A Sensitive Label-free Amperometric CEA Immunosensor Based on Graphene-Nafion Nanocomposite Film as an Enhanced Sensing Platform

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A novel approach to fabricate a label-free amperometric immunosensor for the detection of carcinoembryonic antigen (CEA) was described. Herein, methylene blue (MB), gold nanoparticles (AuNPs) and carcinoembryonic antibody (anti-CEA) were layer-by-layer assembled on the graphene-Nafion nanocomposite film-modified electrode by means of a self-assembling technique and the opposite-charged adsorption. Subsequently, the stepwise self-assembling procedure of the immunosensor was further characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The factors influencing the performance of the resulting immunosensor were studied in detail. The developed procedure showed improved features, including larger amount and higher immunoactivity of the immobilized antibody and repeatable regeneration of the sensor, as well as direct, rapid and simple determination for the antigen without multiple separation and labeling steps. The immunosensor could detect the target protein in a range of 0.5 to 120 ng/mL with a limit of 0.17 ng/mL (at 3σ). Finally, the immunosensing system was evaluated on several clinical samples. Analytical results were found to be in satisfactory agreement with those detected by the enzyme-linked immunosorbent assay (ELISA) method, indicating that this new method was a promising alternative tool for clinical diagnosis.

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Introduction

Human carcinoembryonic antigen (CEA), a glycoprotein having a molecular mass of *ca.* 200 kDa, is one of the most widely used tumor markers for serodiagnosis of colon cancer. An ultratrace amount of CEA dissolved in sera or other biofluids was reported as related to the state of the tumor. Thus, monitoring the level of CEA is particularly helpful when evaluating curative effects or to judge recrudescence or metastasis. The traditional CEA detection methods, including immunodiffusion,¹ enzyme-linked immunosorbent assay (ELISA),² radio-immunoassays (RIAs),³ are useful, though some of these methods require sophisticated instrumentation or radioactive chemicals or a cumbersome labeling process or are time-consuming. The electrochemical immunosensor is an alternative approach for CEA determination with the advantages of low detection limit, fast response, simple design and ease of miniaturization.⁴⁻⁷

The development of a simple and effective strategy for immobilizing bioreagents on or into the electrode is a crucial step in the design and fabrication of electrochemical immunosensors. Recently, graphene and its derivatives have attracted wide attention in the applications of nanoelectronics, biomedicines and biosensors because of their remarkable conductivities, large surface areas and strong adsorption abilities.⁸⁻¹⁰ To construct a graphene-modified electrode, one

finds that stable immobilization of the graphene on the surface of underlying electrode is of great importance. However, the poor dispersion remains one major drawback for its applications. A number of strategies have been developed in order to improve the stability and immobilized amount of the graphene-based sensors. Chen and coworkers proposed a biocompatible sensing strategy based on a graphene and chitosan composite film for immobilizing the hemoglobin protein.¹¹ Zhu's group reported an effective method to increase the solubility by functionalizing graphene with poly(diallyldimethylammonium chloride) (PDDA), a linear positively charged polyelectrolyte, for extending the application in biosensing.¹² Fu *et al.* used graphite platelets (with a thickness under 100 nm) for preparing a glucose biosensor based on the immobilization of glucose oxidase (GOD) with Nafion binder, which acts as both an effective solubilizing agent and a biocompatible matrix.¹³

Thus, the present investigation tried to develop a simple and sensitive strategy by using Nafion dispersed graphene nanosheets as an enhanced sensing platform. Nafion was a kind of well known ion-exchanging polymer with high chemical stability and ion-exchanging property.^{14,15} The Nafion used here played a dual role. The first was to immobilize the graphene nanosheets on the electrode with high dispersion. The second was to adsorb methylene blue (MB), an organic dye belonging to the phenothiazine family that displays excellent mediating ability in electrocatalytic oxidation and reduction,^{16,17} onto the graphene-based surface by ion exchange adsorption. The result demonstrated that the electrode reaction of MB bound to graphene-Nafion film showed a reversible, two-electron transfer process with good stability and reproducibility. Then the

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resulting electrode was used to absorb gold nanoparticles (AuNPs) *via* opposite-charged adsorption. The formed AuNPs monolayer served as a suitable platform for the immobilization of anti-CEA. This work reports the preparation of the immunosensor; we used both the CV and electrochemical impedance spectroscopy (EIS) to characterize the stepwise self-assembling procedure of the immunosensor. In addition, the factors influencing the performance of the resulting immunosensor have been studied in detail.

Experimental

Reagent and materials

Graphene nanosheets were obtained from Pioneer Nanotechnology Co. (Nanjing, China). CEA (from human fluids) and monoclonal anti-carcinoembryonic antigen antibody (anti-CEA), methylene blue (MB), bovine serum albumin (BSA, 96 - 99%), Nafion, gold chloride and tannic acid were obtained from Sigma Chemical (St. Louis, MO). The CEA ELISA kit was purchased from Biocell (Zhengzhou, China). All chemicals and solvents used were of analytical grade and were used as received. Double distilled water was used throughout this study. Gold colloids were prepared using the procedure previously reported, which is based on the reduction of HAuCl_4 with $\text{Na}_3\text{-citrate}$.^{18,19} The resultant gold sols were stored in a brown glass bottle at 4°C. The solution color was claret. The mean size of the prepared Au colloids was about 16 nm, which was estimated from transmission electron microscopy results (the graph is not shown). The preparation of phosphate buffer solution (PBS) of pH 7.4: KH_2PO_4 0.2 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2.9 g, NaCl 8.0 g and KCl 0.2 g were dissolved in 1000 ml doubly distilled water. The working buffer was composed of 0.1 M sodium acetate and acetic acid solution containing 0.1 M KCl of pH 6.5.

Apparatus

Voltammetric measurements were carried out with a CHI 610A electrochemistry workstation (Shanghai CH Instruments, China). A three-compartment electrochemical cell contained a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and the modified glassy carbon electrode (GCE) as working electrode. The size of the Au colloid was estimated from transmission electron microscopy (TEM) (H600, Hitachi Instrument, Japan). The pH measurements were made with a pH meter (MP 230, Mettler-Toledo, Switzerland) and a digital ion analyzer (Model PHS-3C, Dazhong Instruments, Shanghai, China). The AC impedance of the immunoelectrode membrane was measured with a Model IM6e (ZAHNER Elektrick, Germany).

Fabrication of the amperometric immunosensor

A GCE ($\phi = 4$ mm) was polished carefully with two alumina slurries (1.0, 0.3 μm) and then rinsed thoroughly two times with bi-distilled water. Then organic contaminants on the polished electrode were removed with a strong "piranha" oxidant solution for 2 min (1:3 mixture of 30% hydrogen peroxide and concentrated sulfuric acid at *ca.* 100°C) and the surface was then washed with bi-distilled water, later sonicated in bi-distilled water two times and in ethanol once successively and dried in air.

At first, 400 μL 5% Nafion solution was diluted by ethanol solution to obtained 1.0 mL 2% (v/v) Nafion solution by the ultrasonication, then 4.0 mg of graphene nanosheets were added into the resulting solution. The mixture was continuously ultrasonicated for 1 h at room temperature. Next, 5 μL

Fig. 1 The schematic illustration of the stepwise immunosensor fabrication process. (a) Formation of graphene-Nafion nanocomposite film, (b) adsorption of MB, (c) formation of AuNPs monolayer, (d) anti-CEA immobilization, (e) BSA blocking, (f) CEA capturing.

graphene-Nafion solution was coated on the surfaces of GCE. The solvent was evaporated from the surface of the film at room temperature for about 5 min. The resulting electrode was thoroughly rinsed with water. Following that, it was immersed in 5 mL MB solution (2 mM) for about 20 min, then dried in air for more than 2 h. Next, it was immersed in 0.5 mL prepared gold colloids for 2 h after it was rinsed with water thoroughly. Last, the resulting electrode was immersed in an anti-CEA solution at 4°C for more than 12 h. Subsequently, the modified immunosensor was incubated in 0.25 wt% BSA for 30 min at 37°C in order to block out unspecific sites. The finished immunosensor was stored at 4°C when not in use. The schematic diagram of the stepwise self-assembling procedure of the immunosensor is shown in Fig. 1.

Sample preparation

The CEA standard solution was prepared by diluting the CEA antigen standard product, which was obtained from Sigma Chemical (St. Louis, MO), to different concentrations with bi-distilled water. The blood sample of each human subject was obtained from the Assay Center of Kunming General Hospital without dilution, then it was centrifuged at 8000 rpm for 15 min to obtain the serum sample.

ELISA measurements

The serum samples were tested by the ELISA method. Briefly, a 100- μL serum sample was added undiluted to one well of a 96-well microplate, the wells were coated with the anti-CEA as purchased. Plates were incubated for 60 min at 37°C. Then the wells were washed 3 times with 0.3 mL PBS (pH 7.4). Non-specific binding sites were blocked by adding 200 μL per well of 0.25 wt% BSA for 30 min at 37°C. Plates were incubated for 30 min at 37°C and washed as before. Next, the detecting solution of anti-CEA coupled to horseradish peroxidase was added as conjugate. After incubation and washing as before, 90 μL of substrate was added in each well. After incubation for 30 min in the dark at 37°C, absorbance was read at 450 nm (Note: on each plate, one serum sample was included in 3 wells and the mean value was obtained).

Fig. 2 Cyclic voltammograms (CVs) of the different electrodes in working buffer (pH 5.5). (a) Bare GCE, (b) Gp-Nf nanocomposite, (c) MB/Gp-Nf, (d) AuNPs/MB/Gp-Nf, (e) anti-CEA/AuNPs/MB/Gp-Nf modified GCE. The insert shows the comparison of MB/Gp-Nf and MB/Nf modified electrodes. The scan rate was 50 mV/s.

Experimental measurements

The electrochemical characteristics of the modified electrode were characterized by using impedance measurements. The apparatus used for AC impedance measurement consisted of a ZAHNER Elektrick Co. Model IM6e response analyzer linked to a 586 PC. Each impedance measurement was performed in the presence of a 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe in 0.01 M phosphate buffer (containing 0.1 M KCl, pH 6.5). Impedance measurements were performed at the frequency range from 10^{-2} to 10^6 Hz at the formal potential of 220 mV, using an alternating voltage of 10 mV.

Electrochemical experiments were mostly performed in a conventional electrochemical cell containing a three-electrode system consisted of an immunosensor as working electrode, a SCE reference electrode and a Pt foil counter electrode, the potential was swept from -0.7 to 0.3 V (vs. SCE) with a sweeping rate of 0.05 V s^{-1} . Cyclic voltammetric experiments were performed in 5 mL 0.1 M pH 5.5 sodium acetate buffer (working buffer).

Results and Discussion

Characteristics of electrochemistry on electrode surfaces

CV and EIS are effective and convenient tools to monitor the electron transmission procedure of the immunosensor.²⁰ Therefore, they are chosen as a marker to investigate the changes of electrode behavior after each assembly step. The cyclic voltammograms of differently modified electrodes (0.1 M, pH 5.5 sodium acetate buffer) are shown in Fig. 2. No obvious electrochemical peak is found in working buffer at the bare GCE (Fig. 2a); when the bare GCE was coated with graphene-Nafion film (GP-Nf), a decrease in the current can be observed (Fig. 2b) as the Nafion can hinder the transmission of electrons toward the electrode surface. After the modified electrode was soaked in MB solution, a surprising increase in both anodic and cathodic peaks can be seen (Fig. 2c). The change demonstrated that the MB molecules have become entrapped into graphene-Nafion film and effectively transferred electrons. The insert shows the performance of MB/Gp-Nf and

Fig. 3 Electrochemical impedance spectroscopy (EIS) of the different electrodes. (a) Bare GCE, (b) graphene-Nafion nanocomposite, (c) MB/Gp-Nf, (d) AuNPs/MB/Gp-Nf, (e) anti-CEA/AuNPs/MB/Gp-Nf modified GCE. Supporting electrolyte, 10 mM PBS + 0.1 M KCl + 2.5 mM $Fe(CN)_6^{4-/3-}$ solution. The frequency range is at $1 \times 10^{-2} - 1 \times 10^6$ Hz at 25°C (Z' vs. Z'' at 220 mV vs. SCE).

MB/Nf modified electrodes, which confirmed that the employment of graphene could improve the conductivity of the nanocomposite. When AuNPs coupled onto the modified electrode surface, a further increase in anodic and cathodic current peaks (Fig. 2d). The reason is that nanometer-sized gold colloids play an important role similar to that of a conducting wire or electron-conducting tunnel, which makes it easier for the electron transfer to take place. After incubation in an anti-CEA solution, the anodic and cathodic peaks decrease (Fig. 2e), which indicates that the biomacromolecules (anti-CEA) have been immobilized on the electrode surface successfully.

EIS is an effective method to probe the interfacial properties of modified electrodes. In EIS, the semicircle diameter of EIS equals the electron-transfer resistance, R_{et} . This resistance controls the electron transfer kinetics of the redox probe at the electrode interface. Here we choose 2.5 mM ferricyanide as redox probe. Figure 3 illustrates the EIS of the different electrodes. Curve a is almost a straight line, which implied the characteristic of a diffuse limiting step of the electrochemical process on a bare GCE in 2.5 mM ferricyanide solution. After the sample is coated with graphene-Nafion, Fig. 3b shows an obvious semicircle domain, which clarified a very high resistance of the electrode interface. Then the R_{et} decreases as the adsorption of MB continues into the graphene-Nafion film (Fig. 3c). A further decrease of R_{et} could be observed in Fig. 3d as the bonding of AuNPs. When the immunosensor was finally obtained, the interfacial resistance increased, which illustrated that the anti-CEA attached to the electrode surface (Fig. 3e).

Optimization of experimental conditions

The concentrations of Nafion ethanol solution for the graphene dispersion and MB solution are important factors to prepare the biosensor. Nafion is a good membrane to immobilize onto the electrode surface while it would hinder the transmission of electrons. When the concentration was too high, the current response would decline too much while, when too low, the stability of the modified electrode was weak. We employed 2% (v/v) Nafion ethanol solution in the experiment to guarantee both a sufficient large current signal and a significant stability.

Fig. 4 DPV curves with CEA standards (A) and calibration plots for CEA with the immunosensor under optimal conditions (the results were the mean values of three measurements at each concentration).

Then MB molecules should become entrapped into graphene-Nafion membrane and its concentration would influence the saturation. It was found that low concentration of MB solution brought a nice current response but weak stability, and high concentration got the good stability but disappointing current response. In the end, we employed 2 mM MB solution to guarantee both a sufficient large current signal and a significant stability.

The effect of the size of the AuNPs on the current response was studied. As is well known, the interaction between protein molecules and AuNPs is very strong due to the very high surface-to-volume ratio of gold nanoparticles and their high surface energy.²¹ Although the packing of smaller-sized AuNPs-bound anti-CEA is denser than that of the larger-sized AuNPs due to the very high surface-to-volume ratio of AuNPs, the coulomb repulsion becomes stronger as the size of the AuNPs is much smaller.²² The results obtained showed that the immunosensor fabricated with 16 nm AuNPs exhibited a larger response than those of the other sizes. Therefore, a 16-nm AuNPs was chosen in the subsequent work.

The experimental conditions including pH value of assay solution, and incubation time and temperature for the antigen-antibody reaction, were also investigated. Experimental results indicated that the optimal electrochemical responses occurred in pH 5.5 assay solution, and the optimal incubation time was 10 min at $37 \pm 1.0^\circ\text{C}$ (data not shown).

Measurement with the immunosensor

A DPV voltammetric measurement was implemented for detection of various CEA standards under optimal experimental conditions. As expected, the DPV peak currents of the immunosensor decreased with the increasing concentrations of CEA (Fig. 4A). Meanwhile, a calibration plot between the DPV peak current and the CEA concentration was obtained (Fig. 4B). The linear range covered almost two orders of magnitude from 0.5 to 120 ng/mL with a regression equation of the form $y = -0.983x + 146.7$ and a limit of 0.17 ng/mL (at 3σ). The method is thus an absolute means to quantify CEA concentrations in the sample, such success indicated that this immunosensor could be used clinically.

Regeneration and reproducibility of the immunosensor

The regeneration of the proposed immunosensor was tested. We washed the immunosensor with 4 M urea solution for the purpose of breaking the antibody-antigen linkage. In this

experiment, after detecting 30 ng/mL CEA, the immunosensor was immersed in a stirred 4 M urea solution for about 5 min and removed, washed with water, then detected in the same working buffer with the same concentration of CEA. Consecutive measurements were repeated 9 times. A relative standard deviation (RSD) of 2.5% was found.

Selectivity and lifetime of immunosensor

The effect of substances that might interfere with the response of the immunosensor was studied. The 30 ng/mL of CEA solution containing the interfering substances of hepatitis B core antigen, hepatitis B e antigen, glucose oxidase, ascorbic acid, L-glutamate, L-lysine, L-glutamic acid, and bovine serum albumin was measured by the proposed immunosensors. None of them caused any observable interference because the low anodic and cathodic potential.

The long-time stability of the immunosensor was investigated over a 150-day period. When the immunosensor was stored dry at 4°C and measured intermittently (every 3–5 days), no apparent change in the same concentration of CEA was found over 40 days. The stability of the sensor to continuous testing was assessed by 100 circles of CVs in working buffer after bonding with 30 ng/mL CEA. The sensor showed a quite satisfying stability, it retained more than 97.1% of its initial response. Good stability can be attributed to the strong interactions between the AuNPs and anti-CEA. Thus, the bioactivity of anti-CEA immobilized on the AuNPs monolayer could be efficiently retained.

Application of immunosensor in human serum

The feasibility of the proposed method for the detection of CEA was evaluated by applying it to the analysis of human serum. Seven samples of serum from Kunming General Hospital were determined using the immunosensor. According to the shift of the current peak value we could qualitatively identify a positive or negative serum specimen. The results with human serum samples obtained from the immunosensor were compared with those from an established ELISAs technique. The results of ELISAs were determined by the Assay Center of Kunming General Hospital. A good correlation was found between the results of the two methods (Table 1), which shows the good agreement of those two methods. Moreover, the time of the detection procedure was less than 15 min (not including the process of sample pretreatment).

Table 1 Evaluation of clinical serum specimen and method validation

Sample No.	Sample concentration/ng mL ⁻¹ ^a		Relative deviation, %
	Immunosensor	ELISA	
1	14.7	16.5	-10.9
2	54.8	55.7	-1.6
3	46.3	42.9	7.3
4	7.1	6.8	4.4
5	21.8	19.5	11.8
6	75.3	78.7	-4.3
7	84.0	81.9	2.6

a. The average value of three measurements.

Conclusions

We describe a new strategy for developing an amperometric immunosensor based on the AuNPs monolayer adsorbing anti-CEA supported by GCE with graphene-Nafion nanocomposite containing MB as redox probes. This methodology has several attractive advantages, such as lack of need for redox agent in working cell, high stability of AuNPs monolayer formed, easily adsorptive immobilization of antibody on AuNPs monolayer, efficient activity retention of loading immunoreactants as well as the simplicity of use and cost effectiveness. Although the strategy has only been applied to CEA as a model system, it could be readily extended toward the determination of other clinically or environmentally interesting biospecies.

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