An Amperometric Immunosensor Based on a Conducting Immunocomposite Electrode for the Determination of *Schistosoma japonicum* Antigen

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A renewable amperometric immunosensor based on a graphite-paraffin-*Schistosoma japonicum* antibody (SjAb) biocomposite electrode has been prepared for the detection of *Schistosoma japonicum* antigen (SjAg). Competitive ELISA was employed involving HRP-SjAg as a tracer and 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate. The product of an enzyme catalytic reaction was detected at +0.1 V (vs. Ag/AgCl reference electrode) for measuring the amount of HRP-labeled SjAg binding to the electrode surface. The assay conditions were optimized, including the amount of SjAb loading in the electrode and HRP-SjAg in the incubation solution, the pH of the measuring solution and the incubation time. The measuring range was 0.5 – 30 µg/ml under the optimum conditions. Rabbit serum samples of different infection degree were measured, which demonstrated that the immunosensor meets the demands of clinical analysis. It exhibits some advantages, such as simplicity of fabrication, rapidity of measurement, and satisfactory sensitivity and reproducibility.

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Introduction

Schistosomiasis japonicum (Sj), a parasite disease, still threatens human health in some parts of Asia, Africa and Latin America. About 1.6 billion people in 76 countries have been infected with this parasite.¹ Thus, the development of fast, robust, selective, easy-to-use, low-cost methods for the determination of SjAg or SjAb is of importance for health protection and for further medical treatment. Conventional diagnostic procedures of this disease are often time-consuming, complicated and need expensive instrumentation.²⁻⁴ These disadvantages have sparked the development of immunosensors.

Electrochemical immunosensors draw interest owing to the relatively simple and inexpensive equipment required.⁵⁻⁸ For the design of an immunosensor, antibodies for the target analyte must be immobilized on the transducer surface, and immobilized antibodies should be stable enough to retain the bioactivity during a sufficient lifetime. General methods for the immobilization of Ab include physical adsorption, covalent attachment and entrapment in a polymer matrix.⁹⁻¹² Another prerequisite for a successful application of an immunosensor is regeneration of the sensing surface after each assay.¹³ Regeneration of the sensing surface is usually accomplished by using solutions of low pH and high salt concentration. However, such a regeneration process is often incomplete and gives irreproducible results due to strong binding of Ag with Ab. A possible solution to this problem is to develop single-use, low-cost immunosensors.¹⁴⁻¹⁶ Fàbregas *et al.*^{17,18} have

reported renewable amperometric immunosensors based on graphite powder, methacrylate or epoxy resin and an Ab composite. The surface of an immunosensor may be renewed through simple polishing.

Carbon paste electrodes (CPE) are also a good choice for the design of immunosensor filling the above two requirements. CPE can easily be modified with an antibody, and the sensing surface of CPE can be regenerated in a rapid and quantitatively reproducible fashion. Encouraged by the success of constructing of a renewable amperometric immunosensor based on a graphite-paraffin-SjAg biocomposite electrode for the detection of SjAb, we tried to use a graphite-paraffin-SjAb electrode as immunosensor for the determination of SjAg using a competitive ELISA involving HRP-SjAg as a tracer and 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate. The determination of SjAg is necessary for the diagnosis of *schistosomiasis*, especially during the initial stage.

Experimental

Apparatus

A 20-ml electrochemical cell equipped with an immunosensor, an Ag/AgCl reference electrode and a platinum-wire auxiliary electrode was used for the electrochemical measurements. Cyclic voltammetric and chronoamperometric experiments were carried out with a microcomputer-based electrochemical analyzer (Tianjin Lanlike Chemical Electronic Co.) and XJP-821(c) polarographic analyzer (Jiansu Electroanalytical Instruments, Jiansu). All of the electrochemical measurements were performed in a 0.1 mol/l citrate phosphate buffer solution. A Model CSS501 thermostat (Chongqing Experimental Equipment, Chongqing, China) was

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Fig. 1 Configuration of amperometric immunosensor: (1) Teflon nut, (2) Teflon tube, (3) conducting stainless-steel rod, (4) biocomposite, (5) electric wire connected with stainless-steel rod.

used to control the incubation temperature. During amperometric experiments, a magnetic bar was used to stir the solution.

Reagents and buffers

A 32 kDa molecular antigen of *Schistosomiasis japonicum* from an adult worm antigen (AWA) was isolated and purified to homogeneity according to a reported method.¹⁹ SjAb was prepared through immunizing rabbits with 2500 SjAg for 45 days. The antibody in the infected rabbit serum was purified by precipitation from a saturated ammonium sulfate solution, as described in the literature.²⁰ Horseradish peroxidase (HRP) and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma. All other chemicals used were of analytical grade. A blocking buffer solution containing 0.1% BSA w/w and 1 mM EDTA in 0.1 mol/l Tris-HCl (pH 7.4) was used as an incubation solution. The washing solution was a 0.1 mol/l Tris-HCl-0.1 mol/l KCl buffer. A solution of 1% BSA in a Tris-HCl buffer solution was used to dilute the SjAg. All solutions were prepared with doubly distilled water.

Fabrication of a graphite-paraffin-SjAb biocomposite based immunosensor

The configuration of the immunosensor is illustrated in Fig. 1. The immunosensor was prepared as follows: 0.5 g of graphite powder, 15 mg of SjAb and 0.5 g of paraffin were dispersed in a small amount of diethyl ether to obtain a homogeneous carbon paste (CP). The resulting paste was kept at room temperature to evaporate a major part of the ether. The modified CP was packed into a Teflon tube (6 mm i.d. and 10 mm deep). A stainless-steel rod was inserted into the tube to pack the carbon paste and achieve electric contact.

Preparation of HRP-SjAg conjugate

HRP was coupled to SjAg by the standard periodate method described in the literature.¹ HRP (5 mg) was dissolved in 0.5 ml of acetate buffer (0.2 mol/l, pH 5.6), and then oxidized with 0.25 ml of freshly prepared 0.1 mol/l NaIO₄ for 30 min in the dark. Glycol (0.5 ml, 2.5% v/v) was added to the solution and incubated for 30 min to neutralize any excess periodate. Then, SjAg (5 mg) was added and the pH of the solution was adjusted to 9.0 with sodium carbonate buffer. This solution was allowed to stay overnight at 4°C. A freshly prepared 0.1 ml NaBH₄ (5 mg/ml) solution was added to reduce the Schiff base formed between HRP and SjAg for 2 h. The reaction mixture was dialyzed for one day against a 0.1 mol/l phosphate buffer, pH 7.4, at 4°C to remove any non-reacted low-molecular reagents. Further purification was conducted by gel filtration on a Sepharose G-25 column to give the SjAg-HRP conjugate. The conjugate was mixed with glycerol and stored at -20°C until ready to use.

Fig. 2 Cyclic voltammograms of 0.5×10^{-3} mol/l TMB and 1×10^{-3} mol/l H₂O₂ in 0.1 mol/l pH 5 citrate-phosphate buffer (a) recorded in the absence of HRP, (b) recorded 5 min after the addition of 1 µg/ml HRP in solution. Scan rate, 100 mV/s.

Immunoassay procedure

A competitive enzyme-linked immunoassay was performed on the SjAb-modified electrode surface for the determination of SjAg. The immunosensor was incubated in 1 ml of a blocking buffer solution containing 3 µg/ml HRP-SjAg and different concentrations of SjAg for 30 min at 25°C. The free SjAg competed with the HRP-SjAg for binding to an SjAb-coated electrode surface. The amount of HRP-SjAg binding to the electrode surface was inversely proportional to the amount of the analyte in the sample. The immunosensor was thoroughly rinsed with the washing buffer to remove any unbound tracer. The electrode was placed in an electrochemical cell containing 10 ml of 0.1 mol/l citrate-phosphate buffer (pH 5). After adding 100 µl of TMB and H₂O₂ peroxidase substrate to give a final concentration of 5×10^{-4} mol/l and 1×10^{-3} mol/l, respectively, the reduction current signal due to the mediator was recorded at +100 mV versus Ag/AgCl reference electrode as an assay signal.

Renewal of the immunosensor surface

Prior to each immunoassay, a small amount of paste was pushed out of the tip, and the surface of immunosensor was polished manually on ultra fine emery-paper until a smooth surface was achieved. The electrode surface was cleaned with doubly distilled water. When not in use, the immunosensor was stored in a refrigerator at 4°C.

Results and Discussion

Cyclic voltammetric studies

Immunoassays are based on the inherent molecular-recognition capabilities of an antibody for the corresponding antigen. Interactions between Ab and Ag can be measured indirectly via detection of using labeled molecules, such as a radioisotope, an enzyme or a fluorescent tracer. The enzyme-linked label can provide a means to amplify the resulting Ag-Ab complex formation. In the present study, we used HRP-SjAg as a tracer and TMB as an enzyme substrate. We, at first, investigated the effect of HRP on the reaction between TMB and H₂O₂. The cyclic voltammograms were taken in a 0.1 mol/l citrate-phosphate buffer (pH 5) containing 5×10^{-4} mol/l TMB

Fig. 3 Amperometric responses of a BSA-modified electrode (a, b) and the SjAb-modified electrode (c, d) in 0.1 mol/l citrate-phosphate containing 0.5×10^{-3} mol/l TMB and 1×10^{-3} mol/l H_2O_2 . The electrodes were incubated with 3 $\mu\text{g}/\text{ml}$ HRP-SjAg (a, c) or with 3 $\mu\text{g}/\text{ml}$ HRP-SjAg + 2 $\mu\text{g}/\text{ml}$ SjAg (b, d) for 40 min.

and 1×10^{-3} mol/l H_2O_2 with a scan rate of 100 mV/s between the potential ranges -500 to 800 mV. As shown in Fig. 2(a), a two-electron redox behavior was observed for TMB. Figure 2(b) is the cyclic voltammogram recorded 5 min after the addition of 1 $\mu\text{g}/\text{ml}$ HRP. The presence of HRP results in an increase of the cathodic current. This demonstrates that TMB and H_2O_2 are favorable for a sensitive amperometric detection of HRP. The graphite-paraffin electrode offers effective detection of the biocatalytic product.

Immunosensor response to SjAg

To investigate the amperometric response of the immunosensor to the analyte, an SjAb-modified paraffin-graphite composite electrode and a BSA-modified paraffin-graphite composite electrode were incubated with 3 $\mu\text{g}/\text{ml}$ HRP-SjAg in the absence and presence of 2 $\mu\text{g}/\text{ml}$ SjAg for 40 min. As shown in Fig. 3, the BSA-modified paraffin-graphite composite electrode shows a small amperometric signal in the absence (a) or presence (b) of the analyte only due to nonspecific binding. In contrast to the BSA-modified paraffin-graphite composite electrode, the SjAb-modified paraffin-graphite composite electrode has a large amperometric response to HRP-SjAg, as shown in Fig. 3(c), which indicates that HRP-SjAg binds with SjAb immobilized on the electrode surface through specific immunoreaction. The presence of free SjAg in the incubation solution (d) decreases the amperometric response owing to decreasing the amount of HRP-SjAg by competitive binding of SjAg to the immunosensor surface. The decrease in the current was about 40% of the current due to HRP-SjAg-specific binding to SjAb immobilized on the electrode surface (response current substrate base-line current). This demonstrates that the proposed immunosensor exhibits a well-defined response to the analyte. In order to promote an analytical performance of the immunosensor, the effects of several experimental parameters were investigated.

Effect of SjAb loading in an immunosensor

Because the assay performance involves SjAg binding with SjAb immobilized on an electrode surface, it is necessary to define the amount of SjAb incorporated in a carbon-paraffin composite electrode, which influences the amount of tracer that binds to the electrode surface in the incubation step and the

Fig. 4 Effect of SjAb loading in a carbon-paraffin composite electrode on the amperometric response of the immunosensor. Immunosensor was incubated with 3 $\mu\text{g}/\text{ml}$ HRP-SjAg for 40 min.

Fig. 5 Effect of the pH of a citrate-phosphate buffer solution on the amperometric response of an immunosensor. The immunosensor was incubated with 3 $\mu\text{g}/\text{ml}$ HRP-SjAg for 40 min.

amperometric response signal. Immunosensors modified with different amounts of SjAb were incubated in 3 $\mu\text{g}/\text{ml}$ HRP-SjAg for 40 min, and the amperometric responses were measured. The results (Fig. 4) show that the amperometric response increases with raising the SjAb loading in the carbon-paraffin composite electrode. To maximize the signal response and to minimize the used amount of SjAb, a 15 mg loading of SjAb in a carbon-paraffin composite was selected to prepare the immunosensor for subsequent experiments.

Effect of the pH

The pH of the measuring solution influences the activity of the enzyme and catalytic product. In order to optimize the pH of the measuring solution for an immunoassay, the immunosensor was incubated in 3 $\mu\text{g}/\text{ml}$ HRP-SjAg for 40 min, and the amperometric response was measured in a 0.1 mol/l citrate-phosphate buffer of different pH. Figure 5 shows the effect of the pH on the magnitude of the amperometric signal. Because the current response arrives at a maximum value at pH 5.0, we used a pH of 5.0 of a citrate-phosphate buffer for the amperometric measurements.

Fig. 6 Effect of the concentration of HRP-SjAg in the incubation solution on the amperometric response of the immunosensor. The immunosensor was incubated with different concentrations of HRP-SjAg for 30 min.

Fig. 7 Effect of the incubation time on the amperometric response of immunosensor. The immunosensor was incubated with 3 μg/ml HRP-SjAg +5 μg/ml SjAg for different time.

Effect of the HRP-SjAg concentration

The concentration of the HRP-SjAg used in a competitive immunoassay also influences the amperometric response of the immunosensor. To optimize the HRP-SjAg concentration, the immunosensor was incubated with increasing concentrations of HRP-SjAg for 30 min. As shown in Fig. 6, the amperometric response increases with an increase of the concentration of HRP-SjAg up to 3 μg/ml, and then trends to saturate. Thus, 3 μg/ml HRP-SjAg in an incubation solution was selected to maximize the response and to minimize the amount of HRP-SjAg used.

Effect of the incubation time

The effect of the incubation time in which the tracer and the analyte were allowed to competitively bind to the immobilized SjAb was studied. The immunosensor was incubated with a solution containing 3 μg/ml HRP-SjAg and 5 μg/ml SjAg for different times. The results are depicted in Fig. 7, which show that the amperometric signal increased rapidly with the incubation time up to 30 min, and then trended to saturate. Thus 30 min was selected as the optimal incubation time to

Fig. 8 Calibration graph showing current responses versus the concentration of SjAg. The immunosensor was incubated with 3 μg/ml HRP-SjAg solutions containing different concentrations of SjAg for 30 min.

maximize the signal and to minimize the assay time.

Determination of SjAg with the immunosensor

For the determination of SjAg, a competitive immunoassay was performed under optimized conditions. Figure 8 displays the competitive curve. As expected for competitive assays according to the four-parameters-logistic equation of Rodbard,²¹

$$y = d + (a - d)/(1 + (x/c)^b),$$

where a represents the upper asymptote of the curve, b the slope of the curve at the midpoint, c the analyte concentration at the midpoint, d the residual current, x the analyte concentration and y the test signal. A sigmoidal relationship exists between the assay response and the free analyte concentration. The measuring range was 0.5–30 μg/ml and the relative standard deviation was below 6.8% ($n = 4$). This demonstrates that the immunosensor offered a reproducible sensing surface by polishing after each measurement. The applicability of the immunosensor for the detection of the SjAg in rabbit serum has been demonstrated. Four rabbit serum samples with different infection degrees were assayed. The results are given in Table 1, which demonstrate that the immunosensor meets the requirements for clinical analysis for evaluating the infection degree by SjAg in serum and the diagnosis of *schistosomiasis*.

Conclusions

The use of an SjAb-modified paraffin-graphite composite electrode for the determination of SjAg in serum samples was examined in this paper. Competitive ELISA formats were adopted with HRP-SjAg as a tracer. The developed system permits a reliable determination of SjAg in serum. It also offers some advantages, such as simplicity of fabrication, rapidity of measurement and being renewable by simple manipulation.

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Table 1 Determination of SjAg in a rabbit serum sample

Rabbit serum sample	Infection degree ^a	Current/ μ A	Concentration detected/ μ g ml ⁻¹
1 [#]	1000	0.60	1.1
2 [#]	2000	0.56	1.4
3 [#]	3500	0.51	1.9
4 [#]	5000	0.44	3.2

a. The infection degree is designed to the number of *Schistosoma japonicum* in immunizing rabbits experiments.

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