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Fabrication of a novel electrochemical immunosensor based on the gold nanoparticles/colloidal carbon nanosphere hybrid material

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ABSTRACT

The gold nanoparticles/colloidal carbon sphere hybrid material was used for the immobilization of protein, and was developed in biosensing. The hybrid material was fabricated by the assembly of gold nanoparticles onto the surface of colloidal carbon spheres, which constructed a 3D antibody immobilization matrix on the glass carbon electrode and made the immobilized biomolecules hold high stability and bioactivity. After the sandwich-type immunoreaction, the formed HRP-labeled immunoconjugate showed good enzymatic activity for the oxidation of o-phenylenediamine by H_2O_2. The approach provided a linear response range between 5 and 250 ng/mL with a detection limit of 1.8 ng/mL. The immunosensor showed good precision, acceptable stability and reproducibility and could be used for the detection of human IgG in real samples, which provided a potential alternative tool for the detection of protein in clinical laboratory.

1. Introduction

The immunoassay combining the specific antigen–antibody (Ag–Ab) recognition for analytical purposes has been successfully applied to the fields such as food industry [1], environmental protection [2] and clinical control [3,4]. Electrochemical immunoassay has been of high interest due to the rapid and sensitive response as well as the simple and convenient operation.

In the field of electrochemical immunosensors, stability or activity of the immobilized biocomponents on solid support has been a long-standing goal. The emergence of nanomaterials has opened new opportunities for electrochemical immunosensors [5]. Some particular nanomaterials, such as gold nanoparticles (AuNPs) and semiconductor quantum-dots (QDs), have already been widely used due to their good biocompatibility [6–11]. Among them, carbon nanotubes (CNTs) have been extensively used in electrocatalysis and biosensing [12–14]. The insolubility of CNTs in most solvents, however, limits their application in designing CNTs-based biosensing devices. Though oxidation with acids is often used to functionalize carbon surface, the modification technique has some disadvantages, such as low degree of functionalization and the corrosion of the carbon surface [15]. Recently, the colloidal carbon spheres were synthesized through a facile microwave-hydrothermal technique [16]. Compared with other carbon materials, these kinds of material have abundance of functional groups, as well as better biocompatibility, dispersibility, and relatively active surface. Their surface composition and properties can facilitate loading with many nanoparticles (e.g., nobel-metal nanoparticles, QDs, and magnetic nanomaterials), which makes them very attractive in many applications such as catalysis, sensors, and separation. More importantly, the preparation of the carbon sphere is an absolutely environment friendly, low-cost, and fast approach. These advantages indicate that the carbon sphere and carbon sphere-based composite have potential application in biosensing. However, only a few researchers paid their attention to the biocompatibility and biosensing application of these materials. In our previous work, we demonstrated that as-prepared AuNPs/carbon spheres composition can be conjugated with horseradish peroxidase labeled antibody (HRP-Ab_2) to fabricate HRP-Ab_2-AuNPs/carbon spheres bioconjugates as a label for sensitive detection of protein [16].

In this work, we have successfully developed an amperometric immunosensor based on self-assemble of AuNPs/colloid carbon spheres (AuNPs/C) hybrids on glass carbon electrode (GCE), which constructed an effective antibody immobilization matrix. In the case of the preparation of the electrochemical immunosensor, the goat anti-human IgG antibody (Ab_1) was immobilized on the AuNPs/C hybrids. The analytical procedure consists of the immunoreaction of the antigen (HIgG) with Ab_1, followed by binding HRP-labeled mouse anti-human IgG antibody (HRP–Ab_2). The formed HRP-labeled immunoconjugate showed good enzymatic activity for the oxidation of o-phenylenediamine by H_2O_2. The approach provided a linear response range between 5 and 250 ng/mL with a detection limit of 1.8 ng/mL. The high sen-

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sitivity of the biosensor may be ascribed to the high specific surface area and the good conductivity of AuNPs/C hybrids. The immunosensor showed good sensitivity and stability, and could be prepared in mass-production. Compared with our previous report [16], the detection strategy is simple, practical and convenient. Moreover, the use of nanotechnology for diagnostic applications shows great promise to meet the rigorous demands of the clinical laboratory for fast response and cost-effectiveness.

2. Experimental

2.1. Reagents and apparatus

Glucose (analytical purity), $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ was purchased from Beijing Chemical Reagent Factory. Poly (diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW = 200,000–350,000), HIgG enzyme-linked immunosorbent assay (ELISA) kits, lyophilized 99% bovine serum albumin (BSA) and Tween-20 were from Sigma Co. Human IgG (HIgG), goat anti-human IgG (Ab1) and HRP-labeled monoclonal mouse anti-human IgG (HRP-Ab2) were purchased from Zhengzhou Chuangsheng Biochemical Reagents (Zhengzhou, China). All other reagents were of analytical reagent grade and used without further purification. 0.1 M PBS with various pH was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.1 M NaOH and H_3PO_4. Doubly distilled water was used throughout the experiments.

Electrochemical immunoassay measurements were performed on a CHI 660 electrochemical analyzer (Shanghai Chenhua, China) with a conventional three-electrode system comprised of platinum wire as the auxiliary electrode, saturated calomel electrode (SCE) as the reference and a modified GCE as the working electrode. The results of ELISA were measured by a spectrophotometric ELISA reader at a wavelength of 450 nm.

The morphologies of AuNPs/C hybrids were observed by transmission electron microscopy (TEM, JEOLJEM-200CX).

2.2. Fabrication of AuNPs/C hybrids modified GCE

The AuNPs/C hybrids were synthesized according to modifications of literature procedure [16]. Colloidal carbon spheres were prepared by microwave-hydrothermal method. 5 g glucose was dissolved in 40 mL water to form a clear solution, the obtained solution was treated at 170 °C for 20 min in microwave-accelerated reaction system. The brown products were isolated by three cycles of centrifugation/washing/redispersion in water.

The purified colloidal carbon spheres were functionalized with PDDA. First, colloidal carbon spheres were dispersed into aqueous solution of 0.25% PDDA containing 20 mM NaCl. After stirring for 20 min, a homogeneous brown suspension was obtained. Residual PDDA was removed by high-speed centrifugation and the complex was rinsed with water for at least three times. Then, 0.12 g colloidal carbon spheres were dispersed in 100 mL of the Au colloid solution and stirred for 10 min. After centrifugation, the light purple AuNPs/C composites were obtained, while the supernatant liquor was colorless. The composites were further washed with distilled water three times and redispersed in 50 mM pH 7.4 PBS.

The GCE with a diameter of 3 mm was used as the substrate to prepare the AuNPs/C hybrid film. Prior to the preparation procedure, the GCE was successively polished to a mirror finish using 0.3 and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with water. After successive sonication in 1:1 nitric acid, acetone, and doubly distilled water, the electrode was rinsed with doubly distilled water and allowed to dry at room temperature. 4 μL of 5 mg/ml AuNPs/C hybrid solution was dropped on the pretreated GCE and dried in a silica gel desiccator.

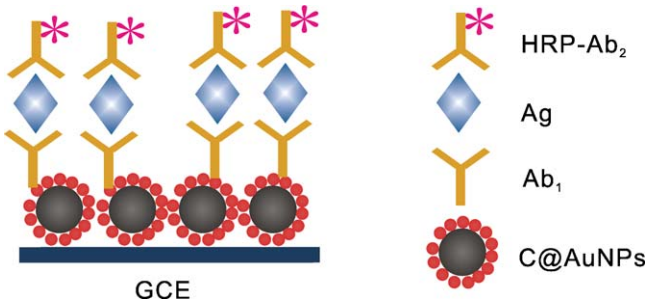

Fig. 1. The analytical scheme of AuNPs/C hybrids modified immunosensor.

2.3. Antibody immobilization and immunoreaction procedure

Ab_1 was immobilized onto the AuNPs/C hybrid modified GCE. 5.0 μL of 0.5 mg/mL Ab_1 solution (50 mM PBS, pH 7.4) was spread onto the hybrids modified GCE surface. The electrode was incubated at 4 °C in a moisture atmosphere to avoid solvent evaporation. After incubation for 15 h, they were rinsed with PBS, 0.05% Tween (PBST) to remove physically absorbed Ab_1. The electrodes were then blocked with 2% BSA and 0.05% Tween-20 solution for 1 h at room temperature, and washed with PBST. After aspiration, Ab_1 modified electrodes incubated with 60 μL of detecting HIgG samples for 50 min at 37 °C. By the binding reaction between Ab_1 and HIgG, the electrodes immersed into the 60 μL of diluted HRP-Ab_2 solution for an incubation of 50 min. Finally, the electrodes were washed thoroughly with water to remove nonspecifically bound conjugations. The way to the immobilization of Ab_1 and the immunoassay procedure were shown in Fig. 1.

2.4. Measurement procedure

The immunosensor was then placed in an electrochemical cell containing 3.0 mL pH 7.0 PBS buffer, 2.0 mM o-phenylenediamine and 3.0 mM H_2O_2, which was deaerated thoroughly with highly pure nitrogen for 5 min and maintained in nitrogen atmosphere at room temperature. In the presence of HRP immobilized on GCE surface, the electroactive species, 2,2′-diaminoazobenzene [17], was firstly produced. The differential pulse voltammetric (DPV) measurements were performed from −0.3 to −0.8 V with the pulse amplitude of 50 mV and the pulse width of 50 ms.

2.5. Commercial ELISA for HIgG.

A commercially available ELISA assay was utilized for method comparison studies. In sandwich ELISA with standard polystyrene 96-well plates, 50 μL of serum sample suspension was incubated at 37 °C for 30 min, and the wells were rinsed 3 times (3 min each) with 0.1 mol/L PBS (pH 7.4) containing 0.5 mol/L NaCl and 1 mL/L Tween 20. Then we added 50 μL of conjugate solution and incubation continued for 1 h. The wells were again rinsed and 50 μL of 3,3′,5,5′-tetramethylbenzidine reagent was added and incubated at 37 °C for 10 min. The enzymatic reaction was stopped by adding 50 μL of 2.0 mol/L H_2SO_4 to each well. The results of ELISA were measured by a spectrophotometric ELISA reader at a wavelength of 450 nm.

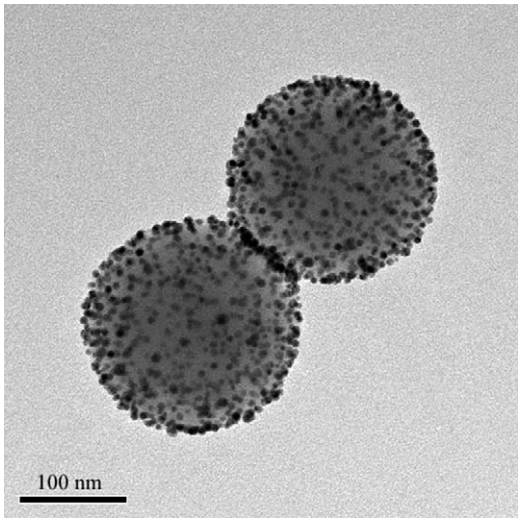

Fig. 2. TEM image of AuNPs/C hybrids.

3. Results and discussion

3.1. TEM of AuNPs/C hybrid material

Fig. 2 shows the typical transmission electron microscopy of AuNPs/C hybrid material. It could be seen that the obtained colloidal carbon spheres with an average diameter of 250 nm are uniform in size and well-dispersed AuNPs decorate the colloidal carbon surface. The prepared AuNPs/C modified electrode was very stable, which was illustrated by the redox process of $[Fe(CN)_6]^{3-/4-}$ in solution phase, the redox peak currents were essentially unchanged after continuously cycling the electrode for 100 cycles or after the electrodes were stored in distilled water for one week.

3.2. Cyclic voltammetric behavior of the immunosensor

It could be verified that the amperometric response of the sensor depended on the immunocomplexes as shown in Fig. 3. No amperometric response was observed at both bare GCE and Ag/Ab1/AuNPs/C/GCE in the potential range in 0.1 M pH 7.0 PBS contained o-phenylenediamine and H_2O_2 (curve a, b). After incubation with HRP-Ab_2, the sensor showed a pair of stable redox peaks,

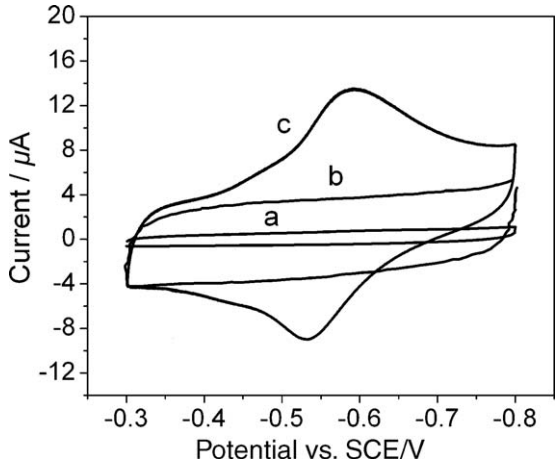

Fig. 3. Cyclic voltammograms of (a) GCE, (b) Ag/Ab_1/AuNPs/C modified GCE in 0.1 M pH 7.0 PBS, and (c) HRP-Ab_2/Ag/Ab_1/AuNPs/C/GCE at in 0.1 M pH 7.0 PBS contained 2.0 mM o-phenylenediamine and 3.0 mM H_2O_2.
Scan rate: 100 mV/s.

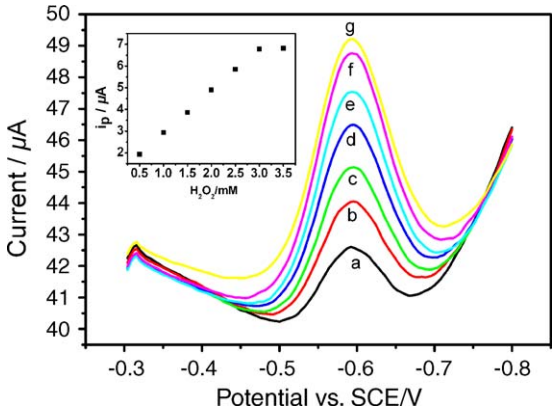

Fig. 4. DPV of HRP-Ab_2/Ag/Ab_1/AuNPs/C hybrids modified GCE in 0.1 M pH 7.0 PBS + 2.0 mM o-phenylenediamine with successive addition of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mM H_2O_2 (from a to g). Inset: the plot of peak current vs. H_2O_2 concentration.

the anodic and cathodic peak potentials were −0.532 and −0.593 V (vs. SCE), respectively (curve c), corresponding to the redox of 2,2′-diaminoazobenzene, the enzymatic product [18]. Here, the 3D structure of composition immobilization matrix could provide a congenial microenvironment similar to that of the protein in a native system and allow the protein molecules more freedom in orientation, thus constructed an effective antibody immobilization matrix and made the immobilized immunocomponents hold high stability and bioactivity. Additionally, the gold nanoparticles were very efficient to increasing the electron-transfer process, and preventing the biomolecules from leaking out because of the strong interaction between gold nanoparticles and mercapto or primary amine groups in these biomolecules [19,20].

3.3. Optimal conditions for enzymatic reaction

With an increasing H_2O_2 concentration in the detection solution, the DPV peak current of HRP-Ab_2/Ag/Ab_1/AuNPs/C/GCE increased and then reached a maximum value (Fig. 4). At low H_2O_2 concentration, both o-phenylenediamine and immobilized HRP were in excess, the rate producing 2,2′-diaminoazobenzene only depended on the concentration of H_2O_2. Thus, its reduction current was proportional to H_2O_2 concentration. When the concentration of H_2O_2 was higher than 3.0 mM, the enzymatic reaction rate became dependent on the amount of the immobilized HRP, resulting in a constant peak current. Thus 3.0 mM H_2O_2 was used for the activity determination of HRP in the immobilized immunoconjugate.

Inhibition of nonspecific binding (NSB) was critical to achieve the best sensitivity and detection limits. Thus, we developed a highly effective blocking procedure utilizing competitive binding of BSA and the detergent Tween-20, and also optimized the concentration of HRP-Ab_2 (1:500). Before exposure to the sample, the sensor was incubated for 1 h with 60 μL 2% BSA + 0.05% Tween-20, then washed with 0.05% Tween-20 in buffer. For measurements, 60 μL different concentrations of HIgG was incubated on the sensor surface, blocking buffer was used to wash, then the sensor was incubated with 60 μL HRP-Ab_2 bioconjugates.

3.4. Amperometric response of immunosensor to HIgG concentration

Under optimal incubation conditions, the DPV peak current of the obtained immunoconjugate in the detection solution containing H_2O_2 and o-phenylenediamine increased with an increasing HIgG concentration in the incubation solution. Fig. 5 shows the

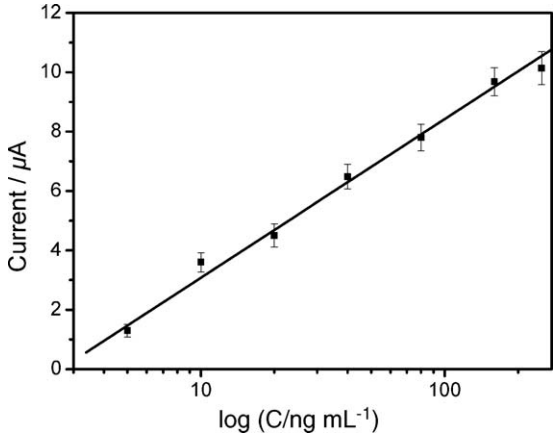

Fig. 5. The resulting calibration curve of HIgG plotted on a semi-log scale.

Table 1
Comparison of serum HIgG levels determined using two methods.

Serum samples	1	2	3
Immunosensor (ng/mL)[a]	5.25	52.6	98.5
ELISA[a]	4.95	49.1	94.2
Relative deviation (%)	6.0	7.1	4.6

[a] The average value of five successive determinations.

plot of backgroundsubtracted peak current vs. the logarithm of the concentration of HIgG. The corresponding calibration plot of response vs. the logarithm of the concentration of the HIgG was linear over the 5.0–250 ng/mL. The linear regression equation is $i_p(\mu A) = -2.0219 + 5.19062 \mathrm{Log}\, C_{HIgG}$ (ng/mL) with a correlation coefficient 0.995 ($n = 6$). The lowest detection limit of HIgG concentration was 1.8 ng/mL. According to the linear equation, we could detect HIgG concentration quantitatively. Higher serum HIgG levels could be detected with an appropriate dilution.

3.5. Precision, reproducibility, stability and regeneration of the immunosensor

The effect of substances that might interfere with the response was studied. Using an incubation solution containing 20 ng/mL C-reactive protein and 20 ng/mL HIgG, the specificity of the proposed immunosensor was examined by detecting the electrochemical response. No significant difference of currents (R.S.D = 8.1%) was observable in the comparison with the result obtained in presence of only HIgG.

The intra-assay precision was estimated by assaying one HIgG level for five replicate measurements. The inter-assay precision, or the fabrication reproducibility, was estimated by determining the HIgG level with five immunosensors made at the same GCE independently. The relative standard deviations of the intra- and inter-assay were 9.6% and 9.8% at the HIgG concentration of 20 ng/mL. After the biosensor was used for three times, the analytical performances did not show an obvious decline, demonstrating that the sensor possessed good stability.

The regeneration of the immunosensor is a key factor for developing a practical device for immunoassay. In this study, the probe was immersed into 0.1 M glycine–HCl (pH 3.0) buffer solution for 15 min, followed by washing with the PBS and water several times. The regeneration property of the immunosensor was investigated. It is found that the as-renewed sensor could be reused for about 6 runs without obvious loss of sensing sensitivity. Even the immunosensor was reused for 10 immunoassay cycles, the sensitivity of the probe still was able to maintain the value of the original 60%.

3.6. Application of the immunosensor in human serum

The feasibility of the immunoassay system for clinical applications was investigated by analyzing several real samples, in comparison with the ELISA method. These serum samples were diluted to different concentrations with a PBS of pH 7.0. Table 1 describes the correlation between the partial results obtained by the proposed immunosensor and ELISA method. It obviously indicates that there is no significant difference between the results given by two methods, that is, the proposed biosensor could be satisfactorily applied to the clinical determination of HIgG levels in human plasma.

4. Conclusions

It has been demonstrated here, AuNPs/C hybrids were directly used to construct an electrochemical immunosensor for the sensitive detection of HIgG. Such 3D composite immobilization matrix was effective for retaining the bioactivity of immobilized biomolecules and supplied a facile methodology for the immobilization of antibody. The proposed immunosensor can be applied to the determination of serum HIgG. The sensor shows good precision and acceptable sensitivity, fabrication reproducibility and storage stability, which could be readily extended toward the measurement of other clinically important markers.

Acknowledgments

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