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Preparation of Immunosensor Based on the SiO_2- Au composite for Lung Cancer-Associated Antigen Determination

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A sensitive immunosensor with gold nanoprobe for the detection of carcinoembryonic antigen (CEA) was synthesized. The immunosensor was prepared by the covalent immobilization of antibodies on Au NPs-PDA-silica/GCE which was firstly prepared by in situ deposition of Au nanoparticles on the polydopamine functionalized silica nanosphere (PDA-silica) modified GCE. Cyclic voltammetry (CV) was employed for investigating the performances of the constructed immunosensor. The immunosensors demonstrated remarkable performance for the detection of clinical lung cancer with high sensitivity of 1080 nA/ng/ml, linear correlation within the concentration ranging from 0.2 to 120.0 ng/ml and low detection limit (S/N=3) of 0.07 ng/ml. The proposed immunosensor can be successfully applied in the determination of CEA concentration through one-step immunoassay, which was signigicant for the diagnosis of clinical lung cancer.

Keywords: Lung cancer; Immunosensor; Carcinoembryonic antigen; Gold nanoparticles; Polydopamine

1. INTRODUCTION

Human lung cancer ranks first in the cancer death among men and women worldwide [1, 2]. For example, among the all cancer deaths in 2008, lung cancer accounted for 31 % and 26 % for male and female, respectively. Lung cancer is caused by the uncontrolled growth and spread of abnormal cells in lung. The abnormal cells are dysfunctional and unable to develop and differentiate into healthy lung tissue, leading to the formation of tumor that hinders oxygen supply from lung to the body by blood circulation system. In addition, the recurrence of cancer and metastasis of tumor cell may still emerge after the therapy. As a tumor marker, carcinoembryonic antigen (CEA) is of great importance to the clinical diagnosis of lung cancer [3-5]. Besides, the tumor severity can be reflected by the

concentration of CEA in serum. Therefore, to determine the level of CEA concentration accurately is highly required for the clinical diagnose of tumor severity.

Numerous immunoassay methods including radioimmunoassay [6], chemiluminescence immunoassay [7] and enzyme-linked immunosorbent assay (ELISA) [8, 9] have been extensively studied for the quantitative determination of CEA. Recently, electrochemical immunosensor has attracted considerable interests owing to many advantages such as simple, direct and specific. Moreover, the electrochemical analysis is more significant in comparison with the above-mentioned conventional techniques as to the features of low cost and time saving [8, 10-14]. The most crucial step of the construction of electrochemical immunosensor is the effective immobilization of immunoreagent on the electrode surface. Therefore, the development of simple and effective immobilization method become a hot research project.

Recently, owing to the large surface area, uniform pore size and good biocompatibility, mesoporous materials have gained a lot of attention accompanied with the development of nanomedicine [15-17]. Mesoporous SiO_2 (MS) that combined the advantages of silica and mesoporous materials have the versatility of combining with other materials such as fluorescent molecules and noble metals [18-20]. Thus, the functionalized MS can be widely applied as platform for immobilization [21, 22].

Another important factor that impacts the sensitivity of the immunosensor is signal amplification. Normally, Au nanoparticles (AuNP) modified with antibodies can be employed as signal transduction medium for electrochemical immunoassays [23]. Owing to the large specific surface area and good biocompatibility, the strong adsorption of antibody on AuNP is observed [24, 25]. Dopamine, a significant catecholaminergic neurotransmitter as the chemical messenger in mammalians [26], can polymerize spontaneously to form the polydopamine (PDA) ploymer at weakly alkaline condition [27, 28]. The obtained multifunctional PDA is a highly versatile platform that can be employed for the immobilization of biological molecules and deposition of metallic nanoparticles as well [29, 30].

In our study, a novel immunosensor contains above three components was successfully synthesized as follows: firstly Au NPs were in situ deposited on the surface of PDA functionalized silica nanospheres (PDA/silica), and then the signal antibodies were immobilized onto the surface of resulting Au NPs-PDA-silica/GCE. The immobilized amount of antibody will influence the sensitivity of the immunosensor greatly. Therefore, in order to improving the sensitivity of the immunosensor, the amount of the antibody was increased by using Au NPs modified GCE owing to the increased binding interaction between antibody and Au NPs.

2. EXPERIMENTAL

2.1. Chemicals

CEA and anti-CEA were supplied by Biocell Company (Zhengzhou, China). Chloroauric acid ($HAuCl_4$), dopamine, Bovine serum albumin (BSA, 96-99%) and human serum albumin (HSA) were supplied by Sigma Aldrich. 50 mM NaH_2PO_4 solution was mixed evenly to 50 mM Na_2HPO_4 solution

in order to obtain phosphate-buffered saline (PBS) with pH of 7.4. Certain Tween-20 (PBST) was added into 50 mL as-prepared PBS with the concentration of 0.05% (w/v) and then used as washing buffer. Certain BSA was added into 50 mL as-prepared PBS with the concentration of 2% (w/v) and then used as blocking solution. Besides, as to the functionalization of PDA, 50 mM Tris-HCl buffer with pH of 8.5 was synthesized.

2.2. Apparatus

The CHI 660 electrochemical workstation (CH Instruments, USA) with three-electrode system including modified working electrode, platinum wire auxiliary electrode and saturated calomel reference electrode (SCE) was employed for all electrochemical tests. The morphology feature of the prepared Au NPs-PDA/silica composite was performed on transmission electron microscope (JEOL 1010). For ELISA analysis, a ELISA kit for CEA determination is based on two monoclonal and one polyclonal antibody developed in Immunotech a Beckman Coulter Company. Format : two step ELISA 96 wells. Pre-analytical step: extraction in 40% ethanol and centrifugation.

2.3. Preparation of gold nanoprobe

According to the previous literature, silica nanosphere was prepared successfully and dispersed homogeneously into 50 mM Tris-HCl buffer with pH of 8.5 [31]. Subsequently, 5.0 mg dopamine was mixed into 1.0 mL of above obtained silica nanosphere dispersion (5.0 mg/mL), and the mixture was stirred at room temperature for 5 h. PDA/silica composite was obtained with centrifugation and washed with water three times. Soon afterwards, Au NPs was in situ deposited on the surface of as-prepared PDA/silica composite. Firstly, certain PDA/silica composite was re-dispersed in 2.0 mL of $HAuCl_4$ containing 10 mg citrate with the concentration of 0.25% (w/v), and then the mixture was stirred for 2 h before centrifugation. The obtained Au NPs modified PDA/silica nanocomposite was washed by water three times and re-dispersed in 2.0 mL water for further use.

2.4. Fabrication of the immunosensor

For the sake of removing adsorbed organic matter, the GCE with diameter of 4 mm was firstly polished with alumina slurries (1.0, 0.3 and 0.05 μm in order) until the mirror-like surface was obtained, then the GCE was rinsed by water and ethanol under ultrasound and dried at room temperature. Subsequently, 25 μL of as-prepared Au NPs-silica composite solution was dropped onto the GCE surface with a pipette and then dried at 4 °C overnight. The resulting Au NPs-PDA-silica/GCE was rinsed by water and then immersed in the anti-CEA solution at 4 °C for 12 h. In order to prevent the non-specific adsorption via the remaining active sites, the resulting anti-CEA- Au NPs-PDA-silica/GCE was immersed in BSA solution with the concentration of 0.25% (w/w) at 35 °C for 1 h. The constructed immunosensor was stored at 4 °C when not in use.

2.5. Electrochemical experiments

Electrochemical experiments were carried out in the electrochemical cell at 35 °C with no stirring. The scanned potential range ranged from -0.2 to 0.6 V. The selected scan rate was 50 mV/s. As to the immunoreaction, the immunosensor was immersed into 0.01 M PBS with pH of 7.0 containing CEA with various concentrations at 35 °C for 20 min. Then the concentration level of CEA was detected in the electrochemical cell with 5.0 mM $[Fe(CN)_6]^{4-/3-}$ solution containing 0.1 M KCl as probe via recording the changes of current response (ΔI) during immunoreaction.

3. RESULT AND DISCUSSION

As can be seen from the TEM image of PDA/silica composite (Fig. 1A), the diameter of PDA/silica composite was 50 nm larger than that of pure silica nanospheres, affirming the successful formation of PDA film via polymerization of monomer dopamine on the surface of silica nanospheres. Fig. 1B showed the TEM image of as-synthesized Au NPs-PDA/silica nanocomposite. A great deal of Au NPs with average size of about 15 nm was observed to be distributed uniformly on the surface of PDA/silica nanocomposite, indicating the successful synthesis of Au NPs modified PDA/silica composite. Owing to the existence of abundant catechol groups in PDA layer, $AuCl_4^-$ can be easily adsorbed onto the surface of PDA layer and then undergo in situ reduction to Au NPs without extra addition of reducing agent.

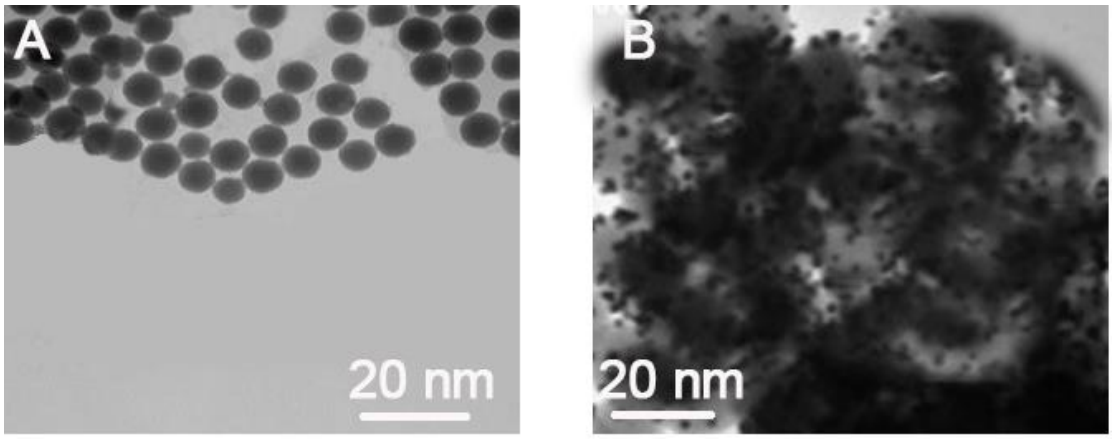

Figure 1. TEM images of (A) PDA/silica composite and (B) Au NPs modified PDA/silica composite.

The successful formation of Au NPs on the surface of PDA/silica composite was further affirmed by the UV-vis spectroscopy. As can be seen form Fig. 2A, no observable absorption peak was found in the spectrum of PDA/silica composite. Nevertheless, an obvious absorption peak centered at 504 nm was observed in the spectrum of both Au NPs and Au NPs modified PDA/silica composite. The observed peak was ascribed to the typical plasmon absorption peak of Au NPs that was synthesized via conventional citrate reduction method [32], indicating that Au NPs were successfully

in situ deposited on the surface of PDA/silica composite. It is worth noting that the absorption peak was red-shift slightly, which was possibly resulted from the surface effect of the core/shell nanospheres. The phenomenon was in consistent with previous reported results [33]. In addition, the presence of Au NPs was affirmed by the EDX spectrum as well (Fig. 2B). A novel gold nanoprobe can then be prepared by the strong interaction between antibody biomolecules and Au NPs, and the obtained nanoprobe can be applied for nonenzymatic electrochemical immunoassay. The in situ deposition method was a simple, green and effective method for the preparation of Au NPs-PDA/silica nanocomposite with high-content Au NPs. Besides, the repeatability and controllability make in situ deposition a promising technique in the application of gold nanoprobe preparation.

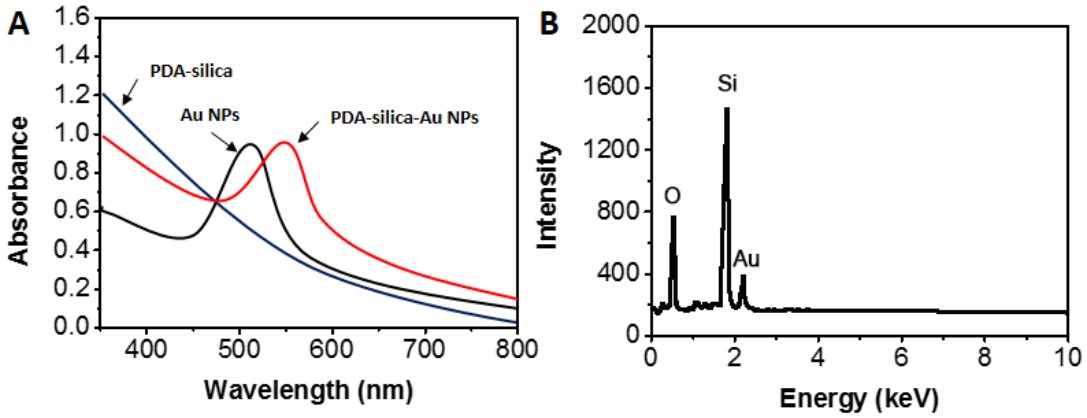

Figure 2. (A) UV-vis spectra of Au NPs, PDA/silica and Au NPs modified PDA/silica composite, respectively. (B) EDX spectrum of the Au NPs modified PDA/silica composite.

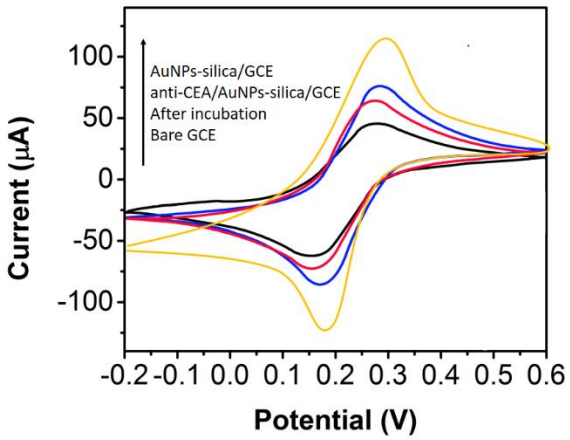

Figure 3. Cyclic voltammograms obtained on different electrodes including bare GCE, AuNPs-silica/GCE, anti-CEA/AuNPs-silica/GCE.

Fig. 3 showed cyclic voltammograms (CVs) obtained on different electrodes with 5 mM $[Fe(CN)_6]^{4-/3-}$ solution containing 0.1 M KCl as electrolyte and 50 mV/s as scan rate. A reversible CV was observed for the bare GCE. The peak current showed a great increase when the obtained Au NPs-PDA-silica/GCE was applied. However, the current response was observed to decrease with the

immobilization of anti-CEA on the surface of electrode, which was resulted from the production of anti-CEA/CEA immunocomplex. The transport of $[Fe(CN)_6]^{4-/3-}$ toward the surface of electrode was then hindered by the formed immunocomplex as inert blocking layer. And the decrease was significant after the immunosensor was immersed in the CEA solution for 20 min.

Various experimental parameters including pH value of supporting electrolyte, incubation temperature and incubation time will affect the amperometric response of as-prepared immunosensors. Herein, the effect of pH of supporting electrolyte varying 6.0 to 8.5 was investigated in detail. The immunosensor was incubated in CEA solution with the concentration of 20 ng/ml and experimental parameters including $Fe(CN)_6^{4-/3-}$ solution (5 mM) containing KCl (0.1 M) as electrolyte and 50 mV/s as scan rate were used. As shown from Fig. 4, the maximum current response was obtained at pH value of 7.0. Therefore, $Fe(CN)_6^{4-/3-}$ solution with pH value of 7.0 was used throughout the electrochemical experiments. Moreover, the neutral environmental is also favorable for real clinical application.

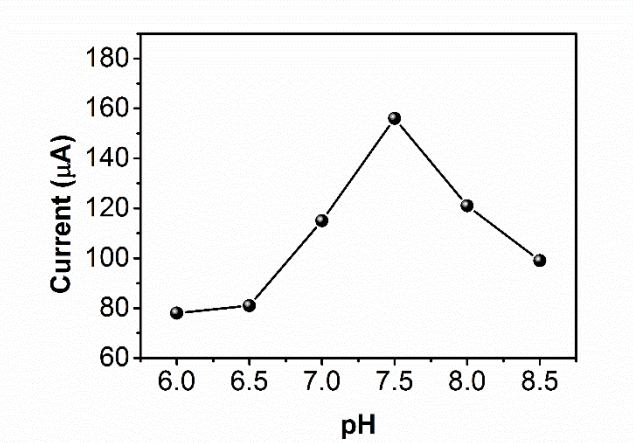

Figure 4. Amperometric response of as-prepared immunosensors in function of the pH value of supporting electrolyte.

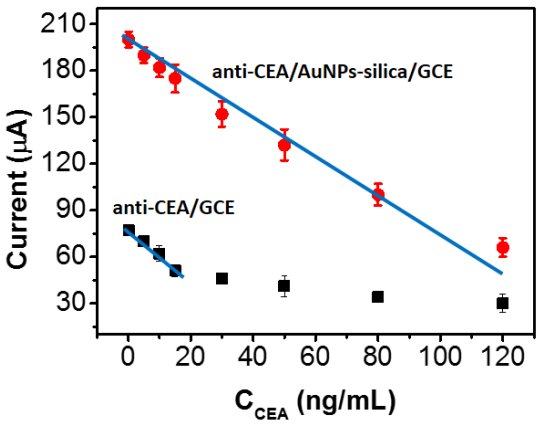

Figure 5. The peak current in function of CEA concentration obtained under optimal conditions on different immunosensors constructed from anti-CEA/GCE and anti-CEA/AuNPs-silica/GCE, respectively.

The peak current response in function of CEA concentration was investigated by applying anti-CEA/GCE and anti-CEA/AuNPs-silica/GCE. As shown in Fig. 5, higher sensitivity and wider response range were obtained on anti-CEA/AuNPs-silica/GCE in comparision with anti-CEA/GCE. As to the determination of CEA by the resulting immunosensors, the peak current was related linearly with CEA concentration ranging from 0.2 to 120.0 ng/ml with high-sensitivity of 1080 nA/ng/ml and detection limit of 0.07 ng/ml. Table 1 shows the performance comparison of our proposed sensor with linteratures.

Table 1. Comparison of proposed CEA immunosensor with previous reports.

Immunosensor	Detection range (ng/mL)	Limit of detection (ng/mL)	Reference
Thiourea modified gold electrode	0.01-10	0.01	34
Conducting long-chain polythiols	0.00001-10	0.0000015	35
Graphene-nafion	0.5-120	0.17	36
MWCNT-NH_2-PdPt nanocages	0.001-20	0.0002	37
anti-CEA/AuNPs-silica/GCE	0.2 to 120	0.07	This work

For the detection of CEA in human serum samples with no purification, the selectivity of the immunosensor to CEA is of great importance. Therefore, the selectivity of the immunosensors was investigated using the complicated CEA solution (40 ng/ml) consisting of possible interferences such as α-1-fetoprotein (40 ng/ml), hepatitis B core antigen (40 ng/ml) and hepatitis B surface antigen (40 ng/ml). CV curves exhibited that the difference of peak current responses was less than 5.5% difference, suggesting the high selectivity of as-prepared immunosensor to CEA. The results here demonstrated that the application of immunosensor in the detection of CEA in serum samples was very promising.

The regeneration performance of the immunosensors is highly critical to the industrial application. The used immunosensor could be completely regenerated by the solvent regeneration method. Firstly, the used immunosensor was immersed into the urea solution (4 M) for about 10 min, and then the immunosensors was taken out and washed with water. The reusability of the immunosensors was affirmed with the relative standard deviation (R.S.D.) of 3.9% calculated by 8 times repeated measurements. This result can compare with several previous reports such as conducting long-chain polythiols constructed immunosensor and graphene-nafion composite constructed immunosensor [35, 36].

It was found that the performance of the anti-CEA/AuNPs-silica/GCE showed a gradual decline after the storage or usage a period of time. Owing to the leakage of $Fe(CN)_6^{4-/3-}$ mediator and slow deactivation of protein, the decreased performance was unavoidable. Herein, the stability tests

about the modified electrode were carried out. The R.S.D. of 50 successive measurements in working buffer was 3.4% as calculated form the CV results. Besides, the acceptable storage stability for immunosensor was obtained with the remaining performance being 91.2% and 80.5% of initial performance after being stored at 4 °C for 60 days and 90 days, respectively. The acceptable storage stability makes the immunosensor a potential technique applied in routine clinical diagnosis for the determination of CEA in human serum.

The accuracy of the constructed immunosensor for CEA determination was evaluated by comparing the obtained results with that achieved with another powerful method namely ELISA. Table 2 showed the obtained results and relative deviations using immunosensors and ELISA. The relative errors between the used two techniques ranged from -7.5% to 9.3%, indicating that the results obtained by immunosensors was in acceptable closeness with that obtained by ELISA. Therefore, the accurate determination of CEA in clinical diagnosis by as-prepared immunosensors was achieved with satisfied results.

Table 2. Experimental results of CEA concentration in serum samples obtained by immunosensors and ELISA techniques

Serum samples	1	2	3	4	5	6
Immunosensor (ng/ml)	0.52	5.15	15.25	48.78	76.66	105.4
ELISA (ng/ml)	0.47	5.13	15.21	49.32	78.52	16.3
Relative deviation (%)	6.9	3.8	6.5	3.3	8.1	2.6

4. CONCLUSION

A novel gold nanoprobe was synthesized by the simple and controllable in situ deposition of Au NPs on the PDA/silica/GCE. The immunosensor constructed with anti-CEA/AuNPs-PDA-silica/GCE can be successfully employed for the determination of CEA conccentration with high sensitivity, low detection limit, satisfied accuracy and good storage stability as well. In conclusion, the proposed immunosensor demonstrated promising potential in the application for practical clinical determination of CEA level.

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