Analytical Biochemistry 482 (2015) 48–54

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Simultaneous electrochemical detection of multiple biomarkers using gold nanoparticles decorated multiwall carbon nanotubes as signal enhancers

Dexiang Feng [a,b], Lihua Li [a,b], Junqing Zhao [a], Yuzhong Zhang [a,*]

[a] College of Chemistry and Materials Science, Anhui Normal University, Wuhu 241000, People's Republic of China
[b] Department of Chemistry, Wannan Medical College, Wuhu 241002, People's Republic of China

ARTICLE INFO

Article history:
Received 17 January 2015
Received in revised form 13 April 2015
Accepted 16 April 2015
Available online 22 April 2015

Keywords:
Gold nanoparticles decorated multiwall carbon nanotubes
Pb^{2+} and Cd^{2+}
Multiplex assay
Carcinoembryonic antigen
α-Fetoprotein

ABSTRACT

In this work, a novel sandwich-type electrochemical immunosensor has been developed for simultaneous detection of carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) based on metal ion labels. Gold nanoparticles decorated multiwall carbon nanotubes (AuNPs@MWCNTs) were used as carriers to immobilize secondary antibodies and distinguishable electrochemical tags of Pb^{2+} and Cd^{2+} to amplify the signals. Due to the intrinsic property of high surface-to-volume ratio, the AuNPs@MWCNTs could load numerous secondary antibodies and labels. Therefore, the multiplexed immunoassay exhibited good sensitivity and selectivity. Experimental results revealed that this sandwich-type immunoassay displayed an excellent linear response, with a linear range of 0.01 to 60 ng mL^{-1} for both analytes and detection limits of 3.0 pg mL^{-1} for CEA and 4.5 pg mL^{-1} for AFP (at a signal-to-noise ratio of 3). The method was successfully applied for the determination of AFP and CEA levels in clinical serum samples.

Precise and early determination of multiplex tumor markers could greatly improve the treatment efficiency of many cancers in clinical diagnosis. Therefore, simultaneous detection of multiplex tumor markers related to a certain cancer in human serum has attracted great attention in the biomedical field. During recent years, various immunoassay methods for simultaneous detection of multiplex tumor markers have been developed [1–5]. Among these methods, electrochemical immunoassay has become one of the predominant analytical methods due to high sensitivity, inherent simplicity, and low cost [6–8].

To realize successfully electrochemical simultaneous multiplexed immunoassays, an important issue is to search distinguishable signal probe as trace labels [9,10] in multiple labels mode. As ideal multiple tags for proteins label, it should meet two demands; one is based on the independent response achieved by each target on the identical sensing interface, and the other is based on distinguishable voltammetric signals to avoid interaction with analytes and the sample matrix [11]. Nowadays, a large number of signal tags such as enzymes, quantum dots (QDs),[1] oligonucleotide, and dyes by loading on carriers for the preparation of labels have been reported in electrochemical immunoassay [12]. Among them, QDs have been proved to be promising due to their comparable size and feasibility for surface modification [13]. However, QD-based labels often not only require a tedious preparation process but also involve a complicated detection step of acid-dissolving process to obtain the electrochemical signals [14,15]. As a result, there is still a great challenge in developing novel probes with a simple preparation process and easy detection steps. Recently, Feng and coworkers developed a novel multianalyte electrochemical immunosensor for ultrasensitive detection of cardiac troponin and fatty-acid-binding protein using metal ions Zn^{2+} and Cd^{2+} functionalized titanium phos-

* Corresponding author. Fax: +86 553 3869303.
E-mail address: zhyz65@mail.ahnu.edu.cn (Y. Zhang).

[1] *Abbreviations used:* QD, quantum dot; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; PDDA, poly(diallyldimethylammonium chloride); AuNPs@MWCNT, gold nanoparticles decorated multiwall carbon nanotube; anti-CEA, CEA antibody; anti-AFP, AFP antibody; BSA, bovine serum albumin; HAc, acetic acid; NaAc, sodium acetate anhydrous; $HAuCl_4{\cdot}4H_2O$, chloroauric acid; PBS, phosphate-buffered saline; PBST, PBS containing Tween; SEM, scanning electron microscopy; $Ab_{2,1}$, anti-$CEA_{2,1}$; $Ab_{2,2}$, anti-$AFP_{2,2}$; SWV, square wave voltammetry; CV, cyclic voltammetry; EIS, electrochemical impedance spectrum; IgG, immunoglobulin G; Glu, glucose; AA, ascorbic acid; ELISA, enzyme-linked immunosorbent assay.

http://dx.doi.org/10.1016/j.ab.2015.04.018
0003-2697/

phate nanospheres as labels [16]. Due to metal ions' highly sensitive electrochemical response and easy signal obtaining process, the metal ions as labels are beneficial as reported [17], but this method still involves time-consuming fabrication of titanium phosphate nanospheres for metal ions exchange. Thus, the development of a metal ion label with simple carriers in electrochemical immunoassay is urgently needed.

Here, we have developed a simple electrochemical immunosensor for simultaneous detection of α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) using Cd^{2+} and Pb^{2+} as multiple labels with a simple carrier. Using poly(diallyldimethylammonium chloride) (PDDA) as linkage reagents, the gold nanoparticles decorated multiwall carbon nanotubes (AuNPs@MWCNTs) nanocomposites with good electric conductivity and biocompatibility [18,7,19] were easily fabricated not only as an ideal simple carrier but also as an excellent signal enhancer. As expected, the immunosensor can achieve simultaneous detection of CEA and AFP in one detection platform with different voltammetric peaks through square wave voltammetry. The peak currents and peak positions were dependent on the concentration and type of the corresponding analytes, respectively. In addition, the peak potentials were not too far, which may save the detection time. The obtained immunosensor exhibited sensitive and stable response for detection of multiple tumor markers and showed great potential in clinical applications.

Materials and methods

Materials

CEA, CEA antibody (anti-CEA), AFP, and AFP antibody (anti-AFP) were purchased from Biocell Biotechnology (Zhengzhou, China) and stored in a refrigerator at 4 °C. Bovine serum albumin (BSA) and PDDA (20% [w/w] in water, MW 200,000–350,000) were purchased from Sinopharm Chemical Reagent (Shanghai, China). MWCNTs with carboxylic acid groups (purity >95%, diameter 20–30 nm, length 10–30 μm) were obtained from Chengdu Institute of Organic Chemistry (Chengdu, China). Acetic acid (HAc), sodium acetate anhydrous (NaAc), $Cd(NO_3)_2$, $Pb(NO_3)_2$, and chloroauric acid ($HAuCl_4{\cdot}4H_2O$) were obtained from Shanghai Chemical Reagent (Shanghai, China). Phosphate-buffered saline (PBS) with various pH values was 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 and then adjusting the pH with 0.10 mol L^{-1} NaOH and H_3PO_4. The washing buffer was PBS (pH 7.0) containing 0.05% (w/v) Tween (PBST). The blocking solution was 1% BSA. The clinical serum samples were obtained from the clinical laboratory of Yiji Shan Hospital (Wuhu, China). Twice-quartz-distilled water was used throughout the study.

Apparatus

All electrochemical measurements were performed on a CHI 650C electrochemical analyzer (CH Instruments, China) with a conventional three-electrode system composed of a platinum auxiliary electrode, a saturated calomel electrode as reference electrode, and a bare Au or modified Au electrode as working electrode.

Electrochemical impedance spectra were performed in 0.1 M PBS containing 5.0 mM $[Fe(CN)_6^{3-/4-}]$ and 0.1 M KCl at pH 7.0. The frequency ranged from 0.1 to 100 kHz, and the amplitude of the alternate voltage was 5 mV.

Morphologies of AuNPs, PDDA functionalized MWCNTs, AuNPs–MWCNTs, and AuNPs@Au were obtained with scanning electron microscopy (SEM) (JEOL JSM-6700F, Hitachi, Japan). The morphologies of the materials were measured on a transmission electron microscope (Hitachi 800).

Preparation of AuNPs@MWCNTs nanocomposites

The colloidal AuNPs of 16 nm diameter were prepared according to the previous protocol (see Fig. S1 in online supplementary material) [20]. The acid-treated MWCNTs were further functionalized with PDDA according to the reported method [21]. The obtained PDDA–MWCNTs (0.5 mg) (see Fig. S2) were dispersed in 5.0 mL of as-prepared colloidal AuNPs and stirred for 20 min, and the AuNPs were assembled on the surface of PDDA@MWCNTs nanocomposites via electrostatic interaction. After centrifugation, the AuNPs@MWCNTs composites were obtained (see Fig. S3), and these were further washed with water and redispersed in 2 ml of 50 mM Tris–HCl solution (pH 9.0) for further use.

Preparation of immunosensing probes

First, the signal anti-$CEA_{2,1}$ ($Ab_{2,1}$) (200 μL, 1 mg mL^{-1}) was added to the above AuNPs@MWCNTs dispersion and stirred at room temperature for 2 h. Second, 400 μL of 1% BSA solution was added to the obtained bioconjugates and allowed to react for 2 h. After centrifugation, the resulting immunocomplex was further washed with 0.01 M PBS (pH 7.0) three times. Finally, the prepared immunocomplex was dispersed in 2 mL of 10 mM $Pb(NO_3)_2$ aqueous solution and shaken overnight; thus, the Pb^{2+}–$Ab_{2,1}$–AuNPs@MWCNTs bioconjugates were synthesized. The Cd^{2+}–anti-$AFP_{2,2}$ ($Ab_{2,2}$)–AuNPs@MWCNTs were synthesized using a similar method.

Fabrication of immunosensor

Before modification, the gold electrode was treated according to our previous reported procedure [22]. The modified electrode was immersed in 0.1 M KNO_3 solution containing 5.0 mM $HAuCl_4$ and electrochemically deposited for 300 s at −200 mV. Thus, AuNPs were distributed on the surface of the gold electrode. The modified electrode was denoted as AuNPs/Au.

Immobilization of $Ab_{1,1}$ and $Ab_{1,2}$ was performed by dropping a mixture of $Ab_{1,1}$ and $Ab_{1,2}$ (10.0 μL, 200 μg mL^{-1}) solution onto the surface of the AuNPs/Au and keeping it in a refrigerator for 12 h at 4 °C. The resulting electrode was washed with PBST and PBS to remove physically absorbed Ab_1. Following that, the modified electrode was incubated with 1% BSA solution for 50 min at 37 °C to block any possible remaining active sites against nonspecific adsorption and was washed several times with PBST and PBS. The obtained immunosensor was stored at 4 °C prior to use.

Electrochemical measurements

A schematic preparation of the immunosensing probes is illustrated in Scheme 1. SEM was performed to characterize the shape of the AuNPs/MWCNTs (inset of Scheme 1A). The preparation procedure of the immunosensors for CEA and AFP determination was as follows. The immunosensor was incubated with the mixture of CEA and AFP solution or serum samples with various concentrations for 50 min at 37 °C, followed by washing with PBST and PBS. Then, it was incubated with a mixture of 1:1 diluted Pb^{2+}–$Ab_{2,1}$–AuNPs@MWCNTs and Cd^{2+}–$Ab_{2,2}$–AuNPs@MWCNTs solution for another 50 min at 37 °C, followed by washing with PBST and PBS. Finally, the electrode was transferred into 0.2 M acetate buffer solution (pH 4.5). Square wave voltammetry (SWV) was used to obtain the response signal of the immunosensor. SWV scanning from 0 to −800 mV (vs. Ag/AgCl) with a pulse amplitude of 25 mV, a pulse frequency of 15 Hz, and a quiet time of 2 s was performed to record the electrochemical responses at −0.54 and −0.71 V for simultaneous quantitative measurement of CEA and AFP.