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A reagentless amperometric immunosensor based on nano-au and carbon nanotubes for detection of alphafetoprotein

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In this paper, carboxyl-ferrocene (Fc-COOH) was explored to label alphafetoprotein antibody (anti-AFP), which was then mixed with AU nanoparticles (nano-Au) and multi-walled carbon nanotubes (MWCNTs) dispersed by chitosan (CS) to form the nano-Au/MWCNTs/anti-AFP-Fc chitosan composite. After that, the composite was immobilized onto the surface of the electrode to prepare a reagentless amperometric alphafetoprotein (AFP) immunosensor. The experimental results indicated that, the proposed immunosensor had good current response to AFP with a linear range from 0.1 to 50 ng/ml with a detection limit of 0.03 ng/ml. Moreover, the electrochemical properties and performance were investigated by the cyclic voltammetry. The results also found that, the immunosensor owned the advantages of high stability, simple preparation and operation and well regeneration performance.

Key words: Multi-walled carbon nanotubes (MWCNTs), Au nanoparticles (nano-Au), chitosan (CS), amperometric immunosensor, alphafetoprotein (AFP).

INTRODUCTION

Alphafetoprotein (AFP) is the most reliable and widely used tumor marker. The detection of serum AFP level plays an important role in the diagnosis, management and predicting relapse of germ cell tumors and original liver carcinoma (Wang et al., 1999; Bad er et al., 2004.). Generally, enzyme-linked immunosorbent assay (ELISA) or immunoradiometric assay (IRMA) are the conventional methods for determination of AFP (Mancal et al., 1988;

Kumakura et al.,1983.). This is due to their less sensitivity, time-consuming or radiation hazards, complicated wash procedure and expensiveness. Due to the specific, simple and direct detection techniques and reduction in size, cost and time of analysis compared with conventional immunoassay techniques, electrochemical immunosensors are of great interest for AFP determination (Zhuo et al., 2009.).

Since the importance of immunoreagent immobilization onto the electrode surface for the immunosensor construction of an electrochemical, searching for an effective and simple immobilization method is of considerable interest (Ramanan et al., 2010), multi-walled carbon nanotubes (MWCNTs) are one of the most promising candidates for immunoreagent immobilization due to their high porosity and surface area, strong adsorption capacity and well-defined electrical properties (Kaminishi et al., 2005; Ji et al., 2010). It is reported that the high surface free energy and existence of inter-tube van der Waals forces may lead to aggregation, which limited their further development in nanodevices construction. Chitosan (CS) is a polysaccharide which

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Abbreviations: AFP, Alphafetoprotein; **anti-AFP,** alphafetoprotein antibody; **Fc-COOH,** carboxyl-ferrocene; **MWCNTs,** multi-walled carbon nanotubes; **nano-Au,** Au nanoparticles; **CS,** chitosan; **ELISA,** enzyme-linked immunosorbent assay; **IRMA,** immunoradiometric assay; **EDC,** N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride; **NHS,** N-hydroxy succinimide; **BSA,** bovine serum albumin; **PBS,** phosphate buffered solution; **CVD,** chemical vapor deposition; **SCE,** saturated calomel reference electrode.

possesses many advantages such as excellent membrane-forming ability, high permeability towards water, good adhesion, biocompatibility and high mechanical strength. More importantly, CS can act as a scaffold for dispersing MWCNTs and incorporating firmly MWCNTs and biomolecules at electrode surface. Thus, MWCNTs/CS composite has been explored as platforms for the development of an electrochemical immunosensor.

Herein, alphafetoprotein antibody (anti-AFP) was first labeled with carboxyl-ferrocene (Fc-COOH) via N-(3-dimethylaminopropyl)-N′- ethylcarbodiimidehydrochloride (EDC) and N-hydroxy succinimide (NHS) as link reagent. Then, it was mixed with Au nanoparticles (nano-Au) and MWCNTs/CS composite to form the anti-AFP-Fc/nano-Au/MWCNTs/CS well-distributed suspension. Next, the obtained composite was dropped onto the surface of the electrode to prepare a reagentless AFP immunosensor, that got rid of the addition of substrates or mediators to the analytic solution. The resulting immunosensor was tested for its response to AFP by cyclic voltammetry. The analytical parameters of the biosensor as well as its reproducibility, stability and selectivity were also evaluated.

MATERIALS AND METHODS

Reagent and chemicals

Anti-AFP (0.312 µg/ml) and AFP were purchased from Biocell Company (Zhengzhou, China). The MWCNTs (>95% purity) synthesized by the chemical vapor deposition (CVD) method were purchased from Chengdu Organic Chemicals Co. Ltd., of the Chinese Academy of Science. The carboxyl-ferrocene (Fc-COOH), chitosan (low molecular weight from the shrimp shell with a degree of deacetylation of 83.3%), gold chloride ($HAuCl_4$), bovine serum albumin (BSA, 96 to 99%), N-hydroxy succinimide (NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC) and sodium citrate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and used without further purification. The 0.1M phosphate buffers (PBS) were prepared by mixing stock standard solution of K_2HPO_4 and KH_2PO_4 and adjusting the pH with KOH. The common chemicals were of analytical grade and used as received. Double distilled water was used throughout this experiment. The nano-Au were prepared as described in the literature (Brown et al., 2000) with sodium citrate as reducing agent and the obtained nano-Au were stored in a dark bottle at 4°C.

Apparatus and measurements

The electrochemical measurements were performed with CH instruments CHI 660C electrochemical analyzer/workstation (Shanghai CH instrumentation, China) with a three-electrode system, which composed of a platinum wire counter electrode, a saturated calomel reference electrode (SCE) and modified gold electrode (Au, diameter 4 mm) for the measurement. All potentials are referred to the SCE reference electrode. All electrochemical experiments were carried out at room temperature. The scanning electron micrographs were taken with a scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan). The pH measurements were made with a pH meter (MP 230, Mettler-Toledo, Switzerland) and a digital ion analyzer (Model PHS-3C, Dazhong instruments, Shanghai, China).

Preparation of gold nanoparticles

Gold nanoparticles (nano-Au) was prepared by reducing $HAuCl_4$ using trisodium citrate according to the literature (Grabar et al., 1995) with a slight modification. First of all, the glassware used to prepare nano-Au were cleaned using freshly prepared aqua regia (HCl/HNO_3 = 3:1) and rinsed thoroughly with doubly distilled water. Then, 1 ml 1% (w/v) $HAuCl_4 \cdot 4H_2O$ solution was added in 250 ml doubly distilled water. Until the mixture solution was heated to a rolling boil, 2.5 ml of 1% (w/v) sodium citrate solution was quickly added to obtained a red wine nano-Au solution, which was then, cooled and stored in a dark glass bottle at 4°C before use.

Fabrication of the MWCNTs and Fc labeled anti-AFP

Prior to use, the MWCNTs were pretreated as follow (Shobha et al., 2008; Luo et al., 2006). Briefly, 25 mg MWCNTs were heated in air at 600°C for 2 h and then, soaked in 6 M HCl solution for 24 h and centrifuged. Then, the MWCNT were chemically functionalized by sonification treatment in a mixture of sulfuric acid and nitric acid (3:1) for 6 h at room temperature, then, washed with deionized water until the pH of the supernatant was 7.0; finally dried under at 60°C overnight for further use.

A 1.0 wt.% CS solution was prepared by dissolving 0.5 g of CS into 50 ml of 1.0% acetic acid solution and intermittently sonicated for 2 h to obtain a homogeneous solution at room temperature. Then, its pH was adjusted to 5.0 using concentrated NaOH solutions. Next, 2 ml of the prepared nano-Au and 2 mg pretreated MWCNTs were dispersed in 2 ml 1 wt.% CS solutions with the aid of ultrasonic agitation for 1 h to obtain a well-distributed suspension.

Fc labeled anti-AFP was obtained by the employment the EDC/NHS as coupling agents. 2 mM EDC and 5 mM NHS were added into 5 mM Fc solution for activating the carboxyl of Fc under continuous stirring for 4 h at room temperature. The 0.4 ml as-prepared activated Fc was mixed with 0.6 ml anti-AFP for 4 h at 4°C for stirring by the formation of an amide link between the carboxyl of ferrocenemonocarboxylic and the amino of anti-AFP (anti-AFP-Fc).

Electrode modification procedure

Prior to the surface modification, the bare gold electrode was polished first with alumina slurry (followed by 0.3 and 0.05 µm) then, cleaned by sequential ultrasonic cleaning in redistilled water, acetone and ethanol for 5 min each and dried in air. Subsequently, the gold electrode was cleaned with piranha solution (H_2SO_4/H_2O_2=7:3) and rinsed thoroughly with doubly distilled water and dried in air.

0.5 ml of the prepared anti-AFP-Fc was added into 2 ml nano-Au/MWCNTs/CS composite solutions. After that, the mixture was stirred for 12 h and stored at 4°C for further use. The cleaned gold electrode surface was covered with 10.0 µl anti-AFP-Fc/nano-Au/MWCNTs/CS mixture and stored at 4°C overnight. In order to remove the unbound antibodies, the anti-AFP-Fc/nano-Au/MWCNTs/CS composite modified electrode was slow dipping into a PBS buffer (pH 7.4) three times. Then, 1% BSA-PBS was incubated with the anti-AFP-Fc/nano-Au/MWCNTs/CS electrode for 20 min to block the unreacted and non-specific sites. Finally, the electrode was thoroughly rinsed with deionized water and dried in air. The schematic principle for fabrication of this immunosensor and the SEM of the anti-AFP-Fc, nano-Au and MWCNTs-CS nano-composite are shown in Figure 1.

Electrochemical studies

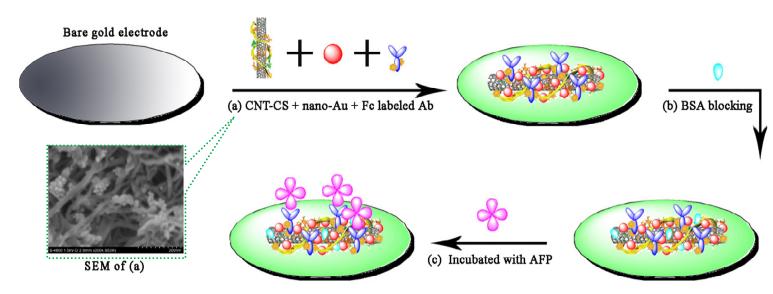Cyclic voltammetric (CV) experiments were performed on a CHI

Figure 1. Schematic representation of construction of the immunosensor: (a) Formation of anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane; (b) blocking with BSA; (c) immunoreactions with AFP.
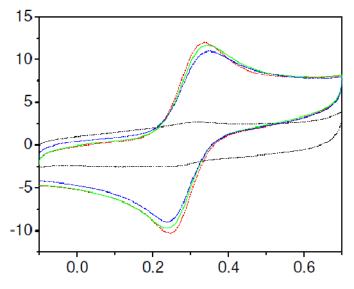
RESULTS AND DISCUSSION

Cyclic voltammetric characterization of modified gold electrode

Figure 2 shows cyclic voltammograms of different electrodes in 0.1 M PBS. The curve in Figure 2 shows the CV wave of bare gold electrode in PBS. Then, a well-defined cyclic voltammogram with quasi-reversible redox waves can be observed at the anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane modified Au electrode (Figure 2b), which indicated the electrochemical activity of the labeled Fc. For the BSA blocking with the modified electrode (Figure 2c), the peak currents decreased (against those of curve b). Subsequently, the immunosensor was incubated with 10 ng/ml AFP standard sample solution; the curve d can be obtained. A further peak currents decrease of curve d indicates the formation of immunocomplex which could retard the electron transfer channels.

In order to investigate the effect of MWCNTs, different kinds of composite membrane with and without, MWCNTs were used to modify the gold electrode. As can be clearly observed, the redox peak current of anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane modified Au electrode (Figure 3a) is remarkably large compared with the anti-AFP-Fc/nano-Au/ CS composite solution coating electrode (Figure 3b), meaning the participating of MWCNTs added to the film may result to a large increase in the response which suggest that, MWCNTs modification leads to a higher electroactive area.

Figure 2. Cyclic voltammograms of different electrodes in PBS (pH = 7.0) at a scan rate of 50 mV/s. (a) bare gold electrode, (b) anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane modified electrode, (c) the modified electrode blocked with BSA and (d) the immunosensor incubated with 10 ng/ml AFP.

660 C electrochemical workstation with a three-electrode system and the potential swept from -0.1 to 0.7 V (vs. SCE) with a sweeping rate of 50 mV/s in 4 ml 0.1 M PBS buffer solution (pH 7.0) containing 0.1 M KCl at room temperature. The proposed method is based on direct immunoassay. The oxidation peak current of CV measurement in PBS was recorded as amperometric response. When AFP was bound with anti-AFP immobilized on the electrode, the antigen-antibody immunocomplex may coat on the surface of the electrode inhibiting the electron-transfer, resulting a in decrease in amperometric response. Therefore, the amperometric response changed proportional to the concentration of AFP after the antibody-antigen reaction.

Conditions optimization of immunoassay

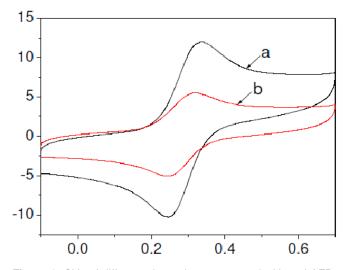The effect of pH on the amperometric responses was

Figure 3. CVs of different electrodes constructed with anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane (a) and anti-AFP-Fc/nano-Au/ CS composite membrane (b).
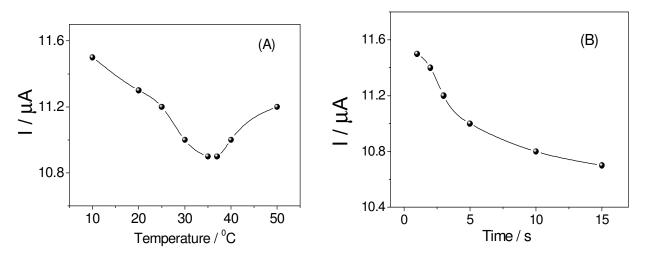

Figure 4. Effect of incubation temperature (A) and incubation time (B) on CV oxidation peak current in pH 7.0 PBS solution.

examined in the pH range of 6.0 to 8.0. The current increased as the medium pH increased from 6.0 to 6.5 and then, decreased at pH values higher than 7.0. It can be found that the responses at pH 6.5 and 7.0 have no significant difference. Thus, we chose pH 7.0 as the optimal pH in the further study.

The temperature effect on the immunoreactions was studied. Briefly, the reaction of the immunosensor with AFP standard solution took place at the temperature of 10 to 50°C for 15 min and then, its amperometric response was examined. The result shows that the immobilized anti-AFP exhibits the highest affinity towards AFP at 37°C (Figure 4a). Thus, 37°C was employed as the optimum incubation temperature in this study.

The influence of immunoreaction time was also

investigated. Using the AFP concentration of 10 ng/ml, the incubation times were 1, 2, 3, 5, 10 and 15 min, respectively. It was found that the amperometric response of the immunosensor decreased with incubation time rapidly up to first 6 min and then, the peak current decreased slowly at about 15 min (Figure 4b). Thus, 15 min was adopted as the optimal incubation time for the immunoassay in the subsequent work.

Performance of the immunosensor

Calibration curve for AFP

Based on the direct immunoassay protocol, the decrease

Table 1. Comparison of different electrochemical immunosensors

Detection methods	Linear range (ng/ml)	Detection limit (ng/ml)	Reference
Cyclic voltammetry	0.2–120.0	0.06	He et al. (2008)
Cyclic voltammetry	1-55	0.6	Lin et al. (2009)
Cyclic voltammetry	0.1-50	0.03	The present work

Table 2. Comparison of serum AFP level determinations by using the two different methods.

Serum sample	ELISA (ng/ml)	Proposed immunosensor (ng/ml)	Relative deviation (%)
1	6.24	6.47	-3.69
2	9.87	10.55	-6.89
3	36.10	35.12	2.71
4	19.80	20.48	-3.43
5	1.42	1.36	4.23

in amperometric response of the immunosensor was inversely related to the different concentration of AFP in standard sample. After the immunosensor was incubated in different concentration of AFP solution for 15 min at 37°C; Figure 4 shows that the amperometric response was proportional to AFP concentration in two ranges from 0.1 to 1.0 and 1.0 to 50 ng/ml, with the correlation coefficient of 0.9980 and 0.9975, respectively. A limit of detection of 0.03 ng/ml was calculated at signal-to-noise ratio of three.

Selectivity

The performance of the immunosensor against interferences is important. Thus, the interference existing in real samples, such as carcinoembryonic antigen (10.0 ng/ml), cancer antigen 125 (10.0 ng/ml), BSA (10.0 ng/ml), were chosen to evaluate the specificity of the immunosensor. When the immunosensor was incubated with the AFP (10.0 ng/ml) solution contained the mentioned interference, no remarkable difference of peak currents was obtained when compared with the presence of only AFP (10.0 ng/ml). Thus, the immunosensor had a good selectivity to AFP.

Regeneration and stability

The regeneration performance of the prepared immune-sensor was carried out using urea solution as regeneration solution to remove the target molecules (AFP) from the sensing layers. It was found that, the immunosensors could be regenerated by immersing in a 4 M urea solution for about 20 min under stirring and then, rinsed thoroughly with PBS after each determination. The results show that it can be repeatedly used for 8 times without significant loss of detection

sensitivity.

Amperometric measurements were carried out with 7 biosensors in a batch for the detection of 10 ng/ml AFP. With the relative standard deviation of 3.7%, 7 electrodes exhibited good reproducibility. The operational stability was also considered by continuous CV scans for 100 circles. The relative deviation of the first circle and the 100th circle was 3.92% showing good stability due to the fact that, the anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane may have a promising potential in fabricating the new kind of immunosensors based on indirect electrochemistry of immunomolecules.

Performance comparison with different electrochemical immunosensors

The performance of different electrochemical immune-sensors has been compared with the present work. As shown in Table 1, the comparative data suggest superiority of the present sensor over some earlier reported immunosensor, especially the characteristics of the detection limit.

Clinical application

In order to evaluate the validity of the proposed method, the fabricated immunosensors were used to determine the AFP in human serum samples. Furthermore, we compared the results obtained from 10 sera with this method and ELISA. Part of the results and the relative deviations between the two methods are shown in Table 2.

Conclusions

The aim of this study is to develop a reagentless

amperometric immunosensor based on anti-AFP-Fc/nano-Au/MWCNTs/CS composite membrane in a direct and simple method. The direct detection of the immunosensor toward AFP has been obtained due to the direct amperometric responses decrease by antigen-antibody immunocomplex generation which could obstruct the path of electron transfer. The immunosensor greatly simplified the immunoassay procedure with acceptable sensitivity. Meanwhile, the nano-Au/MWCNTs/CS composite can efficiently retain the biological activity of protein.

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