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Amplification of antigen–antibody interactions via back-filling of HRP on the layer-by-layer self-assembling of thionine and gold nanoparticles films on Titania nanoparticles/gold nanoparticles-coated Au electrode

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Abstract

A novel amperometric immunosensor with amplified sensitivity for the determination of carcinoembryonic antigen (CEA) has been developed based on the assembly of $\{Thi^+/nano\text{-}u\}_n$ layer-by-layer films by alternate adsorption of negatively charged gold nanoparticles (nano-Au) and positively charged thionine (Thi^+) on Titania nanoparticles/gold nanoparticles composite film formed previously on the electrode *via* self-assembly and deposition method, which provided an interface to assemble carcinoembryonic antibody (anti-CEA). Subsequently, HRP was backing-filled into the CEA-modified electrode surface to amplify the response of the antigen–antibody interactions. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used to monitor and confirm the films growth. Assay conditions were optimized including the incubation temperature and time, the pH of working buffer, the concentration of the H_2O_2 and the electrodeposition time of nano-Au, etc. The reduction current of the immunosensor decreases linearly in the range of CEA from 0.2 to 80.0 ng/mL with a detection limit of 0.07 ng/mL in presence of 0.55 mM H_2O_2 in working solution. Moreover, the immunosensor showed acceptable reproducibility, high sensitivity and long-term stability. Clinical serum samples were assayed with this method and the results were in acceptable agreement with those obtained from the enzyme-linked immunosorbent assays (ELISAs). Therefore, the platform that combines the advantages of nanostructured materials with those of the layer-by-layer self-assembling technique opens the doors to the new and exciting possibilities for the development of immunosensor using different transduction modes.

Keywords: Amperometric immunosensor; CEA; Gold nanoparticles; Horseradish peroxidase; Layer-by-layer; Titania nanoparticles

1. Introduction

Carcinoembryonic antigen (CEA), a glycoprotein with a molecular weight of 180–200 kDa, is one of the most widely used tumor markers. As well known to us, it has been found to exist in the serum of most tumor patients. The serum CEA may be elevated in neoplastic diseases of the lung cancer [1,2], breast cancer [3,4], colon cancer [5], ovarian carcinoma [6,7], etc. Thus, the determination of CEA in serum is very essential to clinical tumor diagnoses.

The immunoassays, based on the measurement of antigen–antibody reactions, have been successfully applied to many fields including food industry, environmental monitoring, biotechnology, pharmaceutical chemistry and clinical diagnostics [8–14]. Many immunoassay methods, such as chemiluminescence immunoassay, radioimmunoassay, fluoroimmunoassay, immuno-turbidimetry and ELISAs, etc., are successfully to meet the increasingly analytical needs but involve the radiation hazards, the complicated wash procedure and expensive instruments [15,16]. These limit the application of those immunoassay techniques

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and make them unfit for fast determination of the analyte. The electrochemical amperometric immunosensors, which combine their simple pretreatment procedure, fast analytical time, precise and sensitive current measurement, and inexpensive and miniaturizable instrumentation, have gained considerable attention [17,18]. Usually, most of amperometric immunoassay techniques are based on the amplification of enzyme-label of either antigen or antibody. Recently, a new strategy for improving the sensitivity of an immunosensor without the sophisticated fabrication procedure *via* back-filling immobilizing of HRP onto the nanoparticle monolayer has been reported in our laboratory [19–21].

Advanced material based on inorganic nanoparticles is currently one of the key research fields of today's materials science. They represent significant fundamental and commercial interests with a wide range of application including optics, electronic and sensors [22,23]. Among these nanomaterials, nano-Au and Titania nanoparticles (nano-TiO_2) have been widely used to construct biosensor due to their excellent ability to immobilize biomolecules such as proteins, enzymes and antibodies [21–28] and at the same time retain the biocatalytic activities of those biomolecules. The layer-by-layer (LBL) assembly of these nanoparticles [29,30] is based on electrostatic alternate adsorption of oppositely charged compounds which was originally developed by Decher and Hong [31], regarded as one of the most convenient, simple and quite universal methods for the construction of ultrathin organized multilayers and opens broad perspectives both in research and in practical applications.

Taking account of the advantages of LBL, nano-Au and nano-TiO_2, the combination of LBL, nano-Au and nano-TiO_2 will be promising. Therefore, we use nano-TiO_2 as a new and effective stabilized agent for immobilization of nano-Au. A nano-Au/nano-TiO_2 composite film was prepared by means of electrodepositing nano-Au onto the nano-TiO_2 modified gold electrode. After deposition, Thi^+ was chose to LBL assembly with nano-Au to form $\{Thi^+/\text{nano-Au}\}_n$ multilayer films, which is based on the electrostatic interaction between positively charged Thi^+ and negatively charged nano-Au monolayer. Subsequently, nano-Au monolayer was used to enlarge valid electrode surface area and to attach anti-CEA, and eventually HRP was used to block the possible remaining active sites of the nano-Au monolayers against the non-specific adsorption binding, which amplified the current signal and improved the sensitivity of the amperometric immunosensor. The formation of multilayer film was characterized by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The electrochemical behaviors and factors influencing the performance of the resulting immunosensor such as the incubation temperature and time, the pH of working buffer, the concentration of the H_2O_2 and the electrodeposition time of nano-Au were investigated in detail. The proposed immunosensor exhibited good accuracy, high sensitivity and long-term stability, which made it to determine CEA in serum samples with satisfactory results. Moreover, the fabrication scheme avoided the addition of an electron transfer mediator to the solution and the separation step. The design of the immunosensor significantly simplified the immunoassay procedure and shortened assay times.

2. Experimental

2.1. Reagent and materials

Anti-CEA and CEA were purchased from Biocell Company (Zhengzhou China). Chloroauric acid ($HAuCl_4$), thionine, bovine serum albumin (BSA, 96–99%) Titania nanoparticles (nano-TiO_2, 5%), horseradish peroxidase (HRP, EC 1.11.1.7, $RZ > 3.0$, $A > 250$ U/mg) and sodium citrate were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents used were of highest quality available and purchased from regular sources. For solution, deionized water with a specific resistance over 18 $M\Omega\,cm^{-1}$ was used throughout this study. Phosphate buffered solutions (PBS) (containing 0.1 M KCl, pH 7.0) were prepared using 0.02 M Na_2HPO_4 and 0.02 M KH_2PO_4. HAc-NaAc buffer solution (0.05 M, containing 0.1 M KCl) at various pH values were prepared by mixing the stock solutions of HAc and NaAc, and then adjusting the pH with NaOH and HAc. All reagents were brought to room temperature before use. Gold nanoparticles were produced by reducing $HAuCl_4$ with sodium citrate at 100 °C for half an hour [32,33]. The mean size of the prepared Au colloids was about 16 nm, estimated by transmission electron microscopy (the graph not shown). The CEA was stored in the frozen state, and its standard solutions were prepared daily with deionized water as in use.

2.2. Apparatus

Cyclic voltammetric (CV) measurements were carried out with a CHI 610A electrochemistry workstation (Shanghai CH Instruments, China). A three-compartment electrochemical cell contained a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and the modified gold electrode ($\Phi = 4$ mm) as working electrode. The size of Au colloid was estimated from transmission electron microscopy (TEM) (H600, Hitachi Instrument, Japan). 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 AC impedance of the immunoelectrode membrane was measured with a Model IM6e (ZAHNER Elektrick, Germany).

2.3. Electrode modification

The Au electrode was first polished to a mirror-like surface with 1.0, 0.3 μm alumina slurry, respectively, followed by rinsing thoroughly with deionized water. The electrodes were successively sonicated in 1:1 nitric acid, ethanol and