposites of PAN with AuNPs or carbon nanotubes have been applied as a promising matrix to improve the sensitivity of the biosensor. For example, the nanocomposites of PAN@CNTs have been used in glucose biosensor and obtained higher sensitivity [21]. Authors explained the reason of the higher sensitivity is that the carbon materials can reinforce the stability of PAN and provide more active nucleation sites [22]. The nanocomposites of PAN@AuNPs were also used in electrochemical DNA biosensor, which enhanced loading of the DNA probe and detection sensitivity [23]. Inspired from the advantage of the nanocomposites of PAN @ CNTs or PAN @AuNPs, we designed three-component composites of AuNPs-PAN@CNTs as trace label, which could combine together the good properties of the nanomaterial and polymer, to fabricate high sensitive electrochemical CEA immunosensor. In this study, the synthesized nanocomposites of AuNPs-PAN@CNTs, which have good biocompatibility and high electrical conductivity, were employed as carrier for HRP and signal antibody immobilization. Dual signal amplification from high content HRP and AuNPs-PANI@CNTs nanocomposites in trace was produced in the sandwich-type immunoassay. Under the optimal conditions, the response signal of the immunsensor was linear with the concentration of CEA ranging from 0.02 to 80.0 ng mL^{-1}, the detection limit is 0.008 ng mL^{-1}.

2. Experimental

2.1. Materials

Carcinoembryonic antigen (CEA), CEA antibody (anti-CEA) and α-fetoprotein (AFP) were purchased from Biocell Biotech. Co., Ltd (Zhengzhou, China) and stored in refrigerator at 4 °C. Aniline was obtained from Accela ChemBio Co., Ltd. (Shanghai, China) and was distilled under reduced pressure before use. Horseradish peroxidase (HRP), thionine and bovine serum albumin (BSA) were purchased from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Multiwalled carbon nanotubes with carboxylic acid groups (CNTs, purity >95%, diameter: 20-30 nm, length: 10-30 μm) were purchased from Nanoport Co. Ltd (Shenzhen, China). Hydrochloric acid (HCl), ammonium persulfate ($(NH_4)_2S_2O_8$, APS), hydrogen peroxide (H_2O_2) and chloroauric acid ($HAuCl_4{\cdot}4H_2O$) were obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). *N*-hydroxysulfosuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Alfa Aesar China Co., Ltd. (Tianjin, China). Phosphate buffer saline (PBS) with various pH values were prepared by mixing the stock solutions of 0.10 mol L^{-1} Na_2HPO_4, 0.10 mol L^{-1} NaH_2PO_4 and 0.10 mol L^{-1} KCl. The washing buffer was pH 7.0 PBS containing 0.05% (W/V) Tween (PBST); Blocking solution was 1% BSA. The clinical serum samples were from the clinical laboratory of the Yiji Shan Hospital (Wuhu, China). Twice-quartz-distilled water was used through the study.

2.2. Apparatus

All electrochemical measurements were performed on a CHI 650C electrochemical analyzer (CH Instruments Inc., China) with a conventional three-electrode system composed of a platinum auxiliary, an silver-silver chloride (Ag/AgCl) (with 3.0 mol L^{-1} KCl) reference, and a bare glassy carbon electrode (GCE) or modified electrode working electrode. All potentials in this work are referenced to the Ag/AgCl.

Electrochemical impedance spectra (EIS) were performed in 0.1 mol L^{-1} PBS containing 5.0 mmol L^{-1} $[Fe(CN)_6^{3-/4-}]$ and 0.1 mol L^{-1} KCl at a pH of 7.4. The frequency ranged from 0.1 to 100 kHz at a formal potential of 0.18 V, and the amplitude of the alternate voltage was 5 mV.

Morphologies of AuNPs-PAN@CNTs and CNTs were obtained by scanning electron microscopy (SEM) using a JEOLJSM-6700F microscope (Hitachi, Japan).

2.3. Preparation of Thi-CNTs nanocomposites

Firstly, CNTs were treated in a 3:1 H_2SO_4/HNO_3 for 12 h at 60 °C [24]. The resulting dispersion were then filtered and washed repeatedly with water until the pH was about 7.0. This procedure shortened CNTs, removed metallic and carbonaceous impurities, and generated carboxylate groups on its surfaces. Next, the carboxylated CNTs were activated for 6 h with NHS/EDC (10 mmol L^{-1}). Subsequently, 10 mg the activated CNTs were added into 12.5 mL of 0.5 mmol L^{-1} thionine and stirred for 24 h. Thus, thionine were covalently bonded to the carboxylate groups of CNTs. The products were washed with water and dried. Finally, 5.0 mg thionine functionalized CNTs (Thi-CNTs) was re-dispersed ultrasonically in 1.0 mL of 0.02% chitosan.

2.4. Preparation of the AuNPs

AuNPs was prepared by reduction of $HAuCl_4{\cdot}4H_2O$ with trisodium citrate methods [25]. Briefly, 50 mL solution of $HAuCl_4$ (0.01%) was brought to the boil, and then 2.0 mL of 1% sodium citrate solution was added, stirring vigorously. The mixed solution was kept boiling for 15 min until the solution became wine red, indicating the formation of AuNPs. Finally, the solution was cooled to room temperature while stirring. The diameter of the AuNPs of about 16 nm was measured by TEM (Seen in supplementary Fig. S1). The AuNPs was stored in dark glass bottles at 4 °C for further use.

2.5. Preparation of HRP labeled signal antibody nanocomposites

Firstly, the PAN@CNTs nanocomposites were prepared according to previous reported protocol [26]. Briefly, 0.5 g CNTs and 0.025 g aniline monomers was sequentially added into 100 mL HCl (1.0 mol L^{-1}), and reacted for 1.5 h in an ice bath. Next, 0.025 g $(NH_4)_2S_2O_8$ was added into the mixture and kept for 6 h. Subsequently, 0.05 mL acetone was added to terminate the aniline polymeric reaction. The products obtained were washed with deionized water and dried under vacuum at 80 °C for 24 h. Thus, PAN@CNTs nanocomposites were obtained.

Secondly, PAN@CNTs nanocomposites were dispersed in 10.0 mL AuNPs solution (1.0 mg mL^{-1}) and stirred for 12 h. During this process, the AuNPs were assembled on the surface of PAN@CNTs nanocomposites via electrostatic interaction (the NH^+ groups of the PAN backbone and negative charged AuNPs). At the meantime, the assembled AuNPs could be controlled by adjusting the concentration of PAN@CNTs.

Thirdly, 1.0 mg of AuNPs-PAN@CNTs nanocomposites were re-dispersed ultrasonically in 2.0 mL of pH 7.0 PBS. Next, 500 μL Ab_2 (2.0 μg mL^{-1}) and 300 μL HRP (0.5 mg mL^{-1}) were injected into the dispersion above and kept it for 2 h. Subsequently, 100 μL 1% BSA was injected into dispersion above to block possible remaining active site and avoid the non-specific adsorption. Finally, the mixture was centrifuged at 15000 rpm for 20 min at 4 °C, and the precipitation was rinsed with washing buffer several times to remove free Ab_2 and HRP. Prior to use, this bioconjugates were stored at 4 °C. In contrast to experiment, HRP-Ab_2-AuNPs-PAN,