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Organic–inorganic matrix for electrochemical immunoassay: Detection of human IgG based on ZnO/chitosan composite

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Abstract

A new strategy to construct amperometric immunosensor for human IgG assay based on ZnO/chitosan composite as sensing platform has been described. This material, which combined the advantages of inorganic species, ZnO and organic polymer, chitosan, can maintain biological activity well. A sequential sandwich immunoassay format was performed on the ZnO/chitosan composite supported by glass carbon electrode (GCE) using goat-anti-human IgG antibody (IgG Ab) and human IgG as a model system. Amperometry was used to determine the amount of horse-radish peroxidase (HRP) fixed on the sensor surface, which was related to the content of the desired human IgG. Assay conditions that were optimized included the amount of labeled antibody, the incubation time and temperature, the pH of the substrate solution, etc. Using hydroquinone as a mediator, amperometric detection at −150 mV (versus SCE) resulted in a detection range 2.5–500 ng mL^{-1}, with a detection limit of 1.2 ng mL^{-1}. The simple manipulations of the construction of ZnO/chitosan composite, as well as low-cost and broad linear range, are the main features of the proposed immunosensing method.

Keywords: Electrochemical immunoassay; Organic–inorganic matrix; Human IgG; ZnO/chitosan composite

1. Introduction

In recent years, various kinds of immunosensors for clinical and environmental purposes [1–3] have been developed. Different immunoassay formats, such as electrophoretic immunoassay, enzyme-linked immunosorbent assay (ELISA), radio immunoassay and dot-immunobinding, have been adopted to meet the increasingly analytical needs. The electrochemical immunosensors, which combine simple, portable, low-cost electrochemical measurement systems with specific and sensitive immunoassay procedures, have gained considerable attention [4–6]. On the basis of the specific reaction of the antibody with the antigen, immunosensors provide a tool for the determination of immunoreagents. Here, the immunologic material is immobilized on a transducer; the analyte is measured through labeled species conjugated with one of the immunoreagents. Enzymes such as cholinesterase [7], horse-radish peroxidase (HRP) [8,9] and alkaline phosphatase [10,11] are used extensively as labels to improve the sensitivity of immunoassay by electrochemical amplification of the signals.

In the design and fabrication of electrochemical immunosensors, the development of a simple and effective strategy for immobilizing bioreagents on or into the electrode, is a crucial step. Entrapment [6], absorption [10] and covalent binding [12] are conventional immobilization methods. However, some of these immobilization methods are relatively complex, requiring expensive reagents or environmentally unattractive solvents, and result in relatively poor stability. Thus, new immobilized schemes and advanced materials that can improve the analytical capacities of sensor devices are highly desired. The inorganic ceramics exhibit relatively high mechanical strength, enhanced thermal stability and negligible swelling in both aqueous and organic solutions compared to most organic polymers [13]. Unfortunately, owing to some drawbacks, especially their brittleness, the practical application of ceramic materials is often limited. Efforts have been made to seek for new materials, which could overcome the cracking caused by sol–gel for biomolecular immobilization in biosensor construction.

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Organic–inorganic composite materials have emerged in recent years. They combine the physicochemical attributes of components and improve their features. Organic components benefit from the formation of defect-free inorganic membranes and make them less brittle. Organic membranes can have their chemical and thermal stability improved by an inorganic phase [14]. These organic–inorganic composite membranes have been used to immobilize enzymes to efficiently retain their activity [13,15,16]. To our knowledge, their utility as a sensing platform in electrochemical immunosensor design has not been yet explored. Therefore, it seems that the use of the organic–inorganic composite material, as a sensing platform in electrochemical immunosensor, is quite promising.

In this paper, we present for the first time a novel immunosensor based on nanoporous ZnO/chitosan inorganic–organic composite film as an immobilization matrix. This material combined the advantages of inorganic species, ZnO, and organic polymer, chitosan. Chitosan was chosen as the material to form the membrane due to its excellent film-forming and adhesion abilities, together with its nontoxicity and biocompatibility [17,18]. Moreover, a chitosn contains amino groups, thus providing a hydrophilic environment, which is compatible with the biomolecules. The immobilization of the antibody is based on the absorption of the nanoporous ZnO. The nanoporous structure of ZnO greatly enhances the active surface available for antibody binding over the geometrical area. To investigate the feasibility of this methodology, goat-anti-human IgG and human IgG were chosen as model systems, while horse-radish peroxidase served as the enzyme label. To quantify the amount of IgG-HRP on the surface of GCE, which is inversely proportional to the amount of the analyte, hydroquinone and H_2O_2 were used as substrates. This matrix can also be used to immobilize other biomolecules.

2. Experimental

2.1. Reagents and solutions

Peroxidase anti-human IgG (HRP-IgG Ab, 1.0 mg mL^{-1}) was purchased from Dingguo Biochemical Reagents (Beijing, China). Hydrogen peroxide (30% v/v aqueous solution) and hydroquinone were obtained from Shanghai Chemical Reagents (Shanghai, China). Goat-anti-human IgG antibody (IgG Ab, affinity purification), normal human reference serum (NHRS, containing 10.9 mg/mL immunoglobulin G (IgG)), bovine serum albumin (BSA) and trishydroxymethyl aminomethane (tris) were supplied by Shanghai Biochemical Reagents (Shanghai, China). Chitosan (CHIT, MW−1×10^{-6}, 75–85% deacetylation) was supplied by Sigma (St. Louis, MO, USA). Nanoporous ZnO was produced by Nano Material Application Engineering Technology Center (Zhejiang, China).

An incubating buffer, 0.1 mol L^{-1} tris–HCl and 1.0 mmol L^{-1} EDTA (pH 7.5), was used. IgG and IgG Ab solutions of the desired concentration were prepared by diluting stock IgG Ab and IgG solutions in the same tris–HCl buffer. A solution of 2% BSA in tris–HCl (pH 7.5) buffer was used as a blocking buffer. The washing solution was 0.1 mol L^{-1} tris–HCl/0.1 mol L^{-1} KCl buffer. The supporting electrolyte of the enzymatic substrate was 0.067 mol L^{-1} phosphate buffer saline (PBS) containing 0.1 mol L^{-1} KCl (pH 7.00). All solutions were prepared with double distilled water.

2.2. Apparatus

Cyclic voltammetric and amperometric analyses were carried out using a CHI 760b Electrochemical Analyzer (Chen Hua Instrument Inc, Shanghai, China). Scanning electron microscope (SEM) image of ZnO/CHIT matrix was taken with a KYKY 2800, using an accelerating voltage of 20 KV (Hitachi, Tokyo, Japan). The three-electrode system consisted of a glass carbon electrode (GCE) (of 4 mm in diameter) as the working electrode, the saturated calomel electrode (SCE) as the reference electrode and a Pt foil as the counter electrode. Cyclic voltammetric experiments were performed in unstirred solutions. Amperometric measurements were carried out in stirred substrate solutions with a steady-state background current that was first obtained before standard H_2O_2 solution was added into the buffer solution. All potentials were measured and reported versus the SCE. A magnetic stirrer and bar provided the convective transport.

2.3. Preparation of ZnO/CHIT solution

An appropriate amount of nanoporous ZnO was dispersed in 0.5% chitosan (0.05 M acetic acid), and the mass ratio of ZnO to chitosan was 1 to 100. The mixture was sonicated for 15 min after stirring for 1 h. Then, a high dispersed colloidal solution was formed.

2.4. Preparation of the immunosensor

A casting solution was obtained by mixing 20 μL of ZnO/chitosan composite solution with 20 μL of IgG Ab solution (56 μg mL^{-1}). An aliquot (10 μL) of this resulting casting solution was pipetted onto the surface of the GCE, being polished before each experiment with 0.05 μm of α-alumina powder, rinsed thoroughly with absolute alcohol and distilled water in ultrasonic bath and dried in the air. The casting solution was allowed to dry at 4 °C overnight. Then, the electrode, loaded with IgG Ab, was introduced into a 2% BSA blocking buffer for 15 min at 25 °C. Rinsed with washing solution, the electrode was immersed in the IgG Ag solution (74 μg mL^{-1}) and incubated for 35 min at 25 °C. Rinsed with washing solution again, the electrode was immersed in the HRP-IgG Ab solution and incubated for 35 min at 25 °C. The immunosensor was, then, rinsed thoroughly with washing buffer and stored in the incubating buffer solution prior to the amperometric measurement.

2.5. Measurement

The amperometric measurements were performed in an electrochemical cell, holding 10 mL of the supporting electrolyte and containing 1.0 mmol mL^{-1} hydroquinone. A three-electrode system was used at the applied potential of −150 mV versus

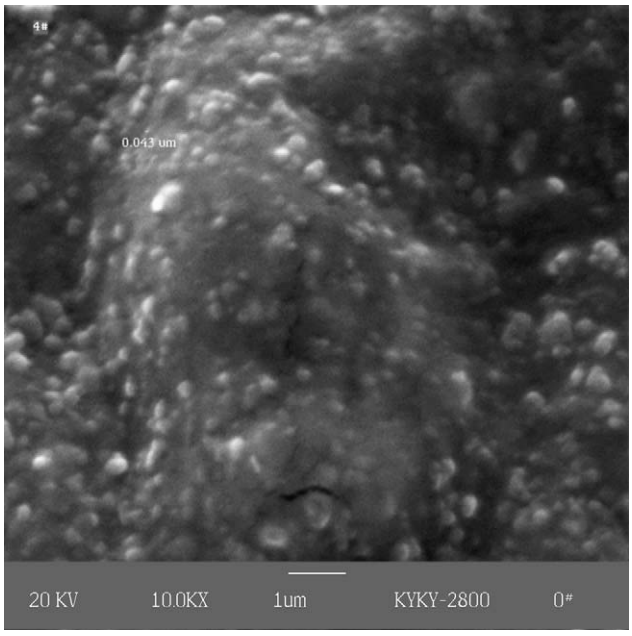

Fig. 1. Scanning electron microphotographs of ZnO/CHIT film. Accelerating voltage, 20 kV.

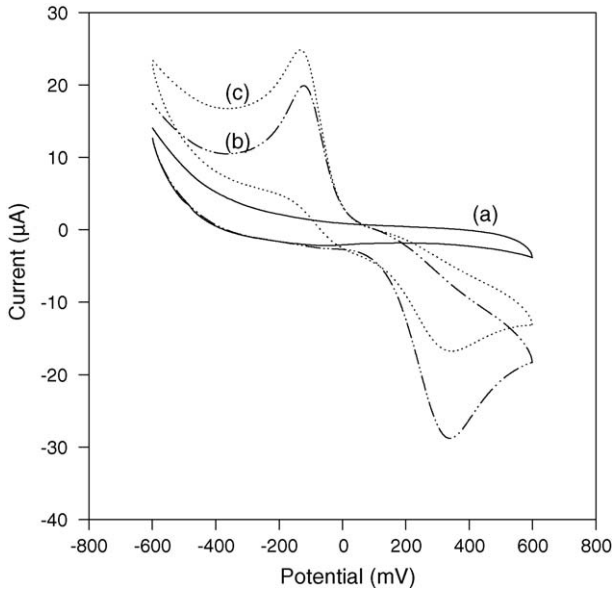

Fig. 2. Cyclic voltammograms obtained with the immunosensor. (a) In a unstirred 0.067 mol L^{-1} PBS (pH 7.00). (b) The same as (a) but containing 1.0 mmol L^{-1} hydroquinone. (c) The same as (b) but containing 2.0 mmol L^{-1} H_2O_2. Scan rate 100 mV s^{-1}.

SCE. After the background current was stabilized, the response was recorded after the addition of H_2O_2.

3. Results and discussion

3.1. Morphology of ZnO/CHIT composite film

Scanning electron microscopy (SEM) can shed light on the microstructure of ZnO/CHIT film. Fig. 1 showing the scanning electron micrographs of ZnO/CHIT film, indicates a uniform porous structure. This provided a significantly enhanced and effective electrode surface for high antibody loading.

3.2. Electrochemical characterization of the immunosensor

The operation of the amperometric immunosensing was based on the use of the antibody labeled with the enzyme HRP and H_2O_2 as the substrates. Hydroquinone, one of the best electron transfer mediator for HRP [19], was chosen as the electron transfer mediator. In the presence of HRP, H_2O_2 oxidizes hydroquinone to benzoquinone. Fig. 2 shows the cyclic voltammograms obtained with the immunosensor in unstirred 0.067 mol L^{-1} PBS (pH 7.0) with and without the addition of hydroquinone and H_2O_2. There was no oxidation/reduction peak when the immunosensor was in unstirred 0.067 mol L^{-1} PBS (pH 7.0) (Fig. 2a). A couple of oxidation/reduction peak, which represents the cyclic voltammogram of hydroquinone, was observed in the presence of hydroquinone (Fig. 2b). However, the presence of 2.0 mmol L^{-1} H_2O_2, the cyclic voltammogram displayed a dramatic enhancement of the cathodic peak current followed by a concomitant decrease of the anodic peak current (Fig. 2c). These phenomena indicate that the HRP attached to the immunosensor surface retained its high enzymatic catalytic activity and the hydroquinone could effectively shuttle electrons from the redox center of HRP to GC.

Amperometric measurements were carried out. Fig. 3 shows typical results obtained with the immunosensor being prepared after incubation in the incubation solution followed by amperometric determination at −150 mV. After the stabilization of background current (a–b); the solution of H_2O_2 is added (b); and the point at which the current reaches its maximum (c). When the amount of analyzed human IgG varied in the incubation solution, the response current also varied. This is the basis of the amperometric assay of human IgG.

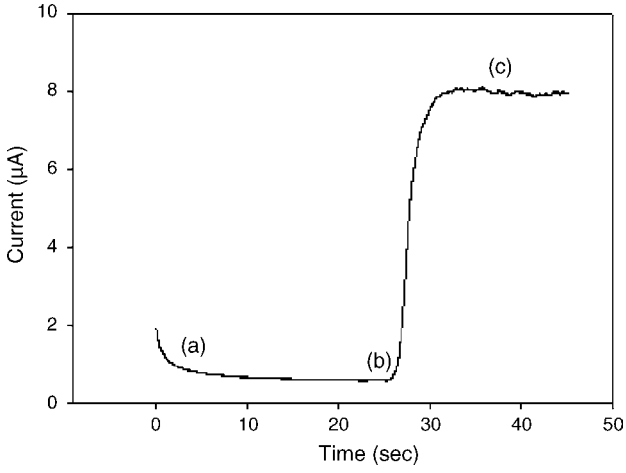

Fig. 3. Current–time curve of the incubated ZnO/CHIT-human IgG immunosensor in 0.067 mol L^{-1} PBS (pH 7.0) containing 1.0 mmol L^{-1} hydroquinone recorded at −150 mV; after the stabilization of background current (a–b); when the solution of H_2O_2 is added (b); and the point at which the current reaches its maximum (c).

3.3. Nonspecific adsorption

Choosing ZnO/CHIT matrix as the support, the nonspecific absorption was investigated using biocomposites with and without BSA blocking. It was observed that the current response reached the values of 0.66, 1.65 and 4.16 μA for composite the matrix (ZnO/CHIT/IgG Ab)-BSA-HRP-IgG Ab, the composite matrix–HRP-IgGAb and for the composite matrix–BSA-IgGAg-HRP-IgGAb immunosensors, respectively. The current due to the nonspecific absorption can be reduced by the addition of BSA. The difference between the response currents of the immunoseneors with and without BSA is obviously related to the reduction of the nonspecific absorption by BSA. The use of BSA seems to reduce the generally accessible surface area besides the available antibody epitopes.

3.4. Optimization of experimental parameters

Parameters of the assay procedure, affecting the response of the immunosensor, were investigated. The response signal of the immunosensor depends on the amount of HRP-IgG Ab conjugate bound to the surface of the immunosensor, which, in turn, corresponds to the concentration of HRP-IgG Ab in the incubating buffer. In order to obtain a maximum response with a minimum amount of HRP-IgG Ab, the immunosensor was incubated with various concentrations of HRP-IgG Ab. The response current increased up to 25 μL of HRP-IgG Ab (in 1 mL of the incubation buffer) and then tended to saturate. Consequently, 25 μL of HRP-IgG Ab (1.0 mg mL^{-1}) solution, added to 1 mL of final incubation solution, were routinely employed in these assays.

The effect of the incubation time on amperometric signals was also investigated (Fig. 4). When the antibodies in the incubation solution reach the antigens at the surface of the immunosensor, it takes time for the contacting species to form compact immunocomplexes. Fig. 4 demonstrates that by increasing the incubation time from 5 to 35 min, the amperometric current increases dramatically and then tends to change only slightly. Therefore, an incubation time of 35 min was adopted in these experiments. One would expect that most of the surface-exposed antigens are binding with the antibodies in the incubation solution, forming compact complexes on the surface of the immunosensor.

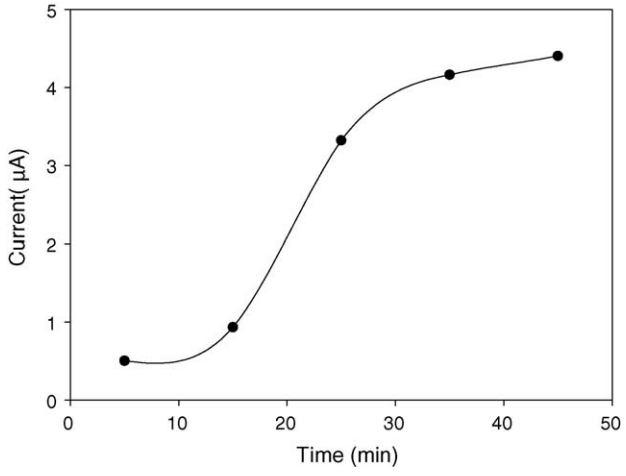

Fig. 4. Effect of the incubation time period. The immunosensor was incubated in solutions containing 25 μL HRP-IgG conjugate at 20 °C.

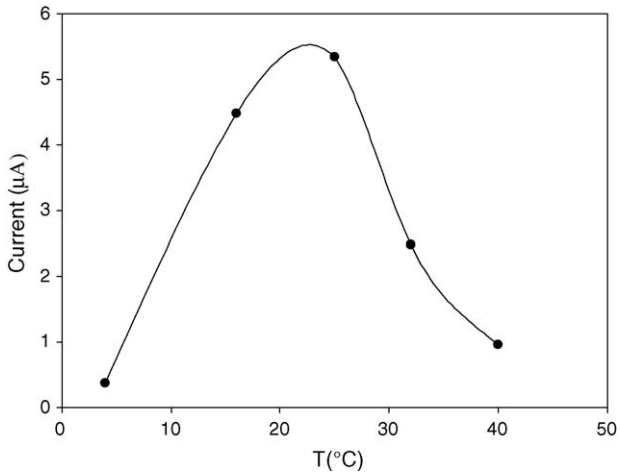

Fig. 5. Effect of the incubation temperature. The immunosensor was incubated for 35 min in incubation solutions containing 25 μL HRP-IgG conjugate.

An additional parameter that affected the assay was the incubation temperature. Various incubation temperatures have been reported in the literature, ranging from 25 to 37 °C [20,21]. As well-known, an optimal temperature of the immunoreaction would be 37 °C. The enzyme used exhibited the best activity at the temperature from 20 to 25 °C. A higher temperature would be harmful to its activity. The effect of the incubation temperature on response current was examined from 4 to 40 °C (Fig. 5). It was found that the signal increases with an increase of temperature up to 25 °C, and then it decreases at higher temperatures. Therefore, 25 °C was chosen as the optimum temperature for the incubation of the immunosensor.

The temperature and pH of the substrate solution, which have a significant influence on the reduction of the enzyme's catalytic product, have been optimized. It is found that the current response achieves its maximum at 26 °C and pH 7.0.

3.5. Calibration curve

Fig. 6 shows the calibration curve obtained using human IgG standards under optimal experimental conditions. The response signal linearly increase with the increase of human IgG concentration in the range 2.5–500 ng mL^{-1} with a correlation coefficient value of 0.993. The linear regression equation is I (μA) = 1.8495 + 0.0191 × (ng/mL). A detection limit is 1.2 ng mL^{-1}.

3.6. Reproducibility and recoveries of the immunosensor

The repeatability of the response current of the immunosensor was investigated at human IgG concentration of 250 ng mL^{-1}. The current response values were 6.53, 6.53, 6.60, 6.16, 6.81 for five successive assays with the variation coef-

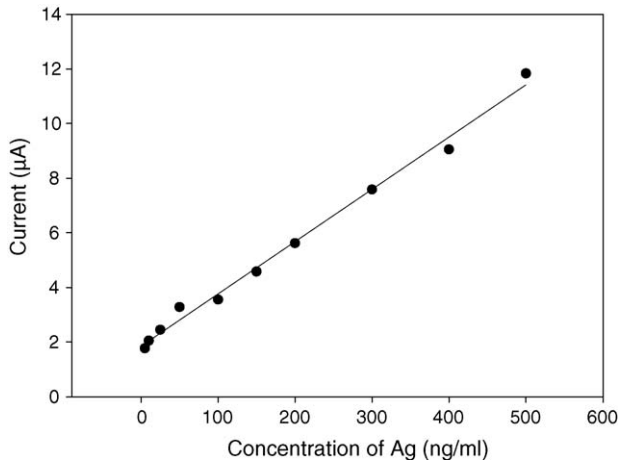

Fig. 6. Current vs. concentration plot for ZnO/CHIT-IgG Ag immunosensor after the sandwich immunoassay. The immunosensor was incubated in 0.1 M tris–HCl/1 mM EDTA buffered solution of pH 7.5 containing different amounts of IgG Ag. These experimental conditions are the optimal.

Table 1
Analysis results of IgG for human serum specimen

Samples IgG	IgG concentration by IgG concentration by ELISA[a] (ng mL^{-1})	EIA[b] (ng mL^{-1})
1	55.5	51.1
2	95.1	92.92
3	235	243.8
4	274	285.5
5	369	363.9

[a] Enzyme-linked immunosorbent assay method.
[b] Electrochemical immunosensing assay method, measured at $V = -150$ mV. As the IgG concentration level of human serum is very high, the human serum specimens have to be diluted to the detection range of the electrochemical immunosensors by using tris–HCl buffer according to the IgG concentration by ELISA.

ficient (R.S.D.) 3.59%. Reproducibility of the immunosensor was tested by measuring 200 ng mL^{-1} human IgG solution. Five immunosensors, made independently, showed the response current values of 5.96, 5.59, 5.65, 5.94, 5.40 μA with an acceptable variation coefficient of 4.20% ($n = 5$).

In order to investigate the possibility of using the prepared electrochemical immunosensor for clinical analysis, some patient human serum samples were examined by the developed electrochemical immunosensor and the results obtained were compared with that by enzyme-linked immunosorbent assay (ELISA). The comparison of results was shown in Table 1. The analytical results show that the developed electrochemical immunosensor is suitable for the applications in clinical area.

4. Conclusions

We have proposed a new designing strategy of an immunoassay based on ZnO/chitosan composite supported by GCE as sensing platform. This organic–inorganic composite matrix combined the advantage of both materials. There are several attractive advantages including simple preparation procedure, efficient activity, retention of loading immunoreagents and satisfactory recovery.

The feasibility of this methodology has been demonstrated with Goat-anti-human IgG antibody (IgG Ab) and human IgG as a model analytical system. By using HRP as the enzymatic label and H_2O_2 as the substrate, the proposed immunosensor can be used to detect human IgG. The results indicated that ZnO/CHIT film is an attractive matrix for the immobilization of biomolecules to fabricate biosensors.

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