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Horseradish peroxidase functionalized gold nanorods as a label for sensitive electrochemical detection of alpha-fetoprotein antigen

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ABSTRACT

In this study, a novel tracer, horseradish peroxidase (HRP) functionalized gold nanorods (Au NRs) nanocomposites (HRP–Au NRs), was designed to label the signal antibodies for sensitive electrochemical measurement of alpha-fetoprotein (AFP). The preparation of HRP–Au NRs nanocomposites and the labeling of secondary antibody (Ab_2) were performed by one-pot assembly of HRP and Ab_2 on the surface of Au NRs. The immunosensor was fabricated by assembling carbon nanotubes (CNTs), Au NRs, and capture antibodies (Ab_1) on the glassy carbon electrode. In the presence of AFP antigen, the labels were captured on the surface of the Au NRs/CNTs via specific recognition of antigen–antibody, resulting in the signal intensity being clearly increased. Differential pulse voltammetry (DPV) was employed to record the response signal of the immunosensor in phosphate-buffered saline (PBS) containing hydrogen peroxide (H_2O_2) and 3,3′,5,5′-tetramethylbenzidine (TMB). Under optimal conditions, the signal intensity was linearly related to the concentration of AFP in the range of 0.1–100 ng ml^{-1}, and the limit of detection was 30 pg ml^{-1} (at signal/noise [S/N] = 3). Furthermore, the immunoassay method was evaluated using human serum samples, and the recovery obtained was within 99.0 and 102.7%, indicating that the immunosensor has potential clinical applications.

As is well known, during the development of tumors, the cells or the organs release specific proteins into the circulation system. The level of these proteins in serum is usually associated with tumor disease. Therefore, they can be used for tumor biomarkers in screening and diagnosis of cancer. Alpha-fetoprotein (AFP) is one of the most important biomarkers. Its normal level in serum is less than 25 ng ml^{-1}, and a higher level is related to liver cancer [1]. Thus, seeking a simple, accurate, and sensitive detection method for AFP is very important for clinical diagnosis. In comparison with other immunoassay methods, including fluorescence, chemiluminescence, and enzyme-linked immunosorbent assay, electrochemical immunoassay has attracted considerable attention due to its high sensitivity, low cost, and simple instrumentation. During recent decades, various electrochemical immunosensors have been reported for tumor biomarkers detection [2–6]. For example, Ju and coworkers [2] fabricated an electrochemical immunosensor based on glucose oxidase functionalized nanocomposites as a trace label. In that work, glucose oxidase and carbon nanotubes (CNTs) were used for signal amplification. As a result, the immunosensor can detect as low as 2.2 pg ml^{-1} AFP. Zhu and coworkers [3] reported a graphene-based electrochemical immunosensor for human immunoglobulin G (IgG) detection. In that work, CNTs and graphene amplified response signals and improved analytical performance of the immunosensor. Recently, Rusling and coworkers [4] published a review about nanomaterial-enhanced electrochemical immunosensors for cancer biomarker proteins and demonstrated the reason why the nanomaterials, including CNTs and graphene, amplify detectable signal and clearly improve performance of immunosensors.

Abbreviations used: AFP, alpha-fetoprotein; CNT, carbon nanotube; Au NP, gold nanoparticle; Au NR, gold nanorod; CTAB, cetyltrimethylammonium bromide; GO, graphene oxidation; HRP, horseradish peroxidase; Ab_2, secondary antibody; PSA, prostate-specific antigen; CEA, carcinoembryonic antigen; $HAuCl_4 \cdot 4H_2O$, chloroauric acid; $AgNO_3$, silver nitrate; BSA, bovine serum albumin; AA, ascorbic acid; $NaBH_4$, sodium borohydride; UA, uric acid; TMB, 3,3′,5,5′-tetramethylbenzidine; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween; TEM, transmission electron microscopy; SEM, scanning electron microscopy; CV, cyclic voltammetry; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; GCE, glassy carbon electrode; Ab_1, capture antibody; H_2O_2, hydrogen peroxide; RSD, relative standard deviation.

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Gold nanoparticles (Au NPs) have been widely employed in the sensing field due to their high surface area and biocompatibility during the past two decades [7–10]. In contrast to Au NPs, gold nanorods (Au NRs) have been widely applied in the optical sensing field due to their higher absorption and light scattering property [11,12]. However, to our knowledge, they have been little reported in the electrochemical sensing field. As is well known, the surface of Au NRs present is positively charged due to cetyltrimethylammonium bromide (CTAB) being positively charged and covered on its surface [13], and the surface of graphene oxidation (GO) present is negatively charged. So, the GO–Au NRs nanocomposites could be easily prepared via electrostatic interaction. Based on the GO–Au NRs nanocomposites as sensing interface, we reported an electrochemical DNA biosensor for sequence-specific DNA detection, and the limit of detection reached 3.5×10^{-15} M [14]. In that work, Au NRs provided good conductivity and biocompatibility and an amplified sensing signal. Based on the advantages of Au NRs, we sought to exploit its application in the immunosensor field. In the current work, we employed CNTs and Au NRs as sensing interface and designed a novel tracer, horseradish peroxidase (HRP)–Au NRs nanocomposites, to label secondary antibody (Ab_2) and developed a sensitive sandwich-type electrochemical immunosensor for AFP detection. The sandwich immunosensor obtained exhibited high sensitivity and selectivity.

Materials and methods

Reagents

Prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), AFP, and antibody were purchased from Biocell Biotechnology (Zhengzhou, China). Multiwalled CNTs with carboxylic acid groups (20–30 nm in diameter and 30 μm in length) were obtained from the Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences. Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), CTAB, silver nitrate ($AgNO_3$), bovine serum albumin (BSA), ascorbic acid (AA), and sodium borohydride ($NaBH_4$) were obtained from Sinopharm Chemical Reagent (Shanghai, China). Uric acid (UA) was purchased from Alfa Aesar (Tianjin, China). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Aladdin (Shanghai, China).

Phosphate-buffered saline (PBS) with various pH values was obtained by adjusting the ratio of the stock solutions of 0.10 M Na_2HPO_4 and 0.10 M NaH_2PO_4. Washing buffer was pH 7.0 PBS containing 0.05% Tween (PBST), and blocking solution was pH 7.0 PBS containing 1% BSA. All chemicals were of analytical grade and used without further purification. All solutions were prepared with twice-quartz-distilled water.

Apparatus

Transmission electron microscopy (TEM; Hitachi-800) was used to obtain the images of the Au NRs. Scanning electron microscopy (SEM; Hitachi JEOL JSM-6700 F, Japan) was used to obtain the morphologies of the CNTs and Au NRs/CNTs nanocomposites.

Electrochemical measurements, including cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS), were performed on a CHI 650C electrochemical workstation (Shanghai Chenhua Instruments, China). The three-electrode system consisted of a glassy carbon electrode (GCE; 3.0 mm in diameter) or the modified electrode as working electrode, a platinum wire as auxiliary electrode, and a silver/silver chloride electrode (Ag/AgCl) as reference electrode. All potential is referenced to the Ag/AgCl in this work.

All measurements were performed at room temperature in a 10.0-ml electrolytic cell with 3.0 ml of solution, from which oxygen was removed by purging with high-purity nitrogen for 10 min, and a blanket of nitrogen was maintained over the solution during the measurements.

Synthesis of Au NRs and preparation of HRP–Au NRs–Ab_2 bioconjugate

Au NRs were synthesized according to our previous protocol [13]. Simply, the gold seed was first synthesized by reducing $HAuCl_4$ with $NaBH_4$. Second, 5.0 ml of CTAB (0.20 M) was added into a mixture solution containing 5.0 ml of $HAuCl_4$ (1.0×10^{-3} M) and 0.20 ml of $AgNO_3$ solution (4.0×10^{-3} M). After that, 70 μl of AA (7.88×10^{-2} M) was injected into the mixture solution above. Subsequently, 12 μl of the gold seed was injected into the growth solution to initiate the growth of Au NRs. During the process, CTAB was assembled on the surface of Au NRs against the agglomeration of Au NRs. Excess CTAB was removed by high-speed centrifugation. Finally, the Au NRs obtained were stored in a freezer for further use.

The HRP–Au NRs–Ab_2 bioconjugate was synthesized according to the literature with a little modification [15,16]. First, 50 μl of HRP (1 mg ml^{-1}) and 50 μl of Ab_2 (5 μg ml^{-1}) were added into 1 ml of Au NRs solution and gently stirred for 3 h. After that, excess HRP and antibody were removed by high-speed centrifugation for 15 min. Next, the bioconjugate obtained was washed with washing buffer and redispersed in 1.0 ml of pH 7.0 PBS containing 1% BSA. Prior to use, this bioconjugate was kept in a refrigerator.

Fabrication of immunosensor

Before modification, the bare GCE was first polished sequentially on a microcloth with three different amounts of alumina powder (1.0, 0.3, and 0.05 μm in diameter). The residual alumina powder was then removed by ultrasonic stirring in 95% ethanol and distilled water for 5 min. Finally, the electrode was rinsed with distilled water and dried in nitrogen.

The preparation procedure of the immunosensor is illustrated in Scheme 1. First, 5.0 μl of CNTs suspensions (0.5 mg ml^{-1}) was dropped onto the surface of the cleaned GCE and dried naturally to form thin film at room temperature. Subsequently, the modified electrode was immersed in the Au NRs solution for 3 h. During the process, the positively charged Au NRs were assembled on the surface of CNTs via electrostatic interaction, and the modified electrode obtained is denoted as Au NRs/CNTs/GCE.

Next, 5.0 μl of capture antibody (Ab_1; 100 μg ml^{-1}) was injected onto the surface of Au NRs/CNTs/GCE and kept for 12 h in a refrigerator. After that, the immunosensor was incubated with 1% BSA solution for 30 min at 37 °C to block out possible remaining active sites, followed by rinsing with PBST. The immunosensor was stored in a freezer for further use.

Measurement procedure

To carry out the immunoreaction and electrochemical measurement, the immunosensor was incubated with the various concentrations of AFP for 30 min at 37 °C, followed by washing with washing buffer solution. Next, the immunosensor was further incubated with HRP–Au NRs–Ab_2 bioconjugate for another 90 min at 37 °C. Finally, it was transferred into 3.0 ml of pH 6.5 PBS containing 15 μM TMB and 20 μM hydrogen peroxide (H_2O_2). DPV was employed to record the electrochemical response of the immunosensor. The reduction peak currents at peak potential of approximately 0.25 V (vs. Ag/AgCl) were recorded for the measurement of AFP. The experiment parameters were as follows: initial potential, +0.6 V; final potential, −0.1 V; pulse amplitude, 0.05 V; pulse width, 0.08 s; sample width, 0.0167 s.