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Ultrasensitive electrochemical immunosensors for multiplexed determination using mesoporous platinum nanoparticles as nonenzymatic labels

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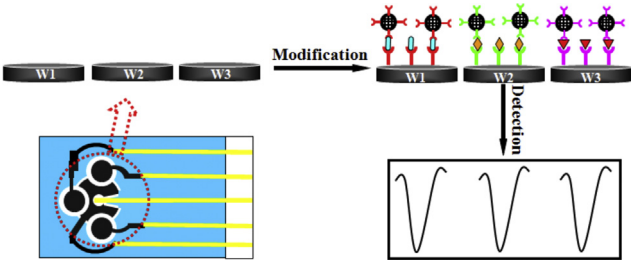
HIGHLIGHTS

- Mesoporous platinum nanoparticles as a new kind of nanomaterial, has high conductivity and electrocatalytic activity.
- The immunosensor array was proposed for simultaneous detection of three kinds of tumor markers related breast cancer.
- This was a unique realization of a nonenzymatic, which did not use natural enzyme or electrochemical redox mediator.

GRAPHICAL ABSTRACT



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ABSTRACT

An ultrasensitive multiplexed immunoassay method was developed at a disposable immunosensor array using mesoporous platinum nanoparticles (M-Pt NPs) as nonenzymatic labels. M-Pt NPs were prepared by ultrasonic method and employed to label the secondary antibody (Ab_2) for signal amplification. The immunosensor array was constructed by covalently immobilizing capture antibody (Ab_1) on graphene modified screen printed carbon electrodes (SPECs). After the sandwich-type immunoreactions, the M-Pt-Ab_2 was bound to immunosensor surface to catalyze the electro-reduction of H_2O_2 reaction, which produced detectable signals for readout of analytes. Using breast cancer related panel of tumor markers (CA125, CA153 and CEA) as model analytes, this method showed wide linear ranges of over 4 orders of magnitude with the detection limits of 0.002 U mL^{-1}, 0.001 U mL^{-1} and 7.0 pg mL^{-1} for CA125, CA153 and CEA, respectively. The disposable immunosensor array possessed excellent clinical value in cancer screening as well as convenient point of care diagnostics.

1. Introduction

Breast cancer is one of the most common malignancies. In accordance with the relevant statistics, the incidence of breast cancer accounts for 7–10% of systemic malignancies, which seriously affects women's physical and mental health and is even life-threatening [1–3]. So how to achieve the early diagnosis of breast cancer has become one of the focuses of clinical research. As known, the patients with breast cancer generally show the positive expression of three tumor markers: carcinoembryonic antigen (CEA), carbohydrate antigen CA125 and CA153 [4]. The traditional immunoassay can only detect single tumor marker with a relatively low detection sensitivity. The simultaneous

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multiplexed immunoassay is more efficient in clinical applications since it can quantitatively detect several tumor markers in a single run with improved diagnostic efficiency [5–8]. Furthermore, the multiplexed immunoassay can enhance detection throughput, shorten analytical time and decrease sampling volume as well as detection cost. Among these techniques, electrochemical immunoarrays prepared by immobilizing capture antibodies or antigens on sputter-deposited [9–12] or screen printed [13–16] electrode arrays have been successfully applied to multianalyte immunoassays. In recent years, various types of nanomaterials have been proposed as labels for signal amplification to enhance the sensitivity of immunosensors. For instance, Merkoçi et al. developed two electrochemical immunoassay methods for detection of CA153 using gold nanoparticles as label [17,18] and got satisfactory results. However, the first method could not monitor several analytes simultaneously [17], while the latter method used different enzymes to achieve the simultaneous detection [18], which needs long analytical time, complex operation and additional temperature control [7,19]. In order to overcome these disadvantages, enzyme mimics and simultaneous multianalyte immunoassays have attracted considerable attention.

Mesoporous metallic structures have extensively used in electrocatalysis [20], chemical sensing [21], and optics [22] due to their bicontinuous nanoscale skeletons and interconnected hollow channels, which are favorable for easy mass transport and high electron conductivity. Notably, mesoporous platinum nanoparticles (M-Pt NPs) display high electrocatalytic activity toward some oxidation reactions [23]. It is also interesting to explore the electrocatalytic performance of M-Pt NPs toward the reduction of small molecules and to use them as enzyme mimics for biosensing application. This study selected M-Pt NPs as labels to develop a novel nonenzymatic immunosensing method by combining with the electrocatalytic reduction of H_2O_2 and a graphene sheet (GS) modified electrode.

GS with a single layer of carbon atoms bonded together in a hexagonal lattice has attracted great attention in the fabrication of immunosensors because of their excellent electronic properties and large surface area [24–27]. Here, a simple, fast, and sensitive multiplexed electrochemical immunosensor array was proposed for simultaneous detection of three kinds of tumor markers related breast cancer based on the combination of M-Pt NPs and GS. To the best of our knowledge, this was a unique realization of a nonenzymatic sandwich-type multiplexed immunoassay, which did not use natural enzyme or electrochemical redox mediator, but took the advantages of the strong catalytic action of M-Pt NPs toward reduction of H_2O_2. The enhanced sensitivity was achieved due to the large surface area of GS for Ab_1 loading, high conductivity of GS for promoting the electron transfer, high catalytic activity and large surface area of M-Pt NPs for accelerating the reduction of H_2O_2. The untrasensitive method showed promising application in clinic screening and diagnostics.

2. Materials and methods

2.1. Apparatus and reagents

Anti-CA125, anti-CA153, anti-CEA, CA125, CA153 and CEA were purchased from E. Star BioTechnology Co., Ltd. (Shanghai, PRC). Bovine serum albumin (BSA, 96–99%) and K_2PtCl_4 were obtained from Sigma (USA). Brij 58 ($C_{16}EO_{20}$, $C_{16}H_{33}(OCH_2CH_2)_{20}$-OH) was purchased from Sangon Biological Engineering Technology & Services CO., Ltd. (Shanghai, PRC). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, PRC). All other chemicals were of analytical reagents grade and used without further purification. Phosphate buffered saline (PBS, 0.1 mol L^{-1} containing 0.1 mol L^{-1} NaCl, pH 7.4) was used as an electrolyte for all electrochemical measurements. Ultra-pure water (18.25 MΩ cm, 24 °C) was used throughout the experiments.

All electrochemical measurements were performed on a CHI 1030B electrochemical workstation (Chenhua, Shanghai, China). Transmission electron microscopic (TEM) images were obtained from a H-800 microscope (Hitachi, Japan). Scanning electronmicroscopic (SEM) images were obtained using field emission SEM (ZEISS, Germany).

2.2. Synthesis of graphene sheet

The GS was prepared by a modified method [28]. Briefly, a mixture of concentrated H_2SO_4/H_3PO_4 (360:40 mL) was added to a mixture of graphite flakes (3.0 g) and $KMnO_4$ (18.0 g), which produced a slight exotherm to 35–40 °C. The resulting mixture was then heated to 50 °C and further stirred for 12 h. Subsequently, the mixture was cooled to room temperature and poured onto ice with addition of H_2O_2 (3 mL, 30%). The filtrate was centrifuged at 8000 rpm for one hour, and the supernatant was decanted away. The remaining solid material was then washed in succession with 200 mL of water, 200 mL of 0.1 mol L^{-1} HCl, and 200 mL of ethanol. For each wash, the mixture was centrifuged at 4000 rpm for 4 h and the supernatant was decanted away. After received solid material was suspended in 10 mL water, it was filtered over a polytetrafluoroethene (PTFE) membrane with a pore size of 0.45 μm and vacuum-dried overnight at room temperature to obtain 5.8 g of product.

2.3. Preparation of M-Pt NPs

M-Pt NPs were synthesized following the method reported previously [23]. In brief, an aqueous solution of K_2PtCl_4 (5 mL, 20 mmol L^{-1}) containing Brij 58 (8.90 mmol L^{-1}) was placed in a small beaker, and then ascorbic acid (5 mL, 0.1 mol L^{-1}) was quickly added. The mixture was left in an ordinary ultrasonic bath with a 56 kHz operating frequency for 10 min. As the Pt deposition proceeded, the color of the reaction solution gradually changed, within 10 min, from transparent light brownish-yellow to brown and then to opaque black. After the reaction, the deposited Pt nanoparticles were collected by centrifugation at 16,000 rpm for 30 min, and the residual Brij 58 was removed by consecutive washing with water. The resulting solid was dried to obtain M-Pt NPs.

2.4. Preparation of M-Pt-Ab_2 bioconjugates

The M-Pt NPs (1 mg) were dispersed in 1 mL of PBS (pH 7.4) by sonication. Then, 10 μg CEA Ab_2, CA125 Ab_2 or CA153 Ab_2 was added into the solution. The mixtures were allowed to react at 4 °C under stirring for 24 h, followed by centrifugation. During this process, Ab_2 could loaded onto M-Pt NPs through the interaction between the amino groups of Ab_2 and Pt NPs [29–31]. The resulting M-Pt-Ab_2 were washed with PBS and then redispersed in 1 mL PBS, which were stored at 4 °C before use.

2.5. Preparation of immunosensor array

Fig. 1 shows the fabrication procedure of the immunosensor array that contains three graphite working electrodes (W1, W2 and W3, diameter: 3 mm). All working electrodes shared the same Ag/AgCl reference and graphite auxiliary electrodes. The insulating layer printed around the working area constituted an electrochemical microcell. Anti-CA125, anti-CA153 and anti-CEA (Ab_1) were immobilized onto the surface of the GS through an amidation reaction between the carboxylic groups on GS surface and the available amine groups of Ab_1, respectively. First, 5.0 μL of 2.5 mg mL^{-1} GS

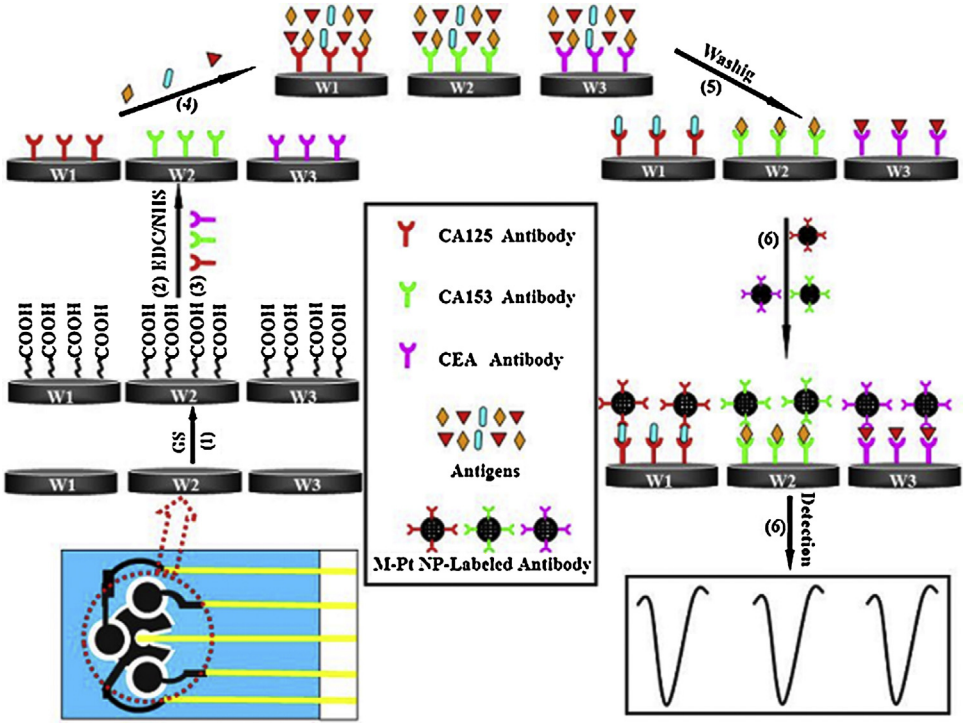

Fig. 1. Schematic representation of preparation of immunosensor array and detection strategy by sandwich-type DPV immunoassay.

was dropped on each working electrode and dried at room temperature. After activation with EDC/NHS (100 mmol L^{-1}, pH 7.4 phosphate buffer saline) for 2 h and washing with water, 5.0 μL of 50 μg mL^{-1} anti-CA125, anti-CA153 and anti-CEA were added onto the corresponding working electrodes W1, W2 and W3, respectively, and reacted at room temperature for 60 min and then 4 °C overnight. Subsequently, excess antibodies were washed with washing buffer. To prevent nonspecific adsorption, the modified working electrodes were incubated in 1% BSA solution at 37 °C for one hour to eliminate the nonspecific binding between the proteins and the electrode surface. After washing with PBS, the immunosensor array was stored at 4 °C before use.

2.6. Simultaneous immunoassay procedure

A sandwich immunoassay was used for simultaneous determination of three cancer markers (CA125, CA153, CEA). The mixture of 50 μL PBS (pH 7.4) and 15 μL of CA125, CA153 and CEA solutions at known concentrations or samples was dropped on the surface of entire immunosensor array and incubated at room temperature for 60 min. After washing with PBS, 5.0 μL M-Pt-CA125 Ab_2, M-Pt-CA153 Ab_2, M-Pt-CEA Ab_2 were dropped to the corresponding immunosensors and incubated for 60 min. The residual reactants were removed by washing with PBS after the incubation process. Then, the electrochemical detection was performed in a 50 μL PBS (pH 7.4) containing 5.0 mmol L^{-1} H_2O_2. The concentrations of multiplexed analytes could be simultaneously determined by monitoring the corresponding signals with CHI 1030B electrochemical workstation.

3. Results and discussion

3.1. Characterization of M-Pt NPs and GS

This work used M-Pt NPs as the label due to their incompact nanohorn structure, larger specific surface area and stronger catalytic activity toward reduction of H_2O_2 than gold nanoparticles or silver nanoparticles. The SEM and TEM images of M-Pt NPs showed a uniform morphology, and every NP consisted of plentiful interconnected nanohorns with around 3 nm in width (Fig. 2a and b). The diameter of the M-Pt NPs was about 30 nm. The incompact nanohorn structure of M-Pt NPs was favorable for mass transport of H_2O_2. The energy-dispersive X-ray spectrum (EDS) showed that the M-Pt NPs consisted of Pt and a small quantity of O and C elements (Fig. 2c). The latter came from the rudimental reducing agent. The TEM and SEM images of the obtained GS showed a wrinkled paper-like structure, which was transparent with irregular size (Fig. 2d and e).

3.2. Electrochemical characterization of immunosensor

The electrochemical impedance measurements in 0.1 mol L^{-1} KNO_3 containing 5.0 mmol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution were used to characterize the conjugation of Ab_2 to the M-Pt NPs. All three M-Pt NPs modified SPCEs showed an electron transfer resistance of around 180 Ω, while the SPCEs modified with three kinds of the resulting M-Pt-Ab_2 at the same amount as that of M-Pt NPs showed a much increased resistance values (Fig. 3), which was due to the insulating properties of the proteins, indicating the successful conjugation of the signal antibody.

The cyclic voltammograms (CVs) of bare SPCEs in oxygen-free PBS did not show any detectable signal (Fig. 4, curves a). After GS was coated on the SPCEs, the background currents increased (Fig. 4, curves b), indicating that GS significantly increased the roughness of electrode surface. The GS/Ab_1/Ag/M-Pt-Ab_2 modified SPCEs for three kinds of antibodies showed greater backgrounds with a weak peak at around −0.3 V (Fig. 4, curves c), which was attributed to the reduction of small quantity of rudimental species in M-Pt NPs and little dissolved oxygen. After adding 5 mmol L^{-1} H_2O_2 in PBS, the CVs showed obvious reduction peak of H_2O_2 (Fig. 4, curves d), which resulted from the electrocatalytic activity of M-Pt NPs toward the reduction of H_2O_2. The peak current of H_2O_2 depended on the amount of M-Pt NPs on the surface of SPCEs, which could be controlled by changing the concentration of antigen for sandwich-type

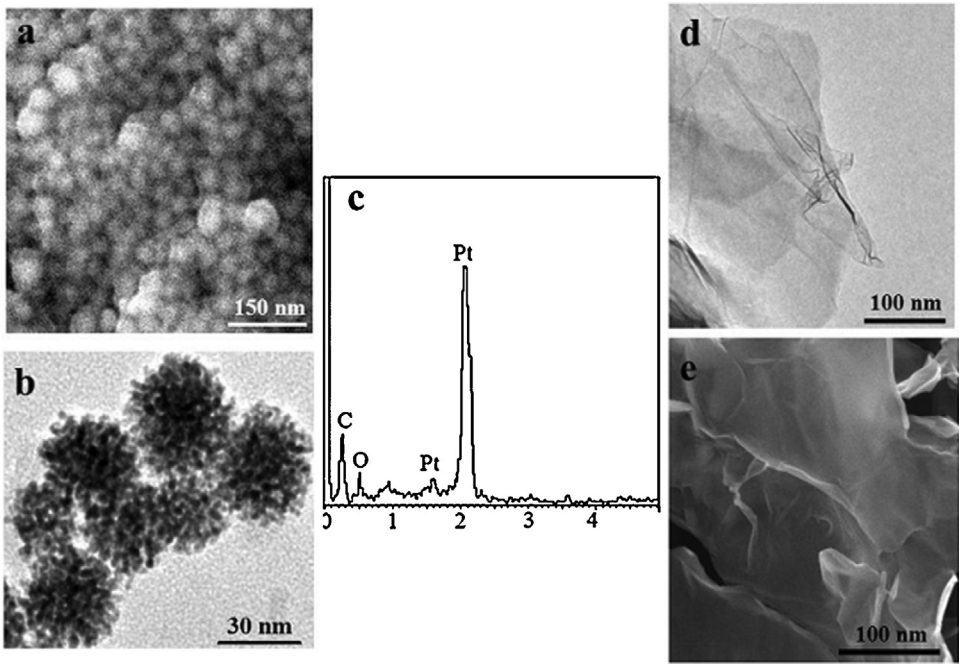

Fig. 2. SEM (a) and TEM (b) images and energy-dispersive X-ray spectrum (c) of M-Pt NPs, and TEM (d) and SEM (e) images of GS.

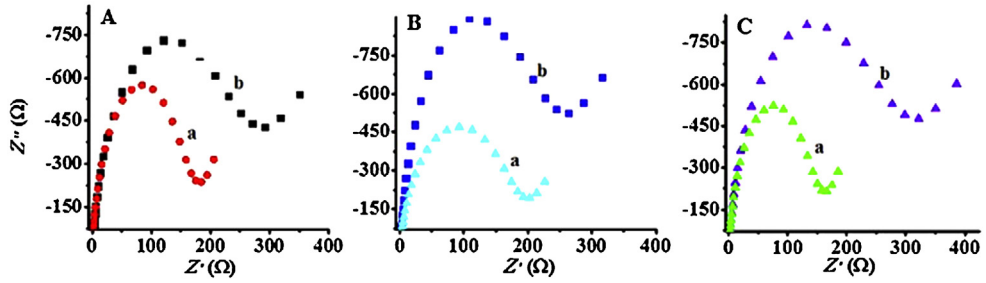

Fig. 3. Electrochemical impedance spectra recorded from 0.1 to 10^5 Hz at M-Pt NPs (a) and M-Pt-Ab_2 (b) modified SPCEs for signal antibodies of anti-CA125 (A), anti-CA153 (B) and anti-CEA (C).

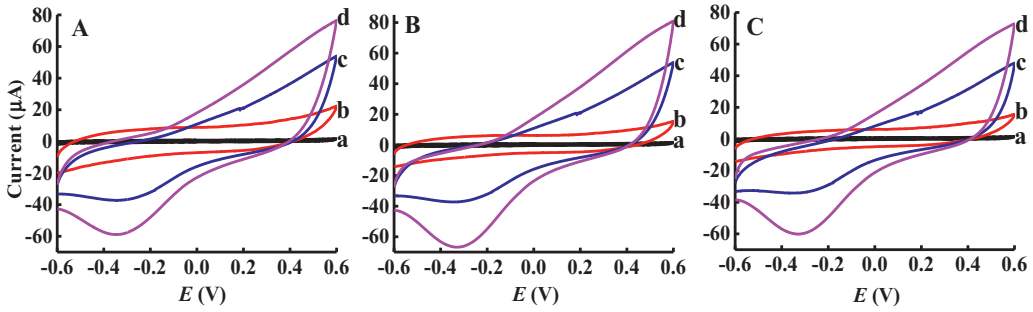

Fig. 4. Cyclic voltammograms of (a) bare, (b) GS modified, (c) GS/Ab_1/Ag/M-Pt-Ab_2 modified SPCEs in oxygen-free PBS and (d) GS/Ab_1/Ag/M-Pt-Ab_2 modified SPCEs in PBS containing 5 mmol L^{-1} H_2O_2 for 5.0 U mL^{-1} CA125 (A), 5.0 U mL^{-1} CA153 (B) and 4.0 ng mL^{-1} CEA (C). Scan rate: 0.1 V s^{-1}.

immunoreactions, leading to a sensitive method for immunoassay of the proteins.

3.3. Condition optimization for immunoassay

The amount of GS for preparation of immunosensors was one important parameter, which affected both the loading of capture antibody (Ab_1) and the electrochemical behaviors of the GS coated electrode. With the increasing concentration of GS, the peak currents at three immunosensors for detection of CA125 (5 U mL^{-1}), CA153 (5 U mL^{-1}) and CEA (4 ng mL^{-1}) increased due to the enhanced loading of Ab_1 for bound more antigens and then M-Pt-Ab_2 (Fig. 5A). However, when the concentration of GS was more than 2.5 mg mL^{-1}, the peak currents gradually decreased due to the increased GS thickness, which was unfavorable to the mass transport of antigen and electron transfer. So 5.0 μL of 2.5 mg mL^{-1} GS was used for preparation of the immunosensors.

The incubation time was another important parameter affecting the analytical performance of immunoassay. At room temperature, the CV responses for CA125, CA153 and CEA increased with the

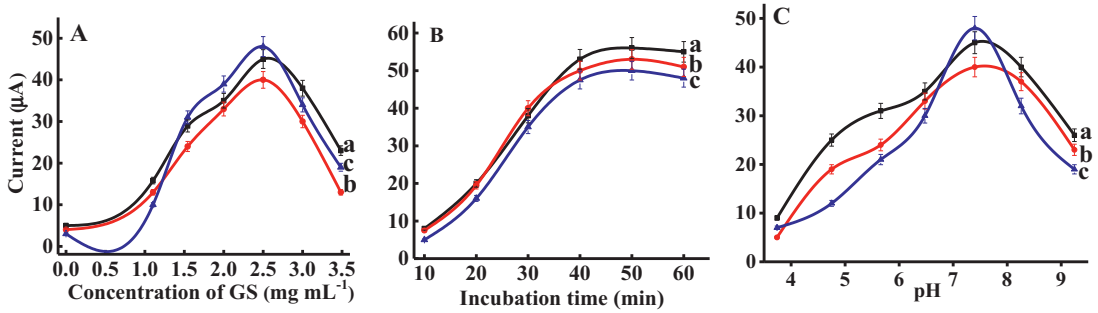

Fig. 5. Effects of (A) GS concentration for preparation of immunosensor array, (B) incubation time and (C) pH of detection solution on CV response for CA125 (a), CA153 (b) and CEA (c).

increasing incubation time and then tended to constant values after 40 min (Fig. 5B), which showed the saturated binding both between the analyte and the capture antibody and between the complexed analyte and M-Pt-Ab_2 on electrode surface. Therefore, an incubation time of 40 min was selected for the immunosensor array.

The reduction of H_2O_2 involved the participation of proton, thus the pH of detection solution was optimized in the pH range from 3.5 to 9.0. As shown in Fig. 5C, the CV response showed a maximum current at pH 7.4. Thus, pH 7.4 PBS was used for immunoassay.

3.4. Immunoassay performance

Under the optimized conditions, the immunoassay performance of the proposed immunosensor array was evaluated for the detection of CA125, CA153 and CEA. As shown in Fig. 6, the differential pulse voltammetric (DPV) peak currents of 5 mmol L^{-1} H_2O_2 at three immunosensors increased with the increase of antigen concentrations (A–C). The plots of peak currents *vs.* the logarithm of antigen concentrations showed good linearity in the ranges of 0.05–20 U mL^{-1} CA125 (R = 0.9988), 0.008–24 U mL^{-1} CA153 (R = 0.9962) and 0.02–20 ng mL^{-1} CEA (R = 0.9927) (D–F). Their limits of detection were 0.002 U mL^{-1}, 0.001 U mL^{-1} and 7.0 pg mL^{-1}, respectively. Compared with previously reported immunosensors (as shown in Table 1), the proposed electrochemical multiplexed immunosensor showed higher sensitivity.

3.5. Evaluation of cross-reactivity and specificity

The proposed electrochemical multiplexed immunoassay method did not need mediator in the detection solution; thus no electrochemical cross-talk occurred among neighboring electrodes. The cross-reactivity among the antibodies and

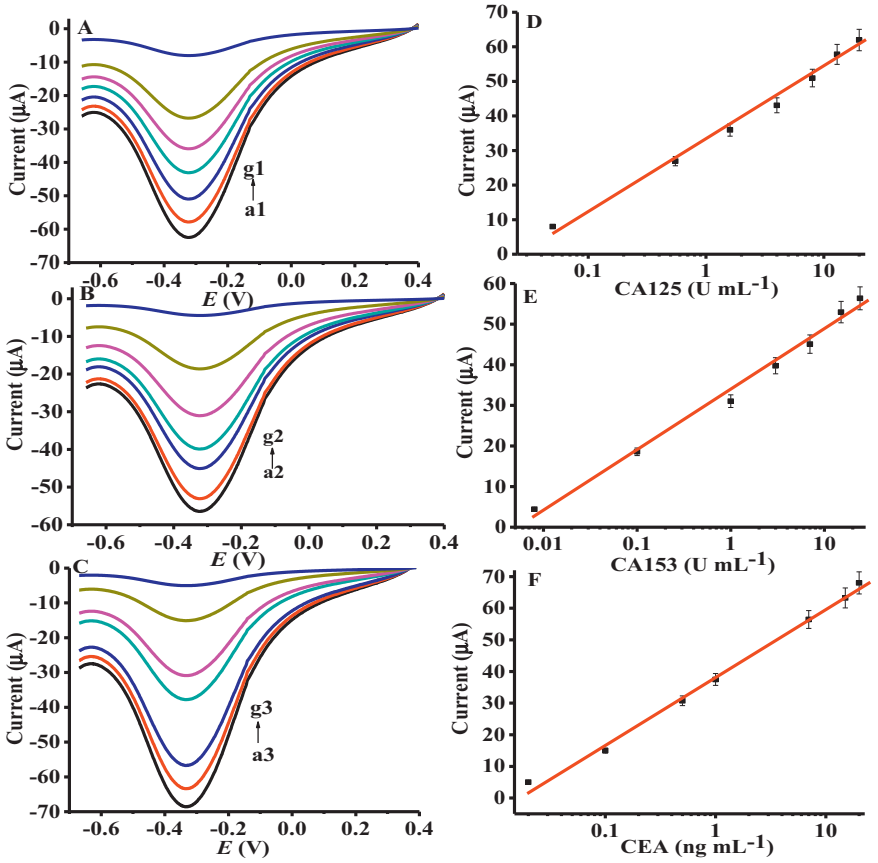

Fig. 6. Differential pulse voltammograms for detection of 20, 15, 8, 4, 2, 0.5 and 0.05 U mL^{-1} CA125 (A from a1 to g1), 24, 15, 8, 4, 1, 0.1 and 0.008 U mL^{-1} CA153 (B from a2 to g2) and 20, 15, 7, 2, 0.5, 0.1 and 0.02 ng mL^{-1} CEA (C from a3 to g4) and their calibration curves (D, E and F).

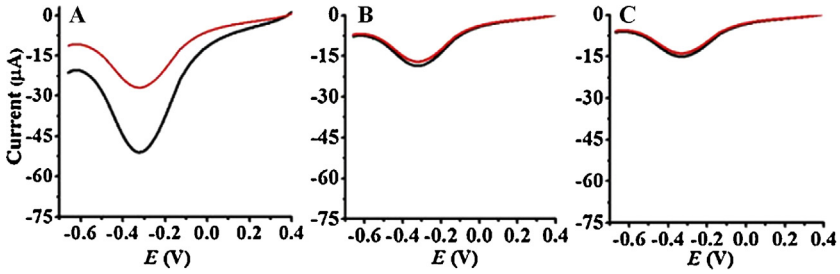

Fig. 7. Cross-reactivity evaluation of the immunosensor array: Differential pulse voltammograms for 1.0 U mL^{-1} CA125 (A), 0.1 U mL^{-1} CA153 (B) and 0.2 ng mL^{-1} CEA (C) before (black) and after (red) addition of 5.0 U mL^{-1} CA125. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
A comparison of the performance of the proposed and referenced immunosensors for tumor markers.

Analyte	Linear range	Detection limit	Reference
CA125 (U mL^{-1})	0.1–450	0.1	[32]
	1.0–60	0.49	[33]
	0.05–20	0.002	This work
CA153 (U mL^{-1})	2.0–100	0.8	[34]
	0.084–16	0.06	[7]
	0.008–24	0.001	This work
CEA (ng mL^{-1})	2.5–120	1.2	[35]
	1.0–80	0.6	[36]
	0.02–20	0.007	This work

nonspecific antigens was studied by incubating the immunosensor in two mixtures containing 1.0 U mL^{-1} CA125, 0.1 U mL^{-1} CA153 and 0.2 ng mL^{-1} CEA, and 6.0 U mL^{-1} CA125, 0.1 U mL^{-1} CA153 and 0.2 ng mL^{-1} CEA, respectively. For the first mixture, the immunosensors could show their corresponding responses (Fig. 7, red curve). After increasing CA125 concentration, the response at the immunosensor for CA125 obviously increased, while the immunosensors for both CA153 and CEA did not show any change (Fig. 7, black curve). Similarly, the change of CA153 and CEA concentrations did not also arouse the difference of response at other immunosensors. Therefore, cross-reactivity at the array was negligible, making it possible for multiplexed immunoassay of the three tumor markers in a single run without interfering with each other. These results also indicated the immunosensors and proposed method were of good specificity.

3.6. Selectivity, reproducibility and stability of immunosensor array

Besides the negligible cross-talk among immunosensors, these immunosensors did not show statistically significant change of DPV responses after alpha fetoprotein or human chorionic gonadotropin was added into the detection solution containing 4.0 U mL^{-1} CA125, 4.0 U mL^{-1} CA153 and 4.0 ng mL^{-1} CEA as interfering substance (Fig. 8). Thus the selectivity of the immunosensor array was acceptable.

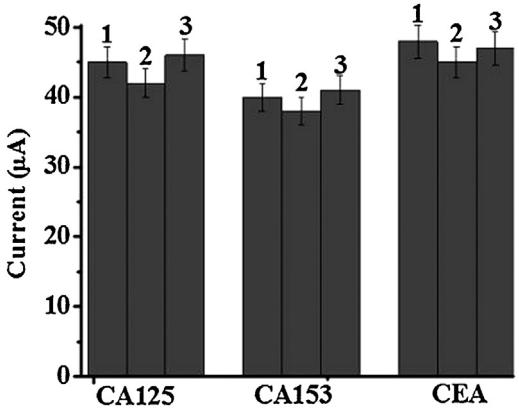

Fig. 8. DPV peak currents for immunosensor array of (1) 4.0 U mL^{-1} or ng mL^{-1} analytes, (2) (1) + 400 ng mL^{-1} alpha fetoprotein, and (3) (1) + 400 ng mL^{-1} human chorionic gonadotropin.

To evaluate the fabrication reproducibility of the immunosensor array, five arrays were used for the detection of a mixture containing 5.0 U mL^{-1} CA125, 7.0 U mL^{-1} CA153 and 2.0 ng mL^{-1} CEA. The relative standard deviations (RSD) of the DPV responses at corresponding immunosensors were 4.3, 2.7 and 5.6%, respectively, which suggested that the reproducibility of the proposed immunosensor array was quite good. After the immunosensor array was stored in pH 7.4 PBS for one month, no obvious change in the detection response to the same CA125, CA153 and CEA concentration was observed. Thus, the stability of the immunosensor array was also acceptable.

3.7. Serum sample analysis

To demonstrate the practical application of the immunosensor array, the detection of CA125, CA153 and CEA in serum samples was performed using a standard addition method. After 5.0 U mL^{-1} CA125, 4.0 U mL^{-1} CA153 and 3.0 ng mL^{-1} CEA were added into serum samples, the average recovery of the immunosensors was 103.6% (n = 5), 99.4% (n = 5) and 98.7% (n = 5), respectively (Table 2). Hence, the developed immunoassay methodology could

Table 2
Results for CA125, CA153 and CEA determination in samples.

Analyte	Content in sample	Addition	Detection content					RSD (%)	Recovery (%)
CA125 (U mL^{-1})	2.0	5.0	6.8	7.3	6.9	6.7	7.2	2.4	99.7
CA153 (U mL^{-1})	2.0	4.0	6.4	5.9	6.3	5.7	5.9	2.4	100.7
CEA (ng mL^{-1})	2.0	3.0	5.1	4.9	4.7	5.3	4.6	2.4	98.4

be satisfactorily applied to the clinical determination of CA125, CA153 and CEA levels in serum samples.

4. Conclusions

This work demonstrated a convenient and effective method for multiplexed immunoassay using a GS-based immunosensor array with M-Pt NPs as nonenzymatic labels. Using CA125, CA153 and CEA as a model panel of tumor markers for breast cancer, the immunosensor array could be simply prepared by covalently immobilizing the capture antibodies onto the surface of a GS-modified SPCE array. The M-Pt NPs showed high electrocatalytic activity toward reduction of H_2O_2 and an incompact nanohorn structure for efficient labeling of signal antibodies and quick mass transport of H_2O_2. The M-Pt NPs as labels possessed the advantages of low cost, simple labeling and good stability. The proposed multiplexed immunoassay method showed high sensitivity, negligible cross-talk, good selectivity and reproducibility, and acceptable stability, providing potential application in clinical diagnostics.

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