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An ultrasensitive sandwich-type electrochemical immunosensor based on signal amplification strategy of gold nanoparticles functionalized magnetic multi-walled carbon nanotubes loaded with lead ions

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In this study, a novel and ultrasensitive sandwich-type electrochemical immunosensor was prepared for the quantitative detection of alpha fetoprotein (AFP), a well-known hepatocellular carcinoma biomarker. Gold nanoparticles (Au NPs) functionalized magnetic multi-walled carbon nanotubes (MWCNTs-Fe_3O_4) were prepared and utilized for the adsorption of lead ions (Pb^{2+}) and the secondary antibodies (Ab_2). The resultant nanocomposites (Pb^{2+}@Au@MWCNTs-Fe_3O_4) were used as the label for signal amplification, showing better electrocatalytic activity towards the reduction of hydrogen peroxide (H_2O_2) than MWCNTs, MWCNTs-Fe_3O_4 or Au@MWCNTs-Fe_3O_4 due to the synergetic effect presented in Pb^{2+}@Au@MWCNTs-Fe_3O_4. Moreover, Au NPs were electrodeposited on the surface of glassy carbon electrode (GCE) for the effective immobilization of primary antibodies (Ab_1). Under the optimal conditions, a linear range from 10 fg/mL to 100 ng/mL and a detection limit of 3.33 fg/mL were obtained. The proposed electrochemical sandwich-type immunosensor shows high sensitivity, good selectivity and stability for the quantitative detection of AFP, holding a great potential in clinical and diagnostic applications.

1. Introduction

Hepatocellular carcinoma (HCC) has been recognized as one of the most common malignant canners (Bertino et al., 2012; Malaguarnera et al., 2010; Wan et al., 2014) and listed among the top three reasons for cancer-related death worldwide (Wang et al., 2008). Approximately 500,000 patients have been diagnosed with HCC worldwide per year. (Bruix et al., 2001). Development of sensitive and accurate detection of disease-related biomarkers is essential for many fields of biomedical and diagnostic research (Guo et al., 2015). Alpha-fetoprotein (AFP) is recognized as the tumor marker for HCC well (Suriapranata et al., 2010). Approximately 60% of HCC patients can be detected when the concentration of AFP is enhanced to a certain level in serum (Goldman et al., 2009; Suriapranata et al., 2010). Therefore, the accurate detection of human serum AFP levels plays an important role in the diagnosis of HCC (Wang et al., 2014a).

In the past few years, a series of methods have been developed for the detection of AFP, such as enzyme-linked immunosorbent assay (Liew et al., 2007), surface plasmon resonance (Teramura and Iwata, 2007), fluoroimmunoassay (Vieira et al., 2005), chemiluminescence (Fu et al., 2006), and chemiluminescence enzyme immunoassay (Wang et al., 2008). In addition to these techniques, electrochemical immunosensors have attacked widespread interest due to the advantages in terms of high sensitivity, rapid detection, excellent selectivity and simple operation (Li et al., 2014; Yu et al., 2013). Therefore, it has been widely applied in many fields like food safety (Jin et al., 2011; Zhao et al., 2007), environmental monitoring (Li et al., 2014; Zhang et al., 2007), as well as clinical diagnostics (Cai et al., 2011; Zhuo et al., 2011).

In order to improve the sensitivity of electrochemical analysis, a variety of nanomaterials are used in the fabrication of the immunosensors to achieve signal amplification (Dong et al., 2013; Wang et al., 2014b; Zhao et al., 2013). Among these efforts, multi-walled carbon nanotubes (MWCNTs) have been extensively applied because of their large specific surface area, excellent conductivity (Tang et al., 2011; Xiang et al., 2010; Zhuo et al., 2011) and good biocompatibility (Chłopek et al., 2006; Lobo et al., 2008). Recently, MWCNTs have been used to build sandwich-type immunosensors by many groups (Cao et al., 2011b; Kavosi et al., 2014; Liu et al., 2011) to promote electron transfer between the electroactive species and the electrode (Cao et al., 2011b; Wang, 2005). It has been found that Fe_3O_4 possesses great auxiliary catalytic activity for the reduction of hydrogen peroxide (H_2O_2)

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(Wang et al., 2014; Wei et al., 2010a, 2010b). Gold nanoparticles (Au NPs) also have been widely used for the construction of electrochemical immunosensors (Li et al., 2006; Tang et al., 2004) as they can facilitate the antibody immobilization with amplified response signal (Cao et al., 2011a, 2011b). The surface functionalization of MWCNTs would provide extra capability to intensify the chemical modification, such as ion adsorption (Niu et al., 2013; Wong et al., 1998). MWCNTs-Fe_3O_4 has large surface area, higher conductivity properties and exceptional adsorption capability to capture the lead ions (Pb^{2+}) (Li et al., 2002). The adsorbed Pb^{2+}, can further promote the redox behavior of H_2O_2, which can be used to amplify the detection signal.

In this research, an innovative sandwich-type electrochemical immunosensor was designed to achieve the quantitative detection of AFP. The synergetic effect presented in gold nanoparticles functionalized magnetic multi-walled carbon nanotubes loaded with lead ions (Pb^{2+}@Au@MWCNTs-Fe_3O_4) can be employed to increase the sensitivity of the immunosensor during the H_2O_2 reduction of (Wu et al., 2013a). Electroplating Au NPs was adsorbed on the modified electrode to immobilize primary antibodies (Ab_1) as the signal amplification platform to construct the immunosensor (Cao et al., 2011b). Due the presence of amino groups (–NH_2), Ab_1 could be effectively conjugated onto the surface of Au NPs by the interaction between Au NPs and –NH_2 on antibodies to construct Au–N (Gao et al., 2013; Han et al., 2013; Wang et al., 2014d). The proposed novel immunosensor strategy would provide a useful technology for the quantitative detection of AFP in human serum due to its high current response, a relatively wide linear range and a low detection limit.

2. Materials and methods

2.1. Reagents and apparatus

AFP antibody (anti-AFP) and AFP antigen were bought from Biocell Co., Ltd. (Zhengzhou, China). Bovine serum albumin (BSA, 96–99%) was purchased from Sigma reagent Co., Ltd. (St. Louis, MO, USA). Multi-walled carbon nanotubes (MWCNTs) were purchased from Alfa Aesar Co., Ltd. (Shanghai, China). $HAuCl_4 \cdot 4H_2O$ were obtained from Sigma-Aldrich Co., Ltd. (Beijing, China). 3-aminopropyl triethoxysilane was purchased from XiBao biological technology Co., Ltd. (Shanghai, China). $K_3[Fe(CN)_6]$ was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Phosphate buffered saline (PBS, pH=7.4) were prepared by mixing Na_2HPO_4 and L KH_2PO_4 stock solution and used as the electrolyte in the process of electrochemical measurements. Ultrapure water (18.25 MΩ cm, 24 °C) was used for all the experimental processes. The level of others chemical reagents were used without further purification. All solutions were stored at 4 °C for further usage.

CHI760D electrochemical workstation was used in the whole process of electrochemical measurement (Shanghai CH Instruments Co., China). Scanning electron microscope (SEM) images were obtained using Quanta FEG250 field emission environmental SEM (FEI, United States). Energy Dispersive X-Ray spectra (EDX) were recorded by JEOL JSM-6700F microscope (Japan). For A.C. impedance measurements, a frequency range of 1Hz–100kHz and AC voltage amplitude of 5 mV were used.

2.2. Preparation of the amino-functionalized MWCNTs-Fe_3O_4

To remove any metal oxide within the MWCNTs, MWCNTs (0.5 g) were added into a mixture of 3.0 M of HNO_3 and 2.0 M H_2SO_4 (3:1, v/v), then kept ultrasonic for 3 h under 40 °C. The MWCNTs were cooled down to room temperature, washed several times with ultrapure water to neutrality, and then dried at room temperature.

MWCNTs-Fe_3O_4 was synthesized according to a published method with some revisions (Morales-Cid et al., 2010). In brief, $FeCl_3 \cdot 6H_2O$ (0.7 g) and MWCNTs (0.2 g) were dispersed in ethylene glycol (37.5 mL). Subsequently, sodium acetate (NaAc, 1.8 g) was added and dissolved under ultrasonic condition at room temperature. Then the solution was transferred into the stainless-steel autoclave. The autoclave was heated to 200 °C and maintained at this temperature for 16 h. The solution was cooled down to room temperature after reaction. The prepared compound sample (MWCNTs-Fe_3O_4) was thoroughly washed to remove the impurities and separated via a strong magnet.

MWCNTs-Fe_3O_4 (0.1 g) and 3-aminopropyl triethoxysilane (0.1 mL) were added into anhydrous ethanol (10 mL). They were heated to 70 °C and kept reflux for 1.5 h in oil bath. Subsequently, magnetic separation with anhydrous ethanol was used to purify the composites. The process was repeated twice to remove impurities. Finally, the prepared amino-functionalized MWCNTs-Fe_3O_4 was stored at 50 °C.

2.3. Preparation of AU@MWCNTs-Fe_3O_4

The preparation of Au NPs was referred to the classical method that $AuCl_4^-$ ions were reduced by the sodium citrate (Frens, 1973). In brief, 100 mL of solution containing $HAuCl_4$ (1 wt%, 1 mL) and ultrapure water (99 mL) was refluxed. Sodium citrate (1.5 mL, 10 mg/mL) was added inside the solution under boiling and maintained stirring for 15 min. A wine red solution of Au NPs was obtained after being cooled to room temperature and stored at 4 °C.

The prepared amino-functionalized MWCNTs-Fe_3O_4 (10 mg) was dispersed in the Au NPs (20 mL) solution. The suspension was stirred for 12 h and magnetic separation. Au NPs could bind with all of amino groups on the surface of amino-functionalized MWCNTs-Fe_3O_4. The sediment was dried and referred as the Au@MWCNTs-Fe_3O_4.

2.4. Preparation of Pb^{2+}@AU@MWCNTs-Fe_3O_4

Au@MWCNTs-Fe_3O_4 (4 mg) was dispersed into lead nitrate solution (4 mL, 1 mg/mL). The solution had been oscillated for 24 h to ensure that Pb^{2+} could be fully absorbed onto the Au@MWCNTs-Fe_3O_4. The Pb^{2+}@Au@MWCNTs-Fe_3O_4 was obtained for further use after magnetic separation.

2.5. Preparation of Pb^{2+}@AU@MWCNTs-Fe_3O_4/Ab_2

Pb^{2+}@Au@MWCNTs-Fe_3O_4 (2 mg/mL) was added to an equal volume of secondary antibodies (Ab_2) (10 μg/mL) in a shaking incubator at 4 °C, the incubation time was 12 h. Due to the presence of amino groups (–NH_2), Ab_2 (protein) could be effectively conjugated onto the Pb^{2+}@Au@MWCNTs-Fe_3O_4 by the interaction between Au NPs and –NH_2 on antibodies to construct Au–N (Gao et al., 2013; Han et al., 2013; Wang et al., 2014d). After magnetic separation, it was configured into different concentrations as required standby and stored at 4 °C. Fig. 1A shows the preparation procedure of Pb^{2+}@Au@MWCNTs-Fe_3O_4/Ab_2.

2.6. Fabrication of the immunosensor

Fig. 1B shows the process of the construction of the sandwich-type electrochemical immunosensor. Generally, the glassy carbon electrode (GCE) was polished by alumina polishing powders with particle size of 1.0, 0.3, and 0.05 μm, respectively, followed by sonication in ethanol and rinsing by ultrapure water. The