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Layer-by-layer multienzyme assembly for highly sensitive electrochemical immunoassay based on tyramine signal amplification strategy

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ABSTRACT

A new sandwich-type electrochemical immunosensor based on nanosilver-doped bovine serum albumin microspheres (Ag@BSA) with a high ratio of horseradish peroxidase (HRP) and detection antibody was developed for quantitative monitoring of biomarkers (carcinoembryonic antigen, CEA, used in this case) by coupling enzymatic biocatalytic precipitation with tyramine signal amplification strategy on capture antibody-modified glassy carbon electrode. Two immunosensing protocols (with and without tyramine signal amplification) were also investigated for the detection of CEA and improved analytical features were acquired with tyramine signal amplification strategy. With the labeling method, the performance and factors influencing the electrochemical immunoassay were studied and evaluated in detail. Under the optimal conditions, the electrochemical immunosensor exhibited a wide dynamic range of 0.005–80 ng mL^{-1} toward CEA standards with a low detection limit of 5.0 pg mL^{-1}. Intra- and inter-assay coefficients of variation were below 11%. No significant differences at the 0.05 significance level were encountered in the analysis of 6 clinical serum specimens and 6 spiked new-born cattle serum samples between the electrochemical immunoassay and the commercialized electrochemiluminescent immunoassay method for the detection of CEA.

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1. Introduction

Tumor markers, usually proteins, are produced by the body in response to cancer growth or by the cancer tissue itself. Sensitive and accurate detection of biomarkers is a fundamental requirement in the fields of modern biomedicine, clinical diagnostics and therapeutic analysis, which often offers the opportunities for the understanding of disease-related biological processes (Weston and Hood, 2004). Immunoassay based on the antibody–antigen interaction is one of the most important analytical techniques in quantitative monitoring of biomarkers due to the highly specific molecular recognition event (Lai et al., 2009). Compared with the conventional immunoassays, *e.g.* by using fluorescence, chemiluminescence, surface-plasmon resonance (SPR), and quartz crystal microbalance (QCM), electrochemical immunosensor has attracted considerable interest because of its intrinsic advantages such as high sensitivity, simple instruments and low power requirements (Li et al., 2008). Various electrochemical immunoassays and immunosensors have been developed for the determination of biomarkers (Lin et al., 2012; Das et al., 2006). However, the increasing demand for early and ultrasensitive screening of cancer biomarkers is pushing the enhancement of detection sensitivity by signal amplification strategies or coupling various detection technologies (Liu et al., 2012).

Nanomaterials provide a promising electrochemical sensing platform, because of the large surface areas for the improvement of mass transport, high loading of receptor molecules for synergistic amplification of the target response, and unique biocompatible, electronic, and catalytic properties for the translation of biorecognition events to an electrochemical response (Xu et al., 2012). In recent years, bovine serum albumin (BSA)-mediated synthesis of inorganic nanomaterials has attracted increasing attention due to their advantages of green reaction processing and multifunctionality of the products (Dickerson et al., 2008; Hu et al., 2013). Nanosilver-doped BSA microspheres (Ag@BSA) with the three-dimensional complex structure can not only keep the protein bioactivity and nanoparticle-based conducting property, but also show good stability (*e.g.*, withstanding a wide range of pH changes) and biocompability, thereby enabling them particularly suitable for biological/biomedical applications (Hu et al., 2012).

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Tyramine signal amplification (TSA) is an enzyme-mediated detection method (Toda et al., 1999). The amplification reagent, tyramine, is a phenolic derivative which can serve as a substrate of horseradish peroxidase (HRP) (Kim et al., 2002). HRP catalyzes reporter-conjugated tyramine and active tyramine deposition at the site of the enzyme reaction, accumulating large numbers of reporter molecules to enhance the signals (Bobrow et al., 1989). As a valid approach, tyramine signal amplification system has been widely applied to immunohistochemistry, and *in situ* hybridization, and more recently to cDNA, pathogen detection, and genotyping arrays (Bobrow et al., 1991; Wang et al., 2011; Anderson and Taitt, 2008; Yuan et al., 2012). To the best of our knowledge, however, there is no report focusing on electrochemical detection of tumor markers through tyramine signal amplification strategy.

Carcinoembryonic antigen (CEA) is a preferred tumor marker to help predict outlook in patients with colorectal cancer (Zhou et al., 2012a). The normal range of blood levels varies between individuals, but levels higher than 3 ng mL^{-1} are not normal. In the present work, we combine the merits of nanolabels and tyramine signal amplification strategy, and devise a new sandwich-type electrochemical immunoassay for the detection of CEA (as a model) by using Ag@BSA microspheres as the labels. Detection antibody and HRP are initially conjugated covalently onto the surface of Ag@BSA microspheres by using glutaraldehyde as the cross-linkage agent, and then the labeling microspheres are used as the trace tags for the detection of CEA on the capture antibody-functionalized probe with a sandwich-type immunoassay format. In the presence of target CEA, the carried HRP can catalyze the deposition of HRP-conjugated tyramine to form a multienzyme system on the probe, thus resulting in the amplification of electrochemical signal relative to hydrogen peroxide system. Enhanced sensitivity for the detection of CEA can be achieved by the increment of HRP loading. The aim of this work is to exploit a new signal amplification strategy for improving the analytical properties of the conventional sandwich-type electrochemical immunoassays.

2. Experimental

2.1. Materials and reagent

Monoclonal rabbit anti-human CEA antibody (anti-CEA, designated as Ab_1) and CEA standards were purchased from Biocell Biotechnol. Co., Ltd. (Zhengzhou, China). Polyclonal rabbit anti-human CEA antibody (MW: 150–200 kDa, lyophilized, designated as Ab_2) was obtained from Beijing Biosynth. Biotechnol. Co., Ltd. (Bioss, China). Silver nitrate ($AgNO_3$), hydrazine monohydrate ($N_2H_4 \cdot H_2O$), bovine serum albumin (BSA), glutaraldehyde (25 wt%), horseradish peroxidase (HRP), tyramine, β-cyclodextrin (CD) and poly-(ethylene glycol) (PEG) were purchased from Sinopharm Chem. Re. Co., Ltd. (Shanghai, China). *N*-(3-dimethyla-minopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and *N*-2-hydroxyethylpiperazine-*N*′-(2-ethanesulfonic acid) were obtained from Sigma-Aldrich (USA). All other reagents were of analytical grade and were used without further purification. Ultrapure water obtained from a Millipore water purification system ($\geq$ 18 MΩ, Milli-Q, Millipore) was used in all runs. Phosphate-buffered saline (PBS) solutions with various pH values were prepared by mixing 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4, and 0.1 M KCl was used as the supporting electrolyte. Clinical serum samples were made available by Fujian Provincial Hospital, China.

2.2. Synthesis of nanosilver-doped BSA microspheres (Ag@BSA)

Ag@BSA microspheres with ~150 nm were prepared according to the literature (Hu et al., 2012). Briefly, silver nitrate aqueous solution (5.0 mL, 50 mM) was initially added into BSA aqueous solution (10 mL, 3.0 mg mL^{-1}) under vigorous stirring at room temperature (RT), and then the mixture was vacuumized and kept static under nitrogen protection for 2 h. Following that, 0.2 mL of hydrazine monohydrate was injected into the vacuumed solution under magnetic stirring. After reaction, the resulting mixture was aged under ambient conditions for 24 h. Subsequently, the suspension was separated by centrifugation at 5000 rpm for 20 min. Finally, the collected solid state products were washed by using distilled water for three times, and dried for further use.

2.3. Conjugation of Ag@BSA microspheres with HRP and Ab_2 detection antibody

For synthesis of HRP and Ab_2-conjugated Ag@BSA microspheres, 100 μL of original glutaraldehyde solution (excess) was initially dropped into 1.0 mL of Ag@BSA suspension under vigorous stirring (1.0 mg mL^{-1}, 0.1 M sodium phosphate buffer, 0.15 M NaCl, pH 6.8), and then the mixture was incubated overnight at RT. Following that, the mixture was centrifuged to remove the excess glutaraldehyde. The obtained sample was redispersed in 1.0 mL of 0.5 M sodium carbonate, pH 9.5. Afterwards, 300 μL of HRP (1.0 mg mL^{-1}) and 10 μL of Ab_2 antibody (1.0 mg mL^{-1}) were injected into the mixture. After gently shaking for 10 min, the mixture was transferred to the refrigerator at 4 °C for further reaction (overnight). During this process, HRP and anti-CEA were covalently conjugated onto the surface of Ag@BSA microspheres (Hermanson, 2008; Xin et al., 2002). Finally, the suspension was centrifuged (5000 rpm) for 10 min at 4 °C. The obtained microspheres (designated as HRP-Ag@BSA-Ab_2) was re-dispersed in 1.0 mL of 0.1 M PBS (pH 7.4) and stored at 4 °C until use.

2.4. Preparation of HRP-tyramine conjugates

HRP-tyramine conjugates were prepared through the typical carbodiimide coupling similar to the literature (Kandimalla et al., 2006). Briefly, 600 μL of 1.0 mg mL^{-1} HRP was dissolved in *N*-2-hydroxyethylpiperazine-*N*′-(2-ethanesulfonic acid) (1.5 mL, 50 mM, pH 9.3) buffer, and the pH of the resulting mixture was adjusted to 7.3 with 3.0 M HCl. 15 mg of NHS and 20 mg of EDC were dissolved in the solution followed by continuous stirring for 45 min. Following that, 600 μL of 5.0 mg mL^{-1} tyramine were added drop by drop into the mixture under continuous stirring at 150 rpm, and left at RT for 12 h. After completion of the incubation, the conjugates were centrifuged for 10 min at 5000 rpm to remove the precipitates. Finally, the obtained conjugates were dialyzed in a dialysis bag against 0.1 M pH 7.4 PBS at RT for 24 h by changing the buffer every 6 h to remove non-conjugated tyramine. The obtained HRP-tyramine conjugates were dispersed into 500-μL PBS (0. 1 M, pH 7.4) (conc. 0.5 mg mL^{-1}) and stored at 4 °C for further use.

2.5. Preparation of the electrochemical immunosensor

A glassy carbon electrode (GCE) with 3 mm in diameter was polished with 0.3 μm and 0.05 μm alumina, followed by successive sonication in distilled water and ethanol for 5 min, and dried in air. The well-polished electrode was cycled in a 0.1 M H_2SO_4 solution 5 times in the potential range from 0 to 2 V. During this process, the anodization of the GCE surface resulted in a multilayer oxide film having –OH groups or –COOH groups (Collier and Tougas, 1987). Following that, 5 μL of CD aqueous solution (50 mg mL^{-1}) was cast onto the surface of the pretreated GCE and dried at RT to form a CD-modified GCE (Zhou et al., 2012b; Tang et al., 2012). After washing with distilled water, 30 μL of Ab_1 antibody (dilution ratio: 1:50) was thrown on the modified

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