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Dual amplification strategy for the fabrication of highly sensitive amperometric immunosensor based on nanocomposite functionalized interface

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ABSTRACT

An effective electrochemical signal amplification strategy based on bilayer enzyme membrane modification and redox probe immobilization was proposed to construct an amperometric immunosensor. At first, porous deposited gold nanocrystals (DpAu) film was performed on bare glass carbon electrode by electrochemical reduction of gold chloride tetrahydrate ($HAuCl_4$) solution and then Prussian blue (PB) was electrodeposited on the surface of DpAu film, which acted not only as redox probe but also as the first amplification membrane layer. Following that, gold colloidal nanoparticles doped chitosan–iron oxide nanocomposite was coated on the surface of PB/DpAu film to avoid the leakage of PB and assemble antibody biomolecules. Finally, horseradish peroxidase (HRP) as the second enzyme membrane was employed to block the possible remaining active sites and avoid the nonspecific adsorption. With the excellent electrocatalytic properties of PB and HRP to the reduction of hydrogen peroxide (H_2O_2), the dual amplification of antigen–antibody interaction and the enhanced sensitivity could be achieved. Under the optimal conditions, the linear range of the proposed immunosensor for the determination of α-1-fetoprotein (AFP) was from 0.05 to 300 ng/mL with a detection limit of 0.02 ng/mL (S/N = 3). Moreover, the immunosensor exhibited good selectivity, stability, reproducibility and regeneration.

1. Introduction

The increased levels of tumor markers in human serum are associated in patients with certain tumors. Therefore, the quantitative detection of tumor markers plays an important role in screening for a disease, in diagnosing a disease, and in determining the prognosis of a disease and are absolutely necessary in clinical assay [1]. The technique used for quantitative determination of tumor markers is usually immunoassay based on highly biospecific recognition interactions between antigen and antibody, which has been successfully applied to many fields such as clinic diagnostics, biotechnology, environmental analysis and food industry etc. Immunosensor as an immunoassay technique is of great interest because of their potential utility as specific, simple, direct detection techniques without need for separation and washing steps and the reduction in size, cost, and time of analysis. In general, there are three types of immunosensor detection devices: electrochemical (amperometric, potentiometric, capacitative or impedance etc.), optical (fluorescence, luminescence, refractive index or surface plasmon resonance etc.), and microgravimetric (quartz crystal microbalance etc.) devices [2]. Among these immunosensors, the amperometric immunosensor is especially promising for its relatively low detection limit and high sensitivity [3,4].

Since most immune protein analytes are not intrinsically able to act as redox partners in an electrochemical reaction, the amperometric immunosensor involves in the immobilization of the indicators with electrochemical redox activities—redox probe on the electrode surface or addition of the redox probe into the analyte solute, such as ferrocene and its derivatives, dyes, potassium hexacyanoferrate etc. [5–7]. To avoid the pollution of the analyte solution, the immobilization of redox probe on the electrode surface has been major strategy to prepare amperometric immunosensor [8]. However, the main problem was that the immobilized redox probe might be leakage from the electrode since most of them were soluble [9]. One promising approach is to coat a substance with excellent biocompatibility and film-forming ability on the substrate to avoid leakage. Prussian blue (PB), a ferric hexacyanoferrate (II) complex, is a classical prototype possess redox activity [10] and is called as the "artificial peroxidase" due to the excellent and selective electrocatalyst for the reduction of hydrogen peroxide (H_2O_2) even in the presence of oxygen [11–13]. In our present work, PB was electrodeposited on the modified electrode to form an electroactive layer as redox probe for the immunosensor and amplify the electrochemical signal via electrocatalytic action for the reduction of H_2O_2.

In the process of immunosensor design, immobilization of biomolecules on the sensing electrode surface has been consid-

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ered to be one of the most important points [14] and so there have been many reports about immunosensor based on different immobilization matrix. Due to its excellent film-forming ability, mechanical strength, biocompatibility, nontoxicity and the presence of reactive amino and hydroxyl functional groups for biomolecules immobilization, chitosan (CS) has been used to construct biosensor. Considering its relatively poor conductivity, many attempts had been made to improve the disadvantage of CS by combining with carbon nanotubes, metal nanoparticles or metal oxide nanoparticles to form nanocomposite for the fabrication of biosensing platform [15]. Iron oxide (Fe_3O_4) nanoparticles, due to their unique properties including biocompatibility, superparamagnetic property, low toxicity and high electron efficiency, could provide a favourable microenviroment for biomolecules immobilization. Furthermore, magnetic nanoparticles as carrier of biomolecule is of great of interest because it is likely to improve delivery and recovery of these biomolecules for biomedical applications. However, the problem of aggregation due to high surface area and magnetic dipole–dipole interactions between particles had limited the application of Fe_3O_4 nanoparticles to biosensing. This problem could perhaps be overcomed by modifying Fe_3O_4 nanoparticles using inorganic semiconductors, conducting polymers or biopolymers etc. [16]. So CS–Fe_3O_4 nanocomposite have recently aroused much interest for the fabrication of biosensor since surface functionalization of magnetic nanoparticles allows biomolecules immobilized on their surface by covalent attachment, self-assembly or embedment method. Lin and Leu [17] have fabricated CS–Fe_3O_4 nanocomposite-based chemical sensor for cathodic determination of hydrogen peroxide. Kaushika et al. have reported glucose biosensor [16] and immunosensor for ochratoxin-A [18] based on CS–Fe_3O_4 nanocomposite. Wang and Tan [19] have reported amperometric immunosensor based on CS–Fe_3O_4 nanocomposite film for estimation of ferritin.

On the other hand, gold colloid nanoparticles (GNPs) play an important role in the field of biosensor because they possess excellent electron transfer efficiency, large specific surface area and strong adsorption ability to biomolecules such as enzyme and antibody (antigen), with well-retained bioactivities and give an environment similar to that of biomolecules in native systems [20,21]. Moreover, GNPs have been reported strongly bound to various functional groups through covalent bonds, including –CN, $–NH_2$, or –SH. Based on the above observation, the gold colloid nanoparticles doped chitosan–iron oxide (CS–Fe_3O_4–GNPs) nanocomposite was synthetized to immobilize biomolecules via the large specific area and strong adsorption ability of GNPs and further enhance the conductibility of CS–Fe_3O_4 nanocomposite in our present work. In recent years, the porous deposited gold nanocrystals (DpAu) as another nanometer-structured gold particles, which were formed by electrochemicall reduction of gold chloride tetrahydrate ($HAuCl_4$) solution, has also been employed to fabricate immunosensor. It could provide a porous and stable surface for the immobilization of biomolecules. Moreover, high specific area of the DpAu layer can amplify the final sensitivity of original flat surface device [22–24].

Usually, most of amperometric immunosensor are based on the amplication of enzyme-label of either antibody or antigen [25,26]. However, the labeling amperometric immunosensor with long preparation time and more reaction steps were needed. In this study, a novel strategy for improving the sensitivity without sophisticated enzyme-label procedure, which employed horseradish peroxidase (HRP, $M_w \approx 44{,}000$) instead of bovine serum albumin (BSA, $M_w \approx 66{,}000$) as blocking agent and "artificial peroxidase"—PB as redox probe, was developed to construct the immunosensor. The assay format avoids the addition of redox probe into the solution and the separation step, which significantly simplifies the immunoassay procedure and shortens assay times. Furthermore, the combination of PB and HRP could result in a dual amplication effect because of their excellent catalytic activity for the reduction of H_2O_2. Another immunosensor employing BSA as blocking agent was also devised to make comparisons. The proposed immunosensor exhibited good accuracy, higher sensitivity and a wider linear range with a lower detection limit. Details of the preparation, characterization, optimal conditions and possible application of immunosensor were investigated as follows.

2. Experimental

2.1. Reagents and materials

AFP and anti-AFP were purchased from Biocell Co. (ZhengZhou, China). Gold chloride tetrahydrate, sodium citrate, Bovine serum albumin (BSA, 96–99%) and HRP were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $K_3Fe(CN)_6$ and $FeCl_3{\cdot}6H_2O$ were purchased from Chemical Reagent Co. (Sichuan, China). Hydrogen peroxide (30%, w/v, solution) was obtained from Chemical Reagent Co. (Chongqing, China). All chemicals and solvent used were of analytical grade and were used as received without further purification. Double distilled water was used throughout all experiments. Phosphate-buffered solution (PBS, 0.025 M) with various pH values were prepared with stock standard solution of K_2HPO_4 and KH_2PO_4. The supporting electrolyte was 0.1 M KCl.

The AFP was stored at 4 °C, and its standard solutions were prepared with PBS when in use. A stock solution of 1 wt% CS was prepared by dissolving 1 g CS flakes in 100 mL 1% HAc by stirring for some time at room temperature. Gold colloidal nanoparticles with mean size of 16 nm (the graph not shown) were prepared by reducing gold chloride tetrahydrate with sodium citrate at 100 °C for half an hour [27].

2.2. Apparatus

Cyclic voltammetric (CV) measurements were performed using a CHI 600A electrochemistry workstation (Shanghai CH Instruments Co., China). A three-compartment electrochemical cell contained a modified glassy carbon electrode (GCE, $\phi = 4$ mm) as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as a reference electrode. All potentials were measured and reported versus the SCE. 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). The morphology of porous DpAu film was studied by means of scanning electron microscopy (SEM, S-4800, Hitachi, Japan). The size and morphology of nanomaterials was estimated from transmission electron microscope (TEM, TECNAI 10, Philips Fei Co., Hillsboro, OR). The electrochemical impedance spectroscopy (EIS) measurements were carried out with a Model IM6e (ZAHNER Elektrick Co., Germany).

2.3. Preparation of gold colloidal nanoparticles doped chitosan–iron oxide nanocomposite (CS–Fe_3O_4–GNPs)

The Fe_3O_4 nanoparticles were first prepared by the co-precipitation of Fe^{2+} and Fe^{3+} under a base condition according the literature with slight modification [28]. 10.0 mL of iron ion solution containing Fe^{2+} and Fe^{3+} with the mole proportion 1:1.75 was added dropwise into 100 mL 6 M NaOH under vigorous mechanical stirring for 30 min at 80 °C. The Fe_3O_4 nanoparticles were collected with a powerful magnet and washed several times with distilled water followed by ethanol and kept in a vacuum oven at 70 °C for drying. Fig. 1a was the TEM image of Fe_3O_4 nanoparticles and the mean size was about 20 nm.