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Layer-by-layer self-assembled multilayer films of multi-walled carbon nanotubes and platinum–Prussian blue hybrid nanoparticles for the fabrication of amperometric immunosensor

Chaofeng Ou, Shihong Chen, Ruo Yuan *, Yaqin Chai, Xia Zhong

College of Chemistry and Chemical Engineering, Key Laboratory on Luminescence and Real-Time Analysis, Ministry of Education, Southwest University, Chongqing 400715, PR China

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ABSTRACT

A label-free amperometric immunosensor for the detection of α-fetoprotein (AFP) based on the layer-by-layer (LBL) assembly of positively charged multi-walled carbon nanotubes–chitosan (MCNTs–CS) and negatively charged platinum–Prussian blue hybrid nanoparticles (Pt–PB) on 3-mercaptopropanesulfonic, sodium salt (MPS) modified electrode is described in this paper. Transmission electron microscopy (TEM) was employed to characterize the morphologies of Pt–PB and Pt–PB–MCNTs–CS. The stepwise LBL assembly process of electroactive species on electrode surface was evaluated by cyclic voltammetry (CV). The immunosensor shows good analytical performance such as low detection limit (0.08 ng ml^{-1}), wide linear range (from 0.1 to 15.0 and from 15.0 to 200.0 ng ml^{-1}), good regeneration, selectivity, stability, and reproducibility for the determination of AFP. Application of the immunosensor to clinical samples demonstrated that results were in good agreement with ELISA.

1. Introduction

α-Fetoprotein (AFP), an oncofetal glycoprotein with a molecule weight of approximately 70 kDa, is well-known as a tumor marker. It is known that the average concentration of AFP is about 25 ng ml^{-1} in healthy human serum [1] and elevated AFP concentration in adult plasma may be an early indication of hepatocellular carcinoma and teratoblastoma [2]. Thus, it is necessary to measure AFP for the clinical diagnosis and even early detection of original liver carcinoma.

Numerous immunoassay techniques have been developed for the detection of AFP, including surface plasmon resonance [3], fluorescence measurement [4], inductively coupled plasma mass spectrometry [5], chemiluminescence assay [6], electrochemiluminescence [7], micellar electrokinetic capillary chromatography [8], liquid-phase binding assay [9], phosphorescence measurement [10], atomic absorption spectrometry [11] and electrochemically based immunosensors [12,13]. Among these methods, the amperometric immunosensors had attracted more interests recently for a higher sensitivity and less complicated instrumentation. Especially, searching for a label-free amperometric immunosensor is of considerable interest in our study since long preparation times and more reaction steps were needed for a labeling amperometric immunosensor [14].

It has been demonstrated that carbon nanotubes (CNTs) have good performances of high surface-to-volume ratios and the ability to promote electron transfer reactions when used as a modifier on electrode in chemical reactions [15,16]. These properties make them extremely attractive for fabricating sensors and biosensors [17–19].

Metal nanoparticles can display following unique advantages over macroelectrodes when used for electroanalysis: enhancement of mass transport, catalysis, high effective surface area and control over electrode microenvironment [20]. Several metal nanoparticles such as Pd [21], Ag [22], Pt [23], Cu [24] and Au [25] have been successfully introduced onto the CNTs. Recently, the nanocomposites consisted of CNTs and metal nanoparticles have been widely applied to biosensors [26–30].

Chitosan (CS) is a naturally occurred biopolymer product found in the exoskeleton of crustaceans. CS has already been utilized in layer-by-layer assembly with certain anionic polymers [31–33] and widely used as biocompatible polymeric immobilization matrix for biosensors and biocatalysis, owing to its affinity with proteins, mechanical stability, biocompatibility, excellent film-forming ability, regenerability and biodegradability [34]. Considering its relatively poor conductivity, CS was usually combined with CNTs, redox mediators and metal nanoparticles for electrochemical biosensing platforms [35].

* Corresponding author. Tel.: +86 23 68252277; fax: +86 23 68254000.
E-mail address: yuanruo@swu.edu.cn (R. Yuan).

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Prussian blue (PB), as a prototype of metal hexacyanoferrates, has been widely used as an electron transfer mediator for analytical applications and has found a wide use in the biosensor development [36–38]. Nevertheless, PB usually shows intrinsic shortcomings of instability for leaking from film for lack of protective films. Recently, Zou et al. [39] fabricated a biosensor based on polyaniline–Prussian blue/multi-walled carbon nanotubes (PANI–PB/MCNTs) hybrid composites, which exhibits excellent response performance to glucose. Furthermore, the PANI–PB/MCNTs modified electrode shows a good stability, as the PANI has good environmental stability to protect the PB film and the MCNTs with high surface area can minimize the leakage due to the hydrolysis of ferric ions and increase the operational stability of the PB.

The layer-by-layer (LBL) assembly technique has been a useful method to generate films with molecular order and stability which involves the alternate adsorption of oppositely charged species [40–42]. This is a simple way to produce multilayer structures with unique mechanical properties and precise control over film composition and thickness.

On the base of above observations and our previous work [30,43], in this research, in order to overcome the relatively poor stability of PB in the film, poor film-forming of Pt and relatively poor conductivity for CS, CS was combined with MCNTs, PB and Pt nanoparticles to prepare $\{MCNTs–CS/Pt–PB\}_n$ multilayer films to immobilize α-fetoprotein antibody (anti-AFP) for detecting of AFP by using LBL assembly technique between positively charged MCNTs–CS and negatively charged Pt–PB nanoparticles. Transmission electron microscopy (TEM) was employed to characterize the morphologies of Pt–PB and Pt–PB–MCNTs–CS. The consecutive growth of $\{MCNTs–CS/Pt–PB\}_n$ multilayers was confirmed by cyclic voltammetry (CV). The performance and factors influencing the performance of the resulting immunosensors were studied in detail. The multilayer assembly of MCNTs–CS and Pt–PB nanoparticles brings a new platform for electrochemical devices. The present immunosensor exhibits good performances in the determination of AFP with high sensitivity, fast response time, wide linear range, and good selectivity, stability, and reproducibility.

2. Experimental section

2.1. Reagents

Chitosan (CS, medium molecular weight), 3-mercaptopropanesulfonic and sodium salt (MPS) were purchased from Aldrich (Milwaukee, USA). The multi-walled carbon nanotubes (MCNTs, >95% purity) were purchased from Chengdu Organic Chemicals Co., Ltd., which is attached to the Chinese Academy of Science. K_2PtCl_6 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-AFP and AFP were purchased from Zhengzhou Biocell Institute, China. All other reagents were of analytical grade and were used without further purification. The working phosphate buffer solutions (PBS) with various pHs were prepared with 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 with 0.1 M KCl supporting electrolyte. Doubly distilled water was used throughout.

2.2. Instruments and measurements

Cyclic voltammetric measurements were carried out on CHI 660A electrochemical analyzer (Shanghai CH Instruments Co., China) with a conventional three-electrode system consisted of a modified gold electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode. All potentials were measured and reported versus the SCE. The size of Pt–PB nanoparticles was estimated from transmission electron microscopy (TEM) (H600, Hitachi Instrument Co., Japan).

2.3. Preparation of MCNTs–CS and Pt–PB nanoparticles

The MCNTs were treated with concentrated nitric acid in order to introduce carboxylic acid groups according to the report [44]. CS stock solution (0.15 wt%) was prepared by dissolving 30 mg CS powder in 20 ml 1.0% acetic acid solution and stirred for 5 h at room temperature until complete dissolution. MCNTs were dispersed in the CS solution with a 30 min sonication to prepare MCNTs–CS suspension.

The Pt–PB hybrid nanoparticles were prepared according to the literature [45]. About 5 ml deionized water, 1 ml 10 mM KCl and 1 ml 2 mM $K_4Fe(CN)_6$ was mixed with vigorous stirring. After 1 ml 2 mM $FeCl_3$ dropped, PB colloid was formed. Then 1 ml 2 mM $K_4Fe(CN)_6$ and 1 ml 2 mM K_2PtCl_6 were added separately. The vigorous stirring was continued for 24 h to obtain Pt–PB colloid solution.

2.4. Fabrication of the immunosensor

The gold electrodes (4 mm diameter) were polished before each experiment with 0.3 and 0.05 μm alumina powders, rinsed thoroughly with doubly distilled water between each polishing step, sonicated with acetone and doubly distilled water, respectively, and then allowed to dry at room temperature.

The cleaned gold electrode was soaked in an aqueous solution of 0.02 M MPS in 0.01 M H_2SO_4 for 2 h. After adsorption, the modified electrode was washed with distilled water. The MPS modified electrode was immersed in the MCNTs–CS containing 3 mg/ml MCNTs for half an hour to obtain a MCNTs–CS/MPS modified electrode. Then, it was alternatively immersed in Pt–PB and MCNTs–CS solution each for half an hour to produce $\{MCNTs–CS/Pt–PB\}_n$ multilayer films. Each immersion was followed by washing and drying with nitrogen. Subsequently, the $\{MCNTs–CS/Pt–PB\}_n$/MCNTs–CS/MPS modified electrode was immersed in anti-AFP solution at 4 °C for about 12 h. Finally, the electrode was incubated in 0.25 wt% bovine serum albumin (BSA) solution about 1 h at 4 °C in order to block possible remaining active sites of the carbon nanotubes, platinum nanoparticles and Prussian blue nanoparticles and avoid the non-specific adsorption. The finished immunosensor was stored at 4 °C when not in use. The schematic diagram of the stepwise self-assemble procedure of the immunosensor is shown in Scheme 1.

3. Results and discussion

3.1. Characterization of hybrid material

Morphologies of PB–Pt nanoparticles and Pt–PB–MCNTs–CS composites were characterized by TEM. Fig. 1A gives the images of Pt–PB nanoparticles and the diameter of them was about 20 nm. The surface of nanoparticles is roughened. After Pt–PB nanoparticles were added into the suspension of MCNTs–CS, Pt–PB–MCNTs–CS composites were obtained (Fig. 1B). As shown, Pt–PB nanoparticles were assembled onto the MCNTs–CS. It is possible that positively charged MCNTs–CS adsorbed negatively charged Pt–PB nanoparticles.

3.2. Electrochemical characteristics of the modified electrode

Pt–PB is negatively charged and can be easily adsorbed by positively charged MCNTs–CS (in pH < 6.3) on MPS film. By alternatively immersing the MPS modified electrode into the MCNTs–CS suspension and Pt–PB colloid solution, visible thin films are formed. The film growth was monitored by CV. Fig. 2 shows the