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A Magnet-Controlled and Renewable Amperometric Immunosensor for Carcinoembryonic Antigen Based on Magnetic Fe_3O_4(core)/Au(shell) Nanoparticles Modified Screen-Printed Carbon Electrode

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A novel amperometric immunosensor for carcinoembryonic antigen (CEA) was fabricated. Firstly, multi-walled carbon nanotubes with carboxyl groups (MCNTs)-thionine (Thi)-Nafion composite was prepared and dropped on the surface of a screen-printed working electrode (SPCE) to prepare the basic electrode (SPCE|MCNTs-Thi-Nafion). Secondly, horseradish peroxidase (HRP) labeled carcinoembryonic antigen antibody (HRP anti CEA) and bovine serum albumin (BSA) were assembled on Fe_3O_4(core)/Au(shell) nanoparticles (GMPs) to form the nanoprobes, which were introduced on the surface of the basic electrode by permanent magnet. Through one-step immunoassay format, the immunosensor was incubated with carcinoembryonic antigen. The formed antigen-antibody complex inhibited partly the active center of horseradish peroxidase labeled on anti carcinoembryonic antigen, which led to a linear decrease in the catalytic efficiency of horseradish peroxidase to the oxidation of immobilized thionine by H_2O_2. Under optimized conditions, the decreased current was proportional to the carcinoembryonic antigen concentration from 0.1-5.0 and 5.0-80 ng mL^{-1} with a detection limit of 0.03 ng mL^{-1} at signal/noise ratio of 3.

Key Words: Renewable amperometric immunosensor, Fe_3O_4(core)/Au(shell) nanoprobes, MCNTs-Thi-Nafion composite, Carcinoembryonic antigen, Screen-printed carbon electrodes.

INTRODUCTION

Carcinoembryonic antigen (CEA) is a protein associated with many kinds of tumors such as colon tumors, breast tumors and so on[1-3]. The normal range for CEA in an adult non-smoker is less than 2.5 ng mL^{-1}. A rising CEA level indicates progression or recurrence of the cancer[4,5]. Therefore, a method for rapid detection of ultra level of CEA is necessary for the early diagnosis of tumor patients. Many immunoassay techniques have been developed for the detection of CEA, including fluoroimmunoassay[6], radioimmunoassay[7], enzyme-linked immunoassay[8] and flow injection immunoassay[9]. Some of these methods have enabled label-free or high-throughput detection of target proteins and some have been successfully implemented in clinical diagnostic procedures in practice[10]. However, methods described above also involve qualified personnel or requiring dangerous or expensive devices[11,12]. The electrochemical immunosensor, especially the amperometric immunosensor, has been one of the attractive analytical tools to achieve the above needs for the early clinical diagnosis of CEA due to its features of fast analysis, sensitive and precise measurement, inexpensive and miniaturized instrumentation[13,14]. However, the development of an electrochemical immunoassay commonly requires the immobilization of antibodies (or in some case antigens) on the transducer surface[15]. In order to ensure their long-term biological activity, it's crucial to search for an effective and simple method to immobilize them on the electrode surface[16]. Moreover, one of the shortcomings for the traditional electrochemical immunosensor is that they must be reconstructed after each determination because the irremovable immune complex formed on the electrode surface influences the next determination[17]. Thus, it should be highly significant to construct the renewable immunosensor so that the immune complex can be readily removed after each determination.

Rencently, due to their special attributes, such as magnetic properties, low toxicity and biocompatibility, magnetic nanoparticles (MNPs) have gained increasing interest and applied in biomolecules immobilization[18]. Gold nanoparticles (nano-Au) can provide a natural environment for bimolecular immobilization and facilitate the electron-transfer of biosensor

because of their high surface area, interesting electrochemical properties and good biocompatibility[19-21]. Therefore, gold-coated magnetic nanoparticles have gained more and more applications in immunoassay[22]. Fan *et al.*[23] taking advantage of a magnetic separation process and the amplification feature of colloidal gold label, used gold-coated magnetic beads for immunoassay development. Qiu *et al.*[24] has reported a dopamine biosensor fabricated by forming the 6-ferrocenylhexanethiol [$HS(CH_2)_6Fe$] functionalized Fe_3O_4(core)/Au(shell) nanoparticles (GMPs) films on the surface of a carbon paste electrode with the aid of a permanent magnet. However, most of these magnetic amperometric sensors were fabricated on conventional electrodes such as glassy carbon electrode (GCE), Au and Pt cylindrical electrodes. The electrodes described above are relatively expensive cost, bulk mass and difficulty regenerating their surface when the immunosensor have been used several times[25]. Moreover, it's not convenient to add or remove the magnet on the long cylindrical electrode to attract the magnetic nanoprobes. Recently, screen-printed carbon electrodes (SPCE) seem to be one of the most promising technologies for massive fabrication of cheap biosensors because of the advantages such as versatility for print, low cost of carbon as electrode material[26]. Amperometric biosensors fabricated *via* SPCE have been commercially used in the fabrication of personal glucose biosensors for diabetics[27]. Above all, SPCE was more convenient to add magnet than the long cylindrical electrode for immobilization of magnetic nanoprobes.

Multiwalled carbon nanotubes (MCNTs) can be modified with thionine (MCNTs-Thi) *via* π-π stacking force and act as a support of the redox mediator as well as the conductor[28] for construction of reagentless electrochemical sensor. Herein, we demonstrated the fabrication of a renewable and magnet-controlled amperometric immunoassay system for dectection of CEA using a renewable MCNTs-Thi-Nafion modified SPCE as the basic electrode (SPCE|MCNTs-Thi-Nafion). Nafion was used as membrane material for the mediator loading, which not only prevented the leakage of thionine, but also improved the antiinterference of the electrodes[29]. Then, a novel bimolecular nanoprobes using GMPs bounded with horseradish peroxidase (HRP) labeled CEA antibody (HRP anti CEA) and bovine serum albumin (BSA) were synthesized. The prepared HRP anti CEA/GMPs/BSA nanoprobes can not only provide a compatible microenvironment for maintaining the activity of the immobilized HRP anti CEA, but also can be attracted on the surface of the basic electrode by a permanent magnet. After each determination, the nanoprobes can be easily washed from the electrodes by removing the magnet, which made the basic electrode renewable only by adding new probe on its surface and effectively simplify the immobilization approach of nanoprobes. Obviously, present method can bring about a reduction in the cost, so its application in real detection is economical, simple and fast. Moreover, the incubation, washing and measurement steps can be easily controlled by the magnet with acceptable reproducibility and good stability.

EXPERIMENTAL

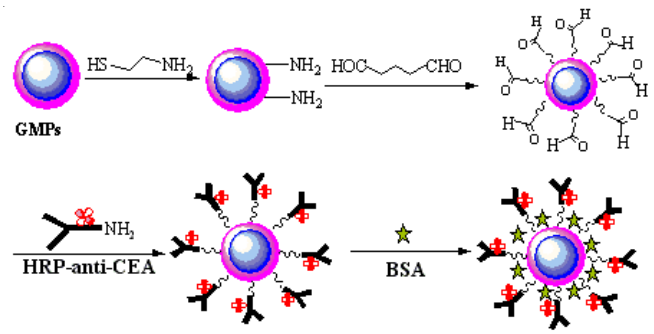
Thionine acetate and BSA were purchased from Sigma Co. Ltd. Nafion (5.0 wt %, Hesen Co. Ltd.), GMPs (particle size: 30 nm, concentration: 5 mg mL^{-1}, Shanxi Lifegen Co. Ltd.). 2-Aminoethanethiol (TCI Shanghai Co. Ltd.), toluene, glutaraldehyde solution (25 %) and H_2O_2 (30 %) were purchased from sinophram chemical reagent Co. Ltd. MCNTs (< 5 nm diameter) were purchased from Shenzhen Nanotech Port Co. Ltd. 0.1M phosphate buffer solution (PBS, pH 6.5) was used as supporting electrolyte. All other reagents were of analytical grade. CEA-ELISA kits, including 48 assay tubes consisted of a series of CEA standard solutions with various concentrations from 5-80 ng mL^{-1} and a soltuion of horseradish peroxidase conjugated murine monoclonal CEA antibody, were purchased from Autobio Diagnostics Co. Ltd. (Zhengzhou, China). All water used was double-deionized water (Milli-Q, Millipore Corporation, USA).

Cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) measurements were carried out on CHI 660B electrochemical analyzer (CH Instruments Co. USA). SPCE were purchased from eDAQ technology Corporation (Spanish). S-3400N scanning electron microscope (Hitachi, Japan) and H-7650 transmission electron microscope (Hitachi, Japan) were employed to assess the morphology of the nanoparticles.

Preparation of MCNTs-Thi and MCNTs-Thi-Nafion composite: The MCNTs were directly purified by ultrasonic techniques according to report[24] in 0.5M HCl for 4 h, then were thoroughly washed with water to get neutral state and centrifuged, finally dried under vacuum at 50 ºC overnight. A mixture of MCNTs (1 mg) and thionine (2 mg) in PBS was sonicated for 12 h, the non-conjugated thionine was removed by centrifugation and washed with water to obtain MCNTs-Thi. Finally, 1 mg MCNTs-Thi was dissolved in a solution containing 100 µL 5 % Nafion and 900 µL 0.1M pH 6.5 PBS and sonicated at room temperature for 0.5 h to prepare MCNTs-Thi-Nafion composite solution.

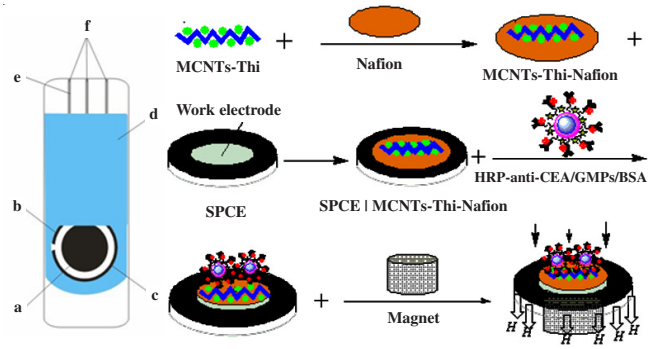
Process of HRP anti CEA and BSA assembled on the surface of GMPs: In this work, 1.5 mL GMPs (0.5 mg mL^{-1}) were mixed with 0.5 mL 5 mM 2-aminoethanethiol with mechanical stirring for 24 h. Then, the precipitate was separated by magnetic decantation and washed with double-deionized water. Afterwards, amino-functionalized GMPs were treated with 2 mL glutaraldehyde in toluene solution for 6 h at room temperature with slightly stirring. The GMPs were then separated by magnet, washed in pH 6.5 PBS and incubated in HRP-anti CEA solution (1.0 mg mL^{-1}) with shaking at 4 ºC for 12 h. Following that, the HRP anti CEA modified GMPs were treated with 3 % BSA at 37 ºC for 1 h to block the nonspecific sites. Finally, the synthesized nanoprobes were stored at 4 ºC when not in use. The schematic procedure of the preparation of the HRP anti CEA/GMPs/BSA is described in **Scheme-I**, which is similar to that reported by Fu[30].

Fabrication of immunosensor: 5 µL MCNTs-Thi-Nafion composite solution was dropped on the well-rinsed working electrode of SPCE. Then 5 µL 0.083 mg mL^{-1} nanoprobes were delivered to the above working electrode to form SPCE|MCNTs-Thi-Nafion/HRP anti CEA/ GMPs/BSA with a magnet attached to the bottom of SPCE working electrode. The immuno-sensor was stored at 4 ºC when not in use. The schematic of the three-electrode SPCE system with carbon working electrode (2 mm in diameter), carbon auxiliary



Scheme-I: Schematic illustration of the preparing procedures of HRP anti CEA and BSA functionalized GMPs

electrode and Ag/AgCl reference electrode and the procedures used for construction of the immunosensor were shown in **Scheme-II**. A nitrogen atmosphere was always maintained in all experiments.



Scheme-II: Schematic diagram of the three-electrode SPCEs system and the procedure for construction of the immunosensor. (a) Work electrode, (b) Ag/AgCl reference electrode, (c) Carbon counter electrode, (d) Insulator, (e) Electrode leader, (f) Joint

Measurement procedure: To carry out the immunoreactions and electrochemical measurement, the immunosensor array was first incubated with a 15 µL drop of the mixture of CEA standard solutions of different concentrations for 15 min at 30 ºC. Before the immunoreactions took place, the current response (I_0) was recorded. After the immunosensor incubated with CEA standard solutions, the current response of the immunosensor (I) decreased due to the immunocomplex blocking the electron-transfer between HRP and thionine. The detection of CEA level was performed by detecting the decrease in amperometric response of the immunosensor to H_2O_2 after the immunoreaction was performed. Therefore, the immunosensor current response ΔI was determined by the following equation: $\Delta I = I_0 - I$ and the percentage decrease (PD) was determined by the equation: $100 \times (I_0 - I)/I_0$.

RESULTS AND DISCUSSION

Characterization of different nanoparticles complex: Fig. 1a-c illustrates the TEM images of the synthesized MCNTs, MCNTs-Thi and MCNTs-Thi-Nafion/GMPs. An obvious difference could be discerned between the micro-structures of MCNTs (Fig. 1a) and MCNTs-Thi (Fig. 1b), MCNTs modified with thionine become thicker than that of MCNTs, which indicates that thionine was successfully conjugated with MCNTs[11]. As compared with the image

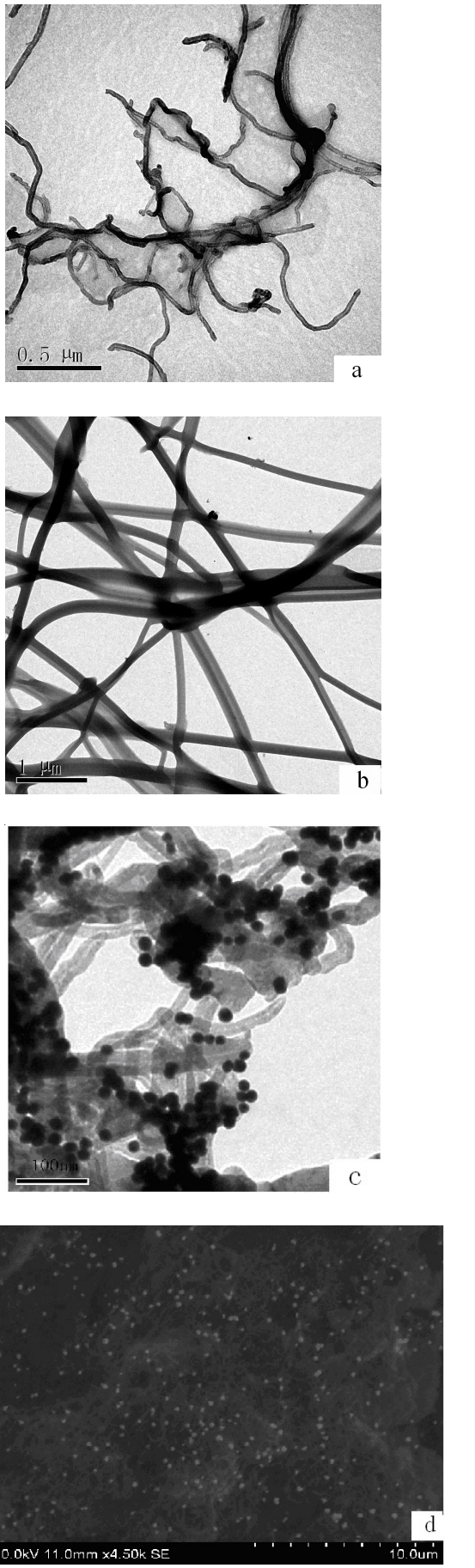


Fig. 1. TEM images of (a) MCNTs, (b) MCNTs-Thi, (c) MCNTs-Thi-Nafion/GMPs, (d) SEM image of SPCE|MCNTs-Thi-Nafion/GMPs

of MCNTs-Thi (Fig. 1b), Fig. 1c shows well dispersed GMPs were readily absorbed onto the MCNTs-Thi-Nafion composite. SEM image also shows that the GMPs were readily absorbed onto the surface of SPCE|MCNTs-Thi-Nafion with a magnet (Fig. 1d). XRFS was also used to confirm if the HRP anti CEA was assembled on the GMPs. The XRFS of nanoprobes showed the characteristic peaks of Fe (kα-6.45 keV), Au (LA-9.7 keV) and S (kα-2.32 keV) (not shown here), indicating the GMPs were modified with HRP anti CEA.

Electrochemical impedance spectroscopy (EIS) of the immunosensor: Electrochemical impedance spectroscopy is an effective method to probe the interface properties of surface-modified electrodes. In the typical impedance spectrum (presented in the form of the Nyquist plot), the semicircle portion at higher frequencies corresponds to the electron-transfer-limited process, whereas linear parts at lower frequencies representing the diffusion-limited process. The semicircle diameter in the impedance spectrum is equal to the electron-transfer resistance (Ret). Fig. 2 illustrates the impedance spectroscopy of the different modified electrodes in presence of 5.0 mM redox probe $Fe(CN)_6^{4-/3-}$. The Nyquist plot obtained at bare SPCE (Fig. 2a) exhibits an almost straight line, characteristic of a diffusion-limited electron-transfer process. When MCNTs-Thi-Nafion was applied to the electrode, the EIS of the resulting film showed a higher Ret, which may be explained by the fact that anionic SO_3^- groups in the MCNTs-Thi-Nafion electrode surface repulsed the negatively charged ferricyanide (Fig. 2b)[31]. After immobilization of GMPs to the electrode surface, the electron transfer resistance obviously decreased again (Fig. 2c), implying that the GMPs act as high electron relay for shuttling electron between the electrochemical probe and the electrode. Subsequently, when HRP anti CEA/GMPs/BSA were immobilized on the surface of MCNTs-Thi-Nafion modified basic electrode, a further increase in electron transfer resistance was observed (Fig. 2d), which indicated that the HRP anti CEA and BSA were successfully assembled on the surface of GMPs and this decreased the ability of transferring electron. After the prepared immunosensor was incubated with CEA antigen solution (Fig. 2e), a larger increase of the resistance was shown. It is ascribed to the antigen-antibody complex layer on the surface of the electrode hindering the access of redox probe towards the electrode surface[32].

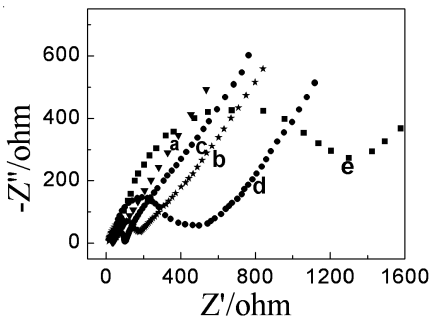


Fig. 2. Electrochemical impedance spectroscopy (EIS) of the different electrodes in the presence of 0.1M PBS (pH 6.5) + 0.1M KCl + 5.0 mM $Fe(CN)_6^{4-/3-}$: (a) bare SPCE, (b) SPCE|MCNTs-Thi-Nafion, (c) SPCE|MCNTs-Thi-Nafion/GMPs, (d) SPCE|MCNTs-Thi-Nafion/HRP anti CEA/GMPs/BSA electrode (the immunose- nsor), (e) the immunosensor incubated in 80.0 ng mL^{-1} CEA

Electrochemical behaviour of different modified electrodes: The electrochemical behaviour of different modified electrodes in 0.1M pH 6.5 PBS were studied by cyclic voltammetry and the results were shown in Fig. 3A. It could be seen that no electrochemical peak was found at the bare electrode (Fig. 3A-a). After modified with MCNTs, a greater background current was observed (Fig. 3A-b) as the MCNTs increased the effective surface area of modified electrode. When MCNTs-Thi-Nafion composite was dropped on the surface of bare electrode, a pair of stable and well-defined redox peaks appeared (Fig. 3A-c), which arose from the redox reactions of thionine. After GMPs were introduced into MCNTs-Thi-Nafion modified basic electrode, the redox peaks current increased dramatically (Fig. 3A-d) as GMPs can act as a conducting wire or an electron communication relay, which increased the electron-transfer efficiency. Compared with that of Fig. 3A-d, the HRP anti CEA/GMPs/BSA immobilized on the basic electrode enhanced the impedance of the electrode surface and the current response was reduced (Fig. 3A-e).

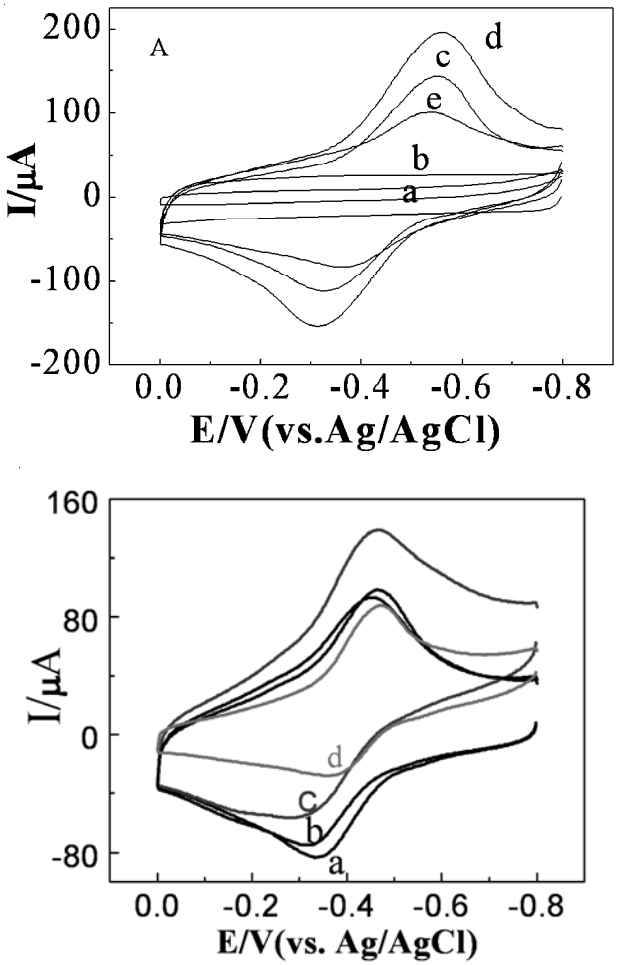


Fig. 3. (A): Cyclic voltammetric responses of different modified electrode in pH 6.5 PBS at scan rate of 100 mV/s. (a) bare SPCE, (b) SPCE|MCNTs, (c) SPCE|MCNTs-Thi-Nafion,(d) SPCE|MCNTs-Thi-Nafion/GMPs, (e) SPCE|MCNTs-Thi-Nafion/HRP anti CEA/GMPs, (B) Cyclic voltammetric responses of different modified electrode in pH 6.5 PBS with and without H_2O_2. (a): SPCE|MCNTs-Thi-Nafion/HRP anti CEA/GMPs, (b) SPCE|MCNTs-Thi-Nafion/HRP anti CEA/GMPs/BSA, (c) (b) +4 mM H_2O_2, (d) (b) incubated with 20 ng mL^{-1} CEA + 4 mM H_2O_2 at 100 mV/s

Cyclic voltammetric response of the immunosensor to H_2O_2: It was widely known that HRP can catalyze the oxidation reaction of thionine by H_2O_2[33]. The cyclic voltammograms obtained with the immunosensor in pH 6.5 PBS with and without H_2O_2 were shown in Fig. 3B. As can be seen, in the absence of H_2O_2, only a pair of oxidation/reduction peak was observed for MCNTs-Thi-Nafion/ HRP anti CEA/GMPs modified SPCE (Fig. 3B-a). Subsequently, the modified electrode was blocked with BSA solution. It could be found that the current response slightly reduced (Fig. 3B-b). The reason was that the BSA protein layers on the electrode hindered the electron transfer. However, after 4 mM H_2O_2 was added to the solution, the reduction peak current significantly increased and the oxidation peak current significantly decreased (Fig. 3B-c) at SPCE|MCNTs-Thi-Nafion/HRP anti CEA/GMPs/BSA (the immunosensor), suggesting an obvious electrocatalytic process, indicating that the HRP conjugated to anti CEA amplified the amperometric response signal of the immunosensor in the presence of H_2O_2. When the immunosensor was incubated with 20 ng mL^{-1} CEA solution, the electrocatalytic current decreased greatly (Fig. 3B-d), indicating the active center of the HRP for the catalytic oxidation of thionine was partly shielded due to the formation of immuno-complex. According to the previous reports[34,35], the mechanism for whole electrode process could be expressed as fllows:

The HRP reduces H_2O_2 to H_2O.

$$H_2O_2 + HRP\ (red) \rightarrow H_2O + HRP(ox)$$

Then the oxidized HRP converts Thi to Thi^+. This total reaction included two separate steps:

$$HRP(ox) + Thi \rightarrow HRP(II) + Thi^{\bullet}$$
$$HRP(II) + Thi^{\bullet} \rightarrow HRP(red) + Thi^+$$

where one electron was donated at a time and $Thi^{\bullet}$ represented the free radical formed during the reaction.

Thi^+ was reduced at the sensor, resulting in a cathodic current.

$$Thi^+ + H^+ + 2e^- \rightarrow Thi$$

Optimization of the immunoassay conditions

Effect of quantity of MCNTs-Thi composite and GMPs on the surface of modified electrode: The effect of MCNTs-Thi concentration incorporated in Nafion film was examined between 0.25 and 2.0 mg mL^{-1} (Fig. 4a). The current response gradually increased from 0.25-1.0 mg mL^{-1}. At higher concentrations over 1.0 mg mL^{-1}, the current response decreased may be due to the leakage of MCNTs-Thi from the Nafion film. Thus, the optimum concentration of MCNTs-Thi was selected as 1.0 mg mL^{-1}. The effect of GMPs concentration was studied between 0.0625 and 0.1 mg mL^{-1} (Fig. 4b). The current response increased from 0.0625-0.083 mg mL^{-1}. Over 0.083 mg mL^{-1}, the catalytic current decreased may be due to the aggregation of GMPs. Thus, the optimum concentration of GMPs was determined as 0.083 mg mL^{-1}.

Effect of pH of the working buffer: A strongly acidic or alkaline environment can destroy the protein microstructure and decrease protein activity. Furthermore, the redox process of thionine requires a proton. Therefore, pH would affect the electrochemical performance of thionine. The effect of solution pH on the immunosensor behaviour at various pH ranged from 5.0-8.0 was investigated. As shown in Fig. 5a, the current response had a maximum value at pH 6.5. Hence, all the experiments were carried out in pH 6.5 PBS throughout this study.

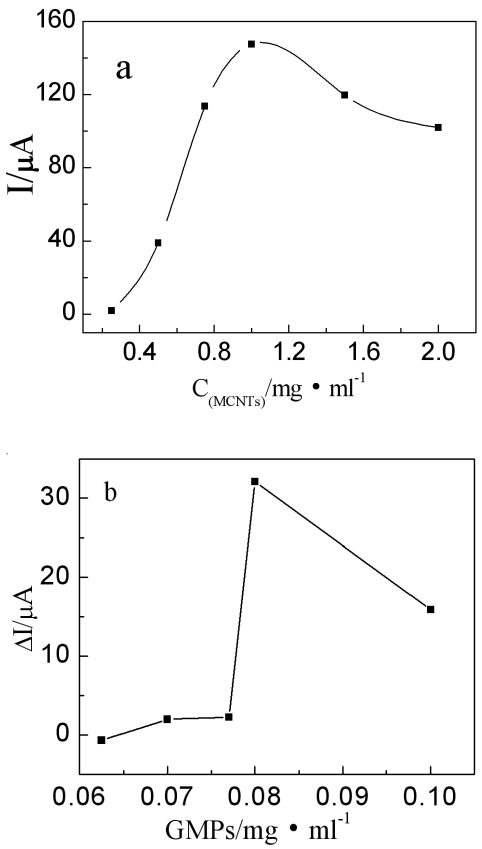


Fig. 4. Optimization of experimental parameters. (a) Influence of cocentration of MCNTs-Thi composite on the fabrication of biosensor. (b) Effect of cocentration of GMPs on the response signals

Optimization of H_2O_2 concentration: The analytical performance of the immunosensor was related to the amount of H_2O_2 in pH 6.5 PBS. Fig. 5b illustrated the current at different concentrations of H_2O_2 ranged from 0.5-5.0. At H_2O_2 concentrations less than 4 mM, the amperometric response increased with the increasing H_2O_2 concentration. When the H_2O_2 concentration was higher than 4 mM, the amperometric response tended to a constant value due to the saturation effect. Thus, 4 mM H_2O_2 was chosen for the immunoassay CEA detection.

Influence of incubation temperature and time: The formation of immunocomplex on electrode surface depends on the incubation temperature and time. The effect of incubation temperature was studied in the temperature ranged from 10-40 ºC. The ΔI increased with increasing temperature and then reached a maximum value at 35 ºC. However, when the temperature was over 40 ºC, it caused irreversible behaviour (denaturizing of proteins). Considering the activity of HRP anti CEA, 30 ºC was chosen as the incubation temperature. The response signals from 3-21 min were collected when the immunosensor was incubated in 20 ng mL^{-1} CEA. The ΔI was rapidly up with the duration of incubation time from 3-15 min and then leveled off slowly, which indicated an equilibrium state reached. Thus, 15 min was chosen to be the optimal incubation time.

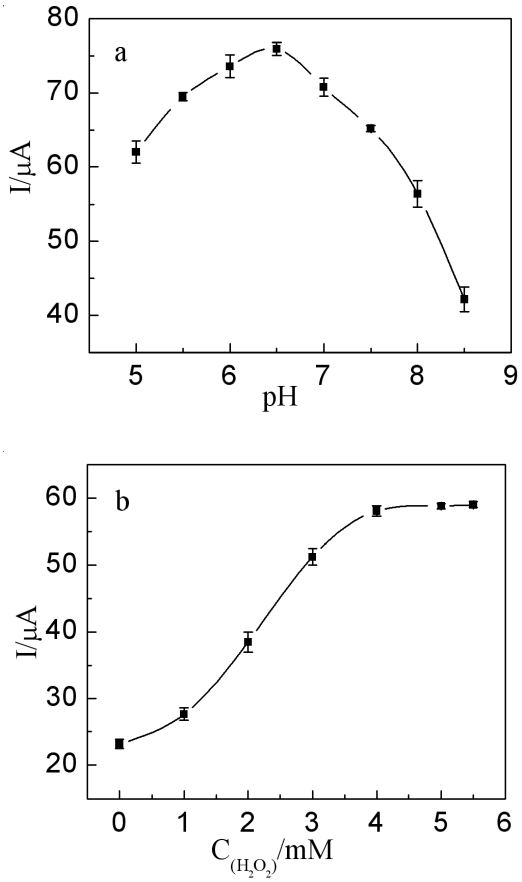


Fig. 5. (a) Influence of the pH on the sensor response. (b) Influence of concentration of H_2O_2 on amperometric response

Differential pulse voltammetry (DPV) response of the immunosensor to CEA: Fig. 6 shows DPV response of the immunosensor to different CEA concentrations under optimal immunoassay conditions. As can be seen, after the antigen reacted with the antibody immobilized on the electrode, the peak currents showed a decrease with the increment of CEA concentration. As presented in the insert of Fig. 6, at high concentration range of CEA, the PD of the DPV peak current did not change significantly, which may be due to that the CEA were almost saturated in the electrode. And the PD was proportional to the CEA concentration in two ranges: (m) from 0.1-5.0 ng mL^{-1} with a regression equation of the form PD = $8.854C_{(CEA)} + 8.369$ and a correlation coefficient of 0.992. And (n) from 5.0-80 ng mL^{-1} with a regression equation of the form PD = $0.2857C_{(CEA)}$ + 48.497 and correlation coefficient of 0.997. The detection limit was 0.03 ng mL^{-1} at a signal-to-noise ratio of 3.

Reproducibility and stability of the immunosensor: The reproducibility of the immunosensor was investigated at CEA concentration of 10 ng mL^{-1}, four immunosensors made independently, showed an acceptable reproducibility with a relative standard deviation (RSD) of 9.8 %.

The storage stability of the CEA immunosensor was tested over a 30 days period. When the CEA electrode was stored at 4 ºC and measured intermittently (every 6 days) in pH 6.5 PBS containning 4 mM H_2O_2, the immunosensor retained 87.7 % of its original response, which means that the immunosensor had a acceptable storage, indicated that this modified method

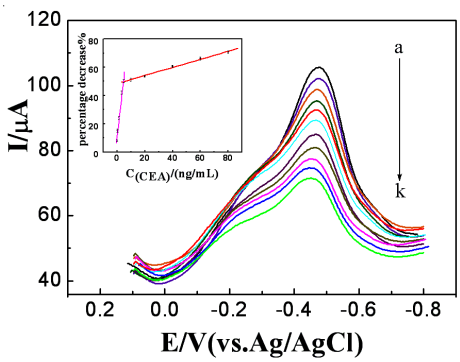


Fig. 6. The DPV responses of the proposed immunosensor in pH 6.5 H_2O_2-PBS solution after incubation with different concentrations of CEA (0, 0.1, 0.5, 1.5, 3.5, 5.0, 10, 20, 40, 60 and 80 ng mL^{-1} from a to k) at 30 ºC for 15 min. Inset: The relationship between the CEA concentration and the percentage decrease of current

was efficient in preventing the leakage of thionine mediator and the magnetic nanoprobes were firmly attached onto the surface of SPCE by magnet.

Interference: Selectivity is an important property of the immunosensor. Possible interfering substances were used to evaluate the selectivity of the present immunosensor. The immunosensors were incubated with 20.0 ng mL^{-1} CEA, respectively containing a-fetoprotein (AFP, 400 ng mL^{-1}), human IgG (HIgG, 1 μg mL^{-1}), carbohydrate antigen 19-9 (CA19-9, 20 ng mL^{-1}), human chorionic gonadotropin antigen (HCG, 20 ng mL^{-1}), BSA (2 μg mL^{-1}), ascrobic acid (AA, 2 μg mL^{-1}), dopamine (DA, 2 μg mL^{-1}) and L-lysine (2 μg mL^{-1}). The peak current responses in the CEA solutions with and without interference showed a difference of less than 4.8 %, indicating that the selectivity of the immunosensor was acceptable.

Regeneration of the immunosensor: In present work, the magnetic nanoprobes were attached on the SPCE surface with an external magnet, thus the external magnet plays an important role in the construction of the immunosensor. After each determination, the modified electrode can be easily regenerated by removing the magnet and rinsing out all the magnetic nanoprobes on the electrode surface, the basic electrode were renewable only by adding new probes on its surface. For five consecutive measurements of CEA (10 ng mL^{-1}) on the regenerated electrode, the RSD of the measured current was 7.0 %.

Application of the immunosensor to serum samples: In order to investigate the possible application of this immunosensor in clinical analysis, four different concentrations of CEA standard solution were added to normal real human serum, the immunosensor was used for determining the above solution and the results were compared with standard ELISA. As shown in Table-1, the relative error between the two methods was from -0.85-9.09 %, which indicated the developed

TABLE-1
COMPARISON OF SERUM LEVELS BY USING TWO METHODS

Added CEA value (ng/mL)	3	5	20	35
ELISA (ng/mL)	2.96	4.96	20.33	34.06
The present immunosensor (ng/mL)	3.23	4.91	20.52	35.55
RSD (n = 3) (%)	10.55	1.85	5.54	5.98
Relative deviations (%)	9.09	-0.85	0.93	4.38

immunoassay might be preliminarily applied for the determination of CEA in human serum for routine clinical diagnosis.

Conclusion

In this study, a magnet-controlled and renewable amperometric immunosensor was constructed by immobilizing HRP anti CEA/GMPs/BSA nanoprobes on MCNTs-Thi-Nafion modified electrodes. The new strategy has following advantages: Firstly, co-immobilization of thionine and HRP anti CEA on SPCE made the immunosensor could directly detect CEA after incubated with CEA solutions without the addition of electron transfer mediator in the analyte solution. Secondly, the use of GMPs as a protien supporter not only increased the electron-transfer efficiency, but also retained excellent biological activity of HRP anti CEA. Finally, after each determination, the nanoprobes can be easily washed form the basic electrode by removing the magnet, which made the basic electrode renewable only by adding new probes on its surface. The developed immunosensor can obviously bring about a reduction on the cost of usage without remobilization of basic electrode, which highlights some clear advantages of simplicity, time-saving, good stability, reproducibility and low cost. Therefore, the study provides a suitable method for the screen determination of low concentration of tumor markers in human serums and can be readily extended for determination of other clinically or environmentally interested biospecies.

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