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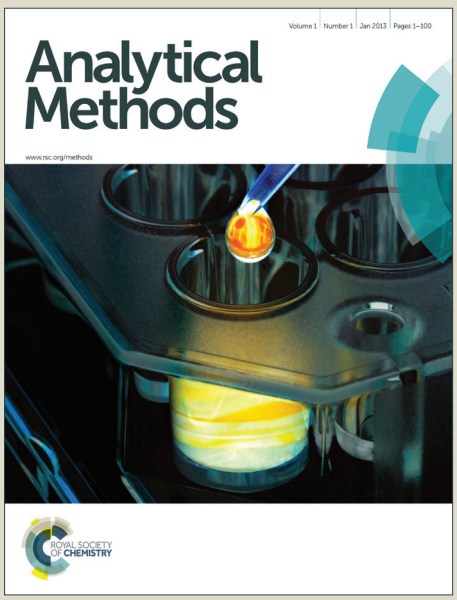

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An electrochemical immunosensor for ultrasensitive detection of HBsAg based on the platinum nanoparticles loaded on the natural montmorillonite

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Abstract

A sensitive and facile electrochemical immunosensor for ultrasensitive detection of Hepatitis B surface antigen (HBsAg) was designed, which was based on platinum nanoparticles decorated amino silanes functionalized montmorillonite (Pt-NH_2-MMT). As luminosilicate clay mineral, the advantages of montmorillonite (MMT), such as large specific surface area, good adsorption/ion exchange capacity and innocuousness, endow Pt-NH_2-MMT with better sensing performance. The platinum nanoparticles (Pt NPs) also play an important part for their good biological compatibility, electron transport rate and catalytic activity. Pt NPs and the available amine group of second HBsAg-antibody could be bounded strongly with each other. And the covalent bond between NH_2-MMT and the carboxyl group of primary HBsAg-antibody could be formed by the activated of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide /N-hydroxysuccinimide. Under optimal conditions, the Pt-NH_2-MMT showed high electrocatalytic activity toward hydrogen peroxide reduction. Under optimal conditions, the immunosensor showed a wide linear response to HBsAg ranging from 0.5 pg/mL to 20 ng/mL with a low detection limit of 2.0×10^{-4} ng/mL. The designed immunosensor displayed high sensitivity, stability and reproducibility for the analysis of HBsAg.

Keywords: Montmorillonite; Hepatitis B surface antigen; sandwitch-type immunosensor; platinum nanoparticles

1. Introduction

As a major health concern worldwide, Hepatitis B virus (HBV) infection causes about one million deaths annually[1]. Hepatitis B surface antigen (HBsAg) is the cornerstone for diagnosis of acute or chronic HBV infection, and a predictive biomarker which largely affect the customize therapy and treatment outcomes of individual patients[2].

In the past few years, various sensing methods have been developed for the HBsAg detection, such as radioimmunoassay[3], enzyme-linked immunosorbent assay[4], chemiluminescence[5] and so on. In addition, electrochemical immunoassay, based on the principle of specific binding of antibody and antigen[6, 7], has been developed for detection of disease in clinical diagnosis. It shows obvious the advantages of high sensitivity, low cost and simple pretreatment procedure[8, 9]. Meanwhile, this technique has been widely applied in product safety[10], environmental detection[11], clinical medicine[12, 13], etc. For the sandwich-type electrochemical immunosensor, many kinds of labels were used to conjugate secondary antibodies for signal amplification[14, 15].

Nanomaterials as the biomarkers could improve the performance of markers and enhance the sensitivity of chemical analysis. Montmorillonite (MMT) is a natural inorganic mineral which has the abundant slit-like mesopores for the plate-like clay layers. The specific surface area of the raw MMT is usually very tiny, which cannot be directly used as the support of catalyst. But, it could increase largely after modifications such as pillared method or acid treatment[16]. Thanks to the great efforts of researchers, MMT is now widely used in catalysis[17, 18], nanocomposites[19],

electrode[20], sensors[21, 22] and adsorption[23], antibacterial[24] and so on. Okutomo et al[25] used the dodecyltrimenthylammonium-magadiite treated by chloro-silicane in this research. The result showed that chloro-silicane could be modified in the face of organic MMT and had little effect on the distance of two layers. From the work of Park et al[26], intercalated/surface-modified MMT could be obtained by using MMT and amino silanes with the treatment of acetic acid. On this basise, we successfully modified the sodium montmorillonites (Na-MMT) with amino silanes as second antibody label. The unique layers structure of MMT could combined nanoparticles, nanosheets, nanowires and other species to construct the biosensor. Noble nanoparticle has excellent biological compatibility and electron transport rate. In addition, the Pt NPs, mimicking natural peroxidases, could perform the good catalytic activity and develop the sensitive electrochemical immunosensor. Saikat Mandal et al[27] proved amine groups bound very strongly to Pt NPs and showed good catalytic activity[28]. The Pt-NH_2-MMT, with large surface area, excellent biocompatibility, good conductivity and remarkable catalytic performance, is a good electrocatalytic material as label in the constructing of electrochemical immunosensor.

With excellent electronic transport ability[29], high specific surface area[30] and good biological compatibility, amination graphene (NH_2-GS) could blind a large of Ab_1 on the surface which has crucial effect on the fabrication of immunosensor. In this research, we designed a sandwitch-type electrochemical immunosensor to achieve the quantitative detection of HBsAg. The primary HBsAg-antibody (Ab_1) was immobilized onto NH_2-GS with the covalent bonding due to the activation of

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/ N-hydroxysuccinimide (EDC/NHS) [31-33], and the second HBsAg-antibody (Ab_2) was bonded to Pt-NH_2-MMT by the Pt NPs. Applying the principle of specific binding of antibody and antigen[34], the current response was directly on the concentration of HBsAg in the sample. For HBsAg analysis with the fabricated immunosensor, a wider linear range from 0.5 pg/mL to 20 ng/mL and a lower detection limit of 0.2 pg/mL were obtained.

2. Experimental section

2.1 Materials and reagents

The HBsAg and HBsAg antibody (Ab) were gained from Shanghai Linc-Bio Science Co. Ltd (Shanghai, China). Bovine serum albumin (BSA, 96-99%) was obtained from Sigma Aldrich (USA). Graphite, chloroplatinic acid (H_2PtCl_6), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 3-aminopropyltrimethoxysilane, and other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Phosphate buffered solutions (PBS) were prepared by compounding the solutions of KH_2PO_4 (0.067 mol/L) and Na_2HPO_4 (0.067 mol/L) to proper pH values. All other chemicals were analytical reagents grade and ultrapure water were used throughout this work.

2.2 Apparatus

All electrochemical tests were performed by the CHI760D electrochemical workstation (Chenhua, China). A conventional three-electrode configuration which contained a glassy carbon electrode (GCE, 4 mm diameter) as working electrode,

saturated calomel electrode (SCE) as the reference electrode and platinum wire as the counter electrode was used. Scanning electron microscope (SEM) was recorded by JEOL JSM-6700F microscope (Japan). And the fourier transform infrared spectroscopy (FTIR) spectrum was obtained from Spectrum One FT-IR Spectrometer (PerkinElmer).

2.3 Synthesis of NH_2-GS

Graphite oxide (GO) was synthesized through the Hummers' method[35], and NH_2-GS was prepared according to a reported method. Typically, 0.6 g graphite flakes and 3.6 g potassium permanganate ($KMnO_4$) were added to flask which was placed with a mixture of concentrated sulfuric acid and phosphoric acid (H_2SO_4:H_3PO_4=72:8 mL) ahead of time. Then, the reaction was kept at 50 ºC and stirred for 12 h to oxidize the graphite flakes and exfoliate the multilayer graphite. After that, it was cooled to room temperature and poured slowly onto the 80 mL of ice which prepared in advanced. And 30% of H_2O_2 was added on the ice dropwise then stirred for 30 min. Subsequently, the obtained brown dispersion was centrifuged at 8000 rpm for 30 min to remove any un-exfoliated graphite oxide (an extremely small amount), then washed with 0.2 mol/L HCl solution three times and aether once. The resultant solid was dried in a vacuum oven at 35 ºC overnight and GO was prepared well.

To prepare the NH_2-GS[35], 100 mg GO was added to 75 mL distilled water and they were sonicated for 1 h. Then, 0.2 mL 3-aminopropyltrimethoxysilane (99 wt%) was added to the mixture, heated to 70 ºC and stirred for 1.5 h. Next, 100 μL

hydrazine (80%) was added into the above dispersion under stirring for 1 h at 100 ºC. After the reaction, the precipitate was filtered and washed with distilled water, then dried at 60 ºC for 24 h for further usage.

2.4 Preparation of amino silanes functionalized montmorillonites (NH_2-MMT)

Na-MMT and NH_2-MMT were prepared according to the reported methods. [36, 37]According to Yan et al's methed [36], 25 g of natural MMT was dispersed in 250 mL 0.5 mol/L NaCl and shaken for 24 h. After that, the clear supernatant was removed, then it was dispersed again in 125 mL 0.5 mol/L NaCl and shaken for12 h. Then, the remaining solid was washed by distilled water until there was free of chloride ions tested by $AgNO_3$ solution. At the end, the product was dried at 50 ºC, then grinded, griddled and saved for further usage.

The NH_2-MMT was prepared by silane coupling agent (3-aminopropyltrimethoxysilane) under the treatment of aceticacid. 1.6 mL 3-aminopropyltrimethoxysilane with 8 mL anhydrous ethanol was mixed and the same volume of acetic acid was dropped in it with stirring. Then 1 g Na-MMT was added to the acetic acid mixture which contained 15 mL anhydrous ethanol and the mixture was stirred for 8 h at 80 ºC. After centrifugation, the obtained NH_2-MMT was further washed with distilled water to neutral pH and dried at 60 ºC under vacuum.

2.5 Preparation of the Pt-NH_2-MMT-Ab_2

The Pt-NH_2-MMT electrocatalyst was prepared by a two-step method[38]. Typically, the nitrogen was passed through a 300 mL solution containing 0.4 mmol/L

$CoCl_2 \cdot 6H_2O$ and 0.4 mmol/L citric acid for 15 min. In this process, the citric acid was used as stabilizator. 20 mL 40 mmol/L sodium borohydride solution ($NaBH_4$) was added to the solution with vigorous stirred for 1 h to insure the $NaBH_4$ was consumed. Under the vigorous stirring, 100 mL 0.8 mmol/L of H_2PtCl_6 was dropped to the mixture and the colour changed to blackish brown. After reacted for 60 min, a solution with 50 mg NH_2-MMT was added to the blackish brown solution and they were stirred for 60 min. At the end, the precipitate was filtered and the filtrate was clear and colorless, washed by distilled water and acetone. Then, the product was dried under vacuum oven at 80 ºC for 12 h.

Fig. 1A shows the preparation procedure of the Pt-NH_2-MMT-Ab_2. The Ab_2 was immobilized onto Pt-NH_2-MMT through the cross-linking reaction between the amino of the Ab_2 and Pt NPs that attached on the NH_2-MMT. Generally, a solution of Pt-NH_2-MMT (2.4 mg/mL, 1 mL) was added to Ab_2 dispersion (10 μg/mL, 1 mL) and under mild shaking for 12 h at 4 ºC. After centrifugation, 1 mL of PBS (pH=7.4) was added to the precipitation, and the Ab_2 could be adsorbed by Pt NPs and loaded on the surface of the MMT. Then the resulting was stored at 4 ºC for further usage.

2.6 Modification of immunosensors

The illustration of the sandwich-type electrochemical immunosensor fabrication procedure is shown in Fig. 1B. GCE was polished carefully with 0.05 μm alumina powder respectively. Afterwards, NH_2-GS solution was dropped onto the surface of GCE. After dried, the electrode was activated with 3 μL 75 mmol/L EDC-15 mmol/L NHS and 6 μL of Ab_1 for 2 h. By the covalent bonding of the carboxyl groups of Ab_1

and amino groups on the NH_2-GS, 6.0 μL 10 μg/mL of Ab_1 was immobilized on the electrode tightly [39]. Then, 3 μL 1 wt% BSA was incubated for 1 h to eliminate nonspecific binding sites. Afterwards, 6 μL HBsAg solutions with various concentrations were modified onto the electrode surface. And washed with the PBS (pH=7.4) to remove unbound HBsAg molecules, after incubated for 1 h. Finally, the prepared Pt-NH_2-MMT-Ab_2 was added onto the electrode surface. After another 1 h, the surface was washed with the PBS (pH=7.4) once more and the prepared electrode was stored at 4 ºC.

2.7 Electrochemical measurements

The potential scanning of amperometric *i-t* curve method was selected at -0.4 V, which was used for all amperometric measurement of the immunosensors. The PBS (pH=7.4) was used for all the electrochemical measurements. When the background current of the amperometric measurement of the immunosensor was stabilized, 10 μL 5.0 mol/L H_2O_2 was injected into 10 mL PBS (pH=7.4) to form a uniform solution (H_2O_2, 5.0 mmol/L) under mild stirring, and the current change was recorded. The current change in response to the immunological reaction was registered as the sensor signal relative to the varying concentrations of HBsAg. The electrochemical impedance spectroscopy (EIS) was scanned in the solution containing 2.5 mmol/L $K_3[Fe(CN)_6]$ and 0.1 mol/L KCl.

3. **Results and discussion**

3.1 Characterization of NH_2-GS, NH_2-MMT and Pt-NH_2-MMT

As shown in Fig. 2A, the NH_2-GS with a rippled, flake-like structure has large surface area to immobilize Ab_1. And the structure of NH_2-MMT is similar with NH_2-GS, which is presented transparent, paper-like and layers structure as shown in Fig. 2B. The sample of Na-MMT (Fig. 2C, curve a) presents the strong absorption peak at 1025 cm^{-1} which is attributed to the silicon-oxygen tetrahedron of Si-O-Si asymmetric stretching vibrations[37]. And the three peaks at 690~890 cm^{-1} are attributed to the Si-C stretching vibrations of organosilicon. Bands at 3624 cm^{-1} and 3432 cm^{-1} are originated from the clay lattice of –OH stretching vibrations and the deformation of adsorbed H_2O of Na-MMT. These are the characteristic absorption peaks of MMT. As shown in Fig. 2C curve b, the clay lattice –OH stretching vibrations and the adsorbed H_2O deformation of peak at 3624 cm^{-1} and 3432 cm^{-1} reduces, for enhancing the MMT hydrophobic with the 3-aminopropyltrimethoxysilane modified. And the new peak at 1557 cm^{-1} associated with the $–NH_2$ bending vibrations. The changes of the characteristic absorption peaks could perform that the 3-aminopropyltrimethoxysilane was attached on the MMT successfully. As shown in Fig. 2D, Pt NPs were adsorbed on the surface of NH_2-MMT which could be used for incubating Ab_2.

3.2 Characterization of the Pt-NH_2-MMT

The sensitivity of the electrochemical immunosensor depends on the high electrocatalytic property of Pt-NH_2-MMT towarding the reduction of H_2O_2. For the purpose of comparison, NH_2-GS, NH_2-MMT and Pt-NH_2-MMT were modified onto the electrodes surface and the results of catalyzing H_2O_2 were shown in Fig.3A.

According to amperometric *i-t* curve, we could find that the current responses of NH_2-GS (curve a) and NH_2-MMT (curve b) were too small to make it out. As expected, Pt-NH_2-MMT (curve c) exhibited a largest current change indicating that the Pt-NH_2-MMT could be a potential material as a label of the immunosensor.

As showed in Fig. 3B, there is no obvious current response in NH_2-MMT (curve a) and Pt-NH_2-MMT (curve b).When they were scanned in the pH=7.4 PBS with the addition of H_2O_2, a dramatic increase of the current is observed, it indicates that the Pt-NH_2-MMT (curve c) has a good electrocatalytic performance towarding the catalysis of H_2O_2.

3.3 Optimization of experimental conditions

In order to obtain best analytical performance for HBsAg, all the steps of the experiment were optimized. And the HBsAg concentration of all the optimization of experimental conditions was 5 ng/mL.

The pH of the PBS plays an important factor in the performance of immunosensors for that the immobilized protein would be damaged by the highly acidic or alkaline surroundings. With this matter, the pH of the PBS was optimized from 4.2 to 9.0 by amperometric *i-t* curve. As shown in Fig. 4A, the pH of PBS for all electrochemical measurement was chosen at 7.4.

The NH_2-GS is used to enhance the specific surface area to connect more Ab_1 and capture more antigens and signal tags, thus the concentration of NH_2-GS in the electrode modification is an important factor on the electrochemical immunosensor. As shown in Fig. 4B, with increasing the concentration of NH_2-GS, the current

response increased from 0.2 to 1.4 mg/mL and decreased from 1.4 to 1.8 mg/mL. Thus, 1.4 mg/mL of NH_2-GS was chosen for all subsequent experiments.

The concentration of Pt-NH_2-MMT is also an important parameter affecting the sensitivity of the immunosensor. It could affect the catalytic performance for the reduction of H_2O_2. As shown in Fig. 4C, with the increase of the concentration of Pt-NH_2-MMT, the current response increased with different growth rate. Obviously, the growth rates from 0.4 to 2.4 mg/mL were faster than it from 2.4 to 3.2 mg/mL. The optional concentration of Pt-NH_2-MMT was 2.4 mg/mL.

3.4 EIS characterization of immunosensor

The impedance of electrode is consisted by the real (Z') and imaginary (Z'') parts.

$$Z = Z' + j\,Z''$$

The Nyquist plots is a plot of -Z'' versus Z', and it is the most commonly data representation method [40]. The impedance spectra consist of a semicircle portion and a linear portion. The semicircle portion at high frequency region is associated with the electrochemical process subjecting to electron transfer, where the diameter corresponds to the resistance [41]. As a result, resistance change could be judged by the diameter change of semicircle portion. Fig. 5 shows the Nyquist plots of the EIS in the process of modifying electrode.

The bare GCE (curve a) exhibited a very small semicircle domain implying a very low electron transfer resistance in the electrolyte solution. After the electrode was modified with NH_2-GS, the resistance is much smaller than that of the bare GCE.

The reason for this observation was that NH_2-GS is an excellent electrically conducting material, which has good electron transfer ability (curve b). The resistance after Ab_1 modified electrodes increased significantly (curve c) demonstrated that Ab_1 was immobilized on the electrode successfully and blocked the electrons transfer. Similarly, the resistance increased after the addition of BSA (curve d), HBsAg（curve e） and Ab_2-Pt-NH_2-MMT (curve f), because the proteide substance modified on the electrode could hinder the electronic conductivity. The results indicated that the electrode was modified successfully.

3.5 Assay performance

Under the optimum conditions, the Pt-NH_2-MMT was used as label to detect different concentrations of HBsAg in PBS of pH=7.4 (Fig. 6A). And the current response increased linearly with the concentrations of the HBsAg in the range from 0.5 pg/mL to 20 ng/mL with a detection limit of 0.16 pg/mL. The calibration curve was ΔI (μA) =26.341+5.576×lgC (ng/mL) and with a correlation coefficient of 0.992 (Fig. 6B). The fabricated immunosensor based on Pt-NH_2-MMT and NH_2-GS has wider linear range lower detection limit. The results indicated that the proposed method could be used to detect HBsAg concentration quantitatively.

3.6 Reproducibility, selectivity and stability

To evaluate the reproducibility of the immunosensor, a series of eleven electrodes were prepared for the detection of 0.05, 0.5 and 5 ng/mL HBsAg (Table. S1). The amperometric responses of the measurement for these electrodes were

showed in Table. S1 and the relative standard deviation (RSD) of the results were less than 5.0%, suggesting that the reproducibility and precision of the proposed immunosensor was quite good.

The selectivity of the immunosensor was performed using BSA, human immunoglobulins (H-IgG), ascorbic acid (Vc) and glucose, as interference substances. The current responses of the immunosensors for the 1.0 ng/mL of HBsAg solution containing 100 ng/mL of interfering substances were measured and the detection results were 0.951, 1.008, 1.029, 0.955. The current variations were from 0.78% to 4.87% and the RSD was 3.92%, indicating that the selectivity of the immunosensor was acceptable.

The stability of the immunosensor was also studied by checking their current responses periodically. The immunosensors were stored at 4 ºC and the current response of immunosensor has no apparent change after one week storage. After two weeks, the current response, compared with the immunosensors without being stored, decreased 5.80%. Indicating find the stability of the immunosensor is at the acceptable range.

3.7 Real sample analysis

In order to evaluate the feasibility of this designed immunosensors, the serum was prepared by standard addition method, which was used HBsAg added into HBsAg-free human blood serum. Then, 0.05, 0.5 and 1.0 ng/mL of HBsAg solutions were added into serum samples (Table 1). The RSD of the results was from 0.888 % and 2.94 % and the recovery was in the range of 98.4 % to 108 %. Consequently, the

novel immunosensor could be clinically acceptable to detect the HBsAg in serum samples.

4. Conclusions

In this investigation, we have designed a novel sandwich electrochemical immunosensor using Pt-NH_2-MMT as labels and NH_2-GS as substrate to detect HBsAg. The NH_2-GS on the electrode not only facilitate electrons to move freely but also provide a large accessible surface area for the immobilization of abundant Ab_1 bound by the covalent bond. The Pt-NH_2-MMT could increase the amount of redox probes to amplificate the response signals. Thus the designed immunosensor exhibited extraordinary electrochemical biocatalysis and sensitivity. The immunosensor fabricated by these materials shows excellent analytical performance for the measurement of HBsAg with low detection limit, good sensitivity, reproducibility and stability. Thus the developed immunosensor could provide significant potential to other protein analysis and diagnosis studies.

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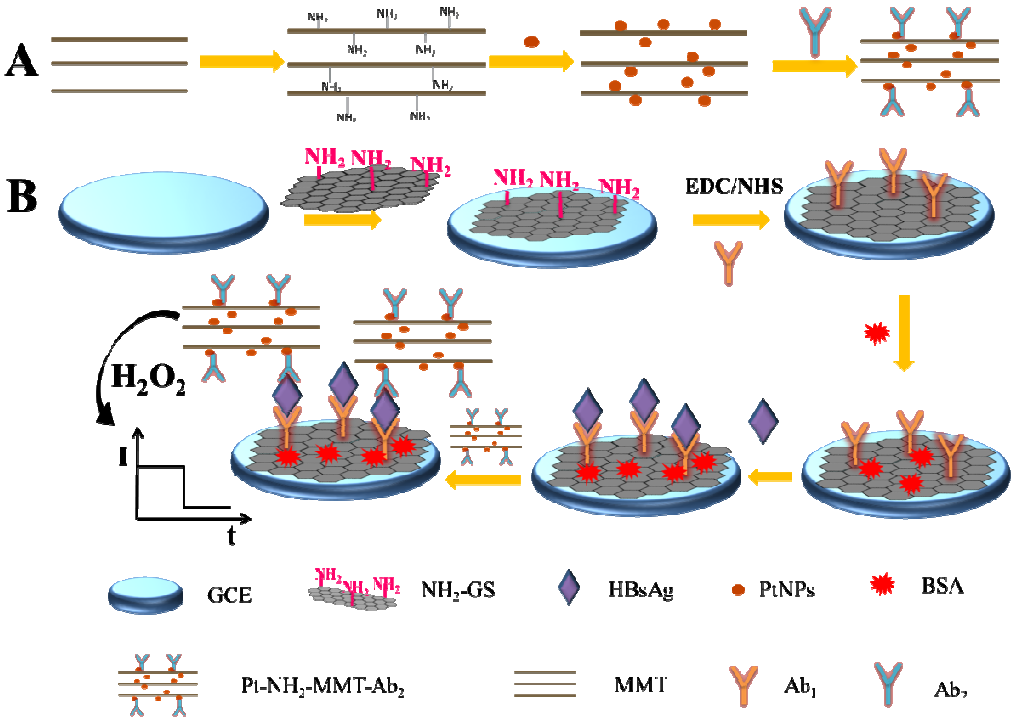

Fig.1 schematic illustration of the stepwise preparation of the Pt-NH_2-MMT (A) and immunosensor (B)

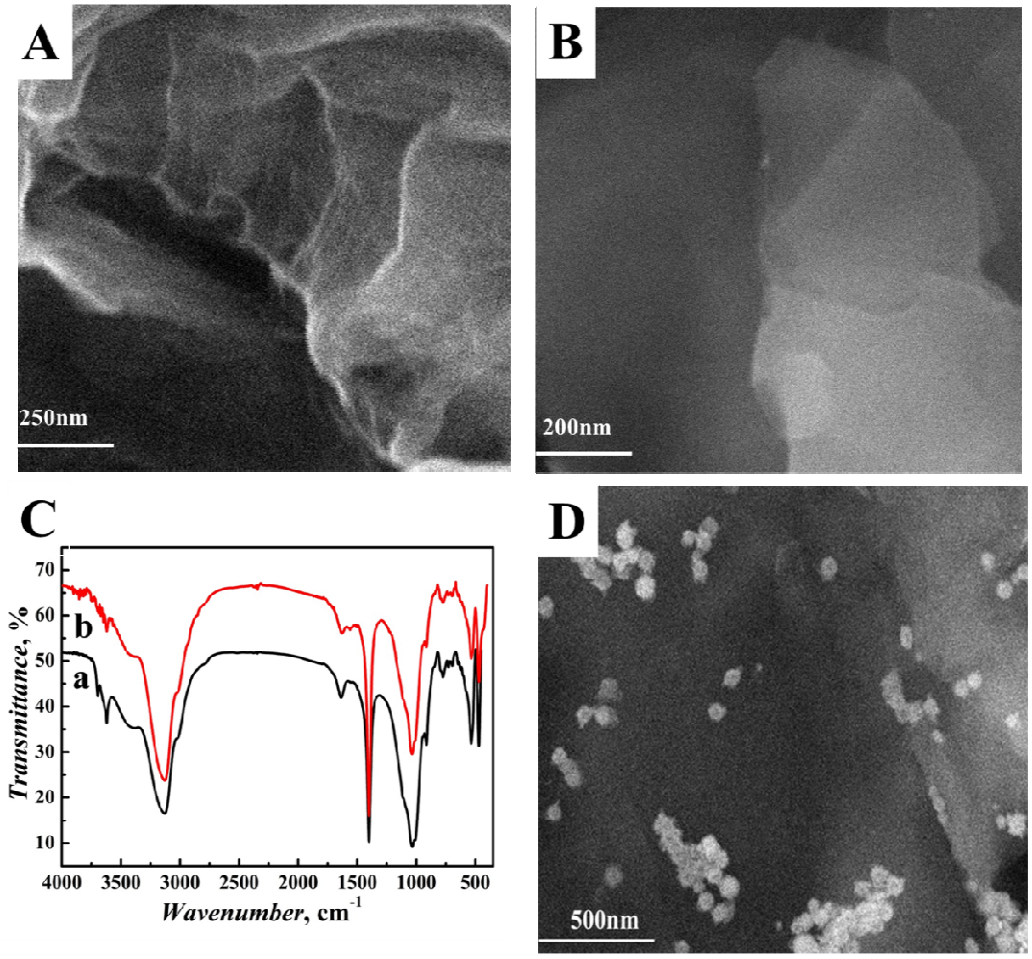

Fig.2 SEM images of NH_2-GS (A), NH_2-MMT (B) and Pt-NH_2-MMT (D), (C) FTIR of Na-MMT (a) and NH_2-MMT (b).

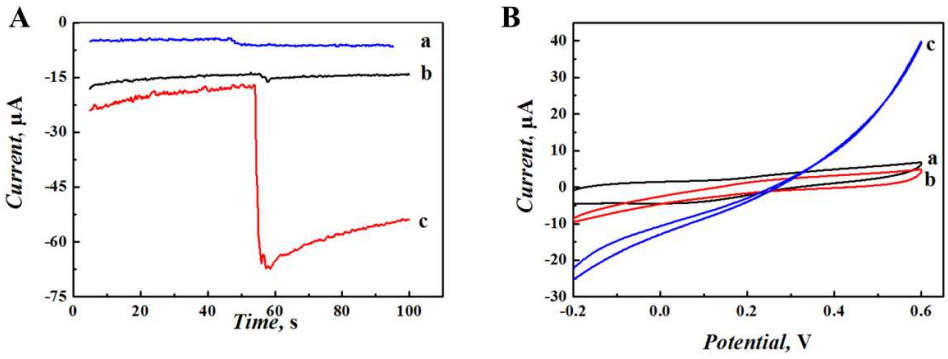

Fig. 3 (A) Ameperometic responses of the immunosensors with different labels: (a) NH_2-GS, (b) NH_2-MMT and (c) Pt-NH_2-MMT, (B) CVs of the immunosensor: NH_2-MMT (a) and Pt-NH_2-MMT (b) as label in PBS at pH=7.4, and Pt-NH_2-MMT (c) with the addition of 5 mmol/L of H_2O_2.

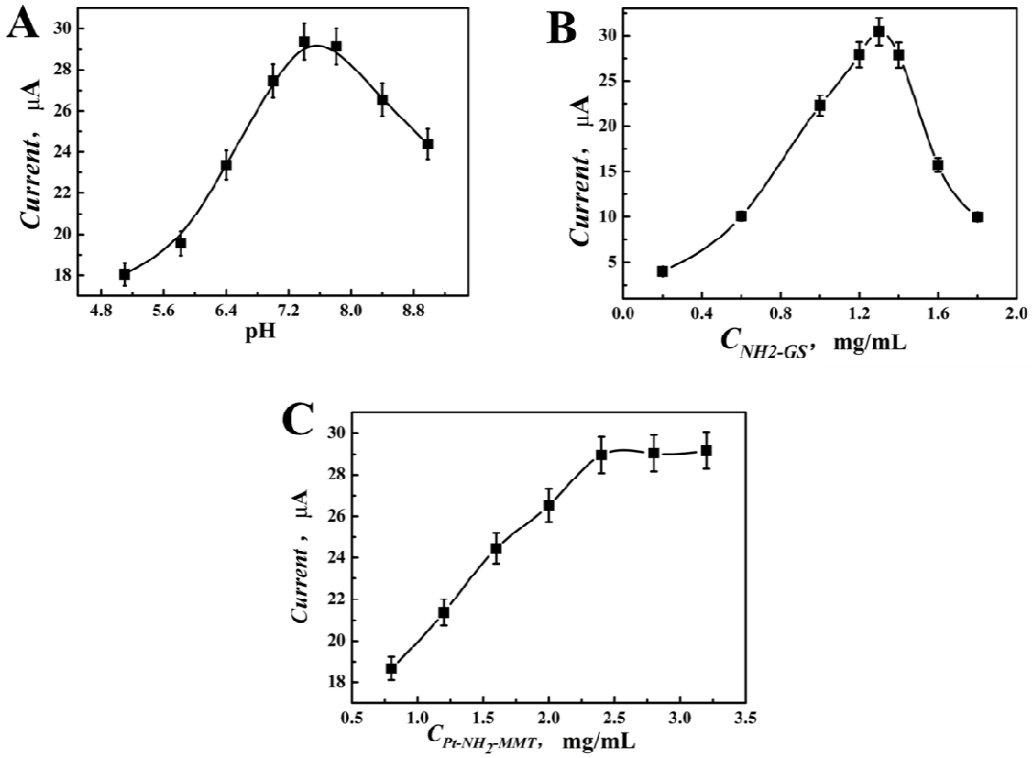

Fig.4 The optimization of experimental conditions with pH (A), NH_2-GS concentration (B) and Pt-NH_2-MMT concentration (C). Error bar=SD (n=3).

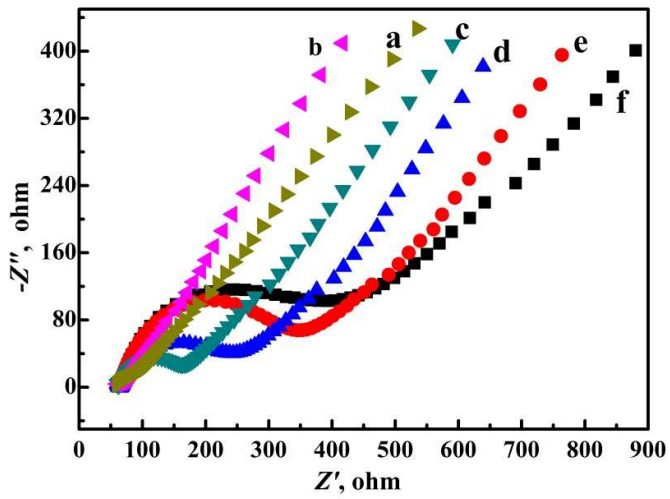

Fig.5 EIS obtained for different modified electrodes in $Fe(CN)_6^{3-/4-}$ containing 0.1 mmol/L KCl solution, (a) GCE, (b) NH_2-GS/GCE, (c) Ab_1/NH_2-GS/GCE, (d) BSA/Ab_1/NH_2-GS/GCE, (e) HBsAg/BSA/Ab_1/NH_2-GS/GCE and (f) Pt-NH_2-MMT-Ab_2/HBsAg/BSA/Ab_1/NH_2-GS/GCE

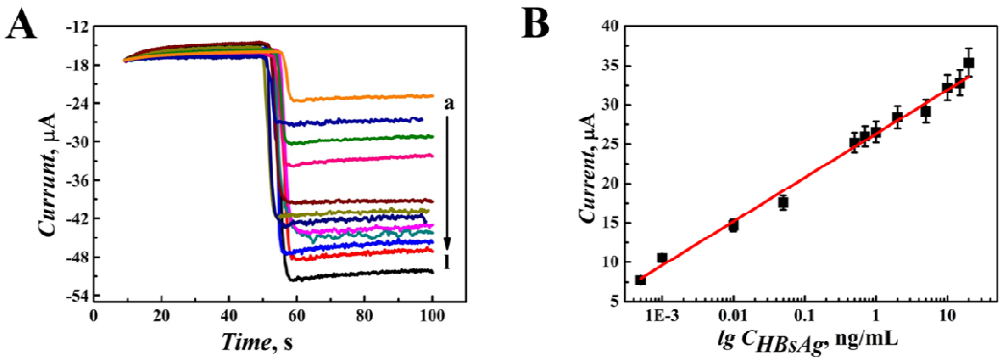

Fig.6 (A) Amperometric response of the immunosensor for the varied concentration of HBsAg in a uniform PBS (5 mmol/L H_2O_2), with the concentration of (ng/mL) (a) 0.0005,(b) 0.001, (c) 0.01, (d) 0.05, (e) 0.5, (f) 0.7, (g) 1.0, (h) 2.0, (i) 5.0, (j) 10.0, (k) 15.0, (l) 20. (B) Calibration curve of the immunosensor toward different concentrations of HBsAg. Error bar=SD (n=5).