Disposable amperometric immunosensor system for rabbit IgG using a conducting polymer modified screen-printed electrode

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Abstract

A disposable and mediatorless immunosensor based on a conducting polymer (5,2,5,2'-terthiophene-3'-carboxylic acid) coated screen-printed carbon electrode has been developed using a separation-free homogeneous technique for the detection of rabbit IgG as a model analyte. Horseradish peroxidase (HRP) and streptavidin were covalently bonded with the polymer on the electrode and biotinylated antibody was immobilized on the electrode surface using avidin–biotin coupling. This sensor was based on the competitive assay between free and labeled antigen for the available binding sites of antibody. Glucose oxidase was used as a label and in the presence of glucose, H₂O₂ formed by the analyte–enzyme conjugate was reduced by the enzyme channeling via HRP bonded on the electrode. The catalytic current was monitored amperometrically at 0.35 V vs. Ag/AgCl and this method showed a linear range of RIgG concentrations from 0.5 to 2 μg/ml with standard deviation ±0.0145 (n = 4). Detection limit was determined to be 0.33 μg/ml.

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1. Introduction

Immunosensors have attracted growing attention with expectation of obtaining quick and highly sensitive immunological response. The extremely high selectivity and affinity of antibody molecules to their corresponding antigens have widely been exploited for analytical purposes mainly as radio-immunoassays (RIAs) or enzyme linked immunosorbent assays (ELISAs). However, these require highly qualified personnel, tedious assay time, or sophisticated instrumentation (Maunaert and Daenens, 1994). Thus, wide ranges of immunosensors combining a classic ELISA format with amperometric, photometric, chromatographic and other detection methods have been developed (Kalab and Skladal, 1997; Rishpon and Ivnitski, 1997). Of these, optical detection methods are most developed in terms of commercial applications (Byfield and Abuknesha, 1994), but the electrochemical detection method using immunoreactions was not applied much by now. However, it appears very promising due to the relatively simple and inexpensive equipment required (Skladal, 1997; Madou and Tierney, 1993; McNeil et al., 1977).

Amperometric immunoassays were initially based on ELISAs and the measurements of electrochemically active products were carried out using redox-enzymes (Tiefenauer et al., 1997). Therefore, amperometric immunosensing requires labeling of either antigen or antibody, since the two reaction partners are electrochemically inert. In most of the cases, an enzyme is used as the label, which allows an enzymatic amplification of the primary signal. In the enzymatic reaction, electrochemically active substances are either consumed or released and electrochemically detected in the second step (Wendzinski et al., 1997; Ghindilis et al., 1997). In the case of competitive assay, generally, an analyte and a labeled analyte compete for a limiting number of immobilized antibody binding sites. The amount of bound conjugate is inversely proportional to the amount of analyte in the sample. Enzyme conjugates are used to produce the electrochemical signals, which can deter-
mine the antigen concentration. Three specific enzymes account for most studies: horseradish peroxidase (HRP), glucose oxidase (GOx), and catalase (Manning et al., 1990). Only conjugated material bound to the electrode surface via the antibody–antigen interaction brings about a catalytic reaction that is coupled to the electrode surface. Unbound material in the bulk solution does not introduce any significant electrocatalytic current (Killard et al., 2001). Therefore, new techniques with shorter preparation times and less reaction steps are needed.

The two main methodologies for immunoassay design are homogeneous and heterogeneous (Tijssen, 1985), which can be applied to an electrochemical sensor. In elegance, homogeneous assays are superior, as they can be performed in solution in a single step. However, the requirement of speed and analytical simplicity cannot be attained with traditional heterogeneous assay formats. Currently, the attachment of antibody to the electrode surface through the specific avidin (or streptavidin)–biotin molecular interaction has been revealed as an effective and reliable approach (Wilchek and Bayer, 1988). In this contribution, however, we have chosen this antibody immobilization protocol and using avidin covalently bonded to the polymer film. When glucose solution is injected, an electrocatalytic current depending on the amount of the bound labeled antigen is measured. Such separation-free, homogeneous sensor formats are relatively quick, easy to handle and so most useful for routine measurements (Duan and Meyerhoff, 1994).

To get the analytical signal in the electrochemical immunosensor system, mostly mediators in sensors have been used to shuttle electrons between the tracer enzyme and the electrode (Ivitski and Rishpon, 1996). The main problem associated with this approach is the leaching of the mediators in the bulk solution. On the other hand, some authors have reported enzyme channeling as immunoassay for mediatorless system using HRP loaded carbon ink, which were printed on the planar electrode (Wright et al., 1995). This approach, however, might cause the denaturation of loaded HRP after a certain time. To overcome these disadvantages, we carried out here a new approach for the immobilization of HRP and streptavidin based on a conducting polymer bearing a carboxylic acid group coated on the screen-printed carbon electrode (SPCE). The similar covalent immobilization of a molecule having amino group using this electroactive polyterthiophene conducting polymer was previously carried out (Lee and Shim, 2001). Therefore, the technique to immobilize HRP and avidin on the conducting polymer coated SPCE also brings the advantages over that.

In the present work, the proposed conducting polymer–HRP–avidin:biotin–anti-R1gG immunosensor has been applied to determine R1gG using a competitive binding assay with GOx labeled R1gG according to the bio-electrocatalytic scheme. Moreover, the application of these SPCE is mainly based on their attractive advantages such as disposability, low cost, and ease of preparation. In the present experiment, the experimental parameters affecting the responses of the sensor were optimized in terms of pH, temperature, conjugate concentration, and incubation time on the competitive assay.

2. Materials and methods

2.1. Reagents

2.1.1. General reagents

A terthiophene monomer bearing a carboxyl group, 5,2′-5′2′-terthiophene-3-carboxylic acid (TTCA) was synthesized (Lee et al., 2002). 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide, EDC were used as received from Fluka. α-D-Glucose was purchased from Aldrich (USA). The buffer for the immunochemical reaction was a 10 mM phosphate buffer solution (PBS, pH 7.4) made from 10 mM disodium hydrogen phosphate (Sigma, USA) and 10 mM sodium dihydrogen phosphate (Aldrich Chemical Company Inc.). Unless otherwise stated, all immunochromes were prepared in PBS.

2.1.2. Biological reagents

HRP (EC.1.11.1.7, 180 U/mg) and GOx (166 U/mg) were used as received from Toyobo, Japan. The streptavidin from Streptomyces avidinii, biotinylated anti-rabbit IgG (antibody developed in goat), rabbit IgG from rabbit serum as antigen and casein from bovine milk used in this work, all of them were obtained from Sigma. Sephadex G-25 medium was received from Pharmacia, Sweden. Glutaraldehyde and lysine were obtained from Sigma. All other chemicals were of analytical grade and used without further purification. All experimental solutions were prepared in doubly distilled water obtained from a Milli-Q water system (18 MΩ/cm), Millipore S.A. 67120 Molshem, France.

2.2. Apparatus

Amperometric measurement and cyclic voltammetric experiments were performed with a model EG&G, PAR 273 Potentiostat/Galvanostat. All electrochemical measurements were performed in the three electrodes system. The carbon inks used in screen-printing (Lot No. 100328) were purchased from Jujo Chemical, Japan. The working electrode having a diameter of approximately 3.14 mm was printed on a polystyrene-based film using a BANDO industrial, Korea (model BS-450HT) screen-printer. The schematic layout of SPCE is shown in Fig. 1.
2.3. Process of the preparation of RIGG-GOx conjugate

A two-step procedure for conjugation of rabbit IgG to GOx described by Tijssen was followed (Tijssen, 1985). The optimized reactions are as follows: The GOx (10 mg dissolved in 0.2 ml 0.1 M phosphate buffer at pH 6.8) and glutaraldehyde were incubated at room temperature for 18 h. Excess glutaraldehyde was removed by passing through a sephadex G-25 column equilibrated with 0.9% NaCl and then 5 mg rabbit IgG in 0.2 ml of 0.5 M sodium carbonate buffer at pH 9.5 was added to it. This mixture was incubated for 24 h at 4 °C and blocked the remaining activated groups with 0.1 ml 1 M lysine (neutralized to pH 7.0) for 2 h followed by overnight dialysis against PBS (pH 7.4). Finally, the conjugate was filtered through a sterile Millipore membrane (0.20 µm) and the filtrate was then stored at −20 °C until required.

2.4. HRP and streptavidin immobilization on the polymer coated SPCE

The SPCE was dipped in 1 mM TTCA monomer containing solution prepared in 1:1 of di(propylene glycol) methyl ether and tri(propylene glycol) methyl ether and the polymer growing was obtained by potential cycling three times from 0.0 to 1.6 V (vs. Ag/AgCl) at 100 mV/s in a 10 mM PBS (pH 7.4). The TTCA polymer (TCAP) coated SPCE was immersed in 10.0 mM 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide EDC containing 10 mM PBS (pH 7.4) for 4 h at room temperature. The EDC-attached electrode was washed and then incubated in a 1 mg/ml of HRP in 10 mM PBS at pH 7.0 for 20 h at 4 °C. After washing again with the same buffer, glucose was added to a 10 mM PBS of pH 7.4 and the steady-state response of the amperometric system was recorded. Calibration plots were detained by plotting $i_p$ values in the presence of enzyme substrate (glucose) minus the background $i_p$ values (where no analyte was present) in a buffer solution.

2.6. RIGG assay protocol and electrochemical measurements

After the immunosensor had been incubated, RIGG was determined using competitive approach, i.e. RIGG competed with the RIGG-GOx tracer for the antibody binding sites immobilized on the electrode surface. Bound enzyme label activity is inversely related to the amount of RIGG present. The activity of the GOx label was measured using an amperometric technique to quantify the antigen–antibody on the surface of the sensor. A potential of −0.35 V vs. Ag/AgCl was applied to monitor the catalytic current in a cell and the cell volume was 1 ml. After the background current was stabilized, a standard solution of glucose was added to a 10 mM PBS of pH 7.4 and the steady-state response of the amperometric system was recorded. Calibration plots were detained by plotting $i_p$ values in the presence of enzyme substrate (glucose) minus the background $i_p$ values (where no analyte was present) in a buffer solution.

3. Results and discussion

3.1. Electrochemical characterization of the sensor using cyclic voltammetry

The anodic electropolymerization of the carboxyl terthiophene monomer coated SPCE in a PBS of pH 7.0 is shown in Fig. 2b. Cyclic voltammograms (CVs) also recorded for the bare SPCE in the same buffer solution (Fig. 2a). During the first anodic scan from 0.0 to +1.6 V on the TTCA monomer coated electrode in a
buffer solution at the scan rate of 100 mV/s, the oxidation peak of the monomer appeared at around +1.35 V. For a comparative study with a glassy carbon disk electrode, the monomer oxidation peak appeared at 1.3 V vs. Ag/AgCl and the reverse scan to the positive direction showed a small cathodic peak at 1.1 V, which corresponded to the reduction of the polymer film formed on the electrode. The peak currents at 1.1 and 1.3 V increased with increasing the cycle number indicating the formation and growth of polymer as reported in earlier communication (Lee et al., 2002).

It is well known that EDC catalyzes the formation of amide bonds between carboxylic acids and amines by activating the carboxylic groups as shown in Fig. 3. The EDC reaction proceeds at room temperature and pH 7.0. Covalent binding of enzyme occurs via lysine residues under formation of amide groups with the activated carboxylic terminal groups of the conducting polymer coated on the SPCE. The streptavidin immobilization was followed by the same procedure. Therefore, both HRP and streptavidin immobilization on the conducting polymer modified screen-printed electrode is simple and gentle. Fig. 4 represents the CVs of a TTCA polymer modified SPCE (curve a), and a TCAP|HRP modified SPCE (curve b) in PBS, pH 7.0 at 20 mV scan rate. As shown in Fig. 4, the HRP modified electrode shows a pair of redox peaks for the direct electron transfer behavior of immobilized HRP at about 50 and −20 mV. In a comparative study for the glassy carbon electrode, peak currents vary linearly with the scan rate, indicating that an electrode process is due to the adsorptive species. It is noted that no corresponding peak is observable at either a bare SPCE or a TCAP modified SPCE electrode in the same potential range. Thus, it suggested that this pair of peak results from the redox process of the electroactive sites of immobilized HRP on the TCAP|SPCE.

The immunoassay protocol have used the well established avidin–biotin interaction for antibody immobilization. Therefore, streptavidin (i.e. avidin) was immobilized on the TCAP|HRP modified electrode and then anti-RIgG (antibody) was attached to TCAP|HRP|avidin modified electrode surface using biotin conjugate. Fig. 5 shows the voltammetric behavior of (a) TCAP|HRP|avidin and (b) TCAP|HRP|avidin:biotinylated-anti-RIgG modified elec-

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**Fig. 2.** CVs of (a) bare SPCE and (b) polymer growing on SPCE in 10 mM PBS, pH 7.0 at scan rate 100 mV/s.

**Fig. 3.** Schematic representation of the formation of amide bonds between lysine residues of the HRP and terminal activated carboxyl functional group (activated by EDC) of TCAP coated on screen-printed electrode.

**Fig. 4.** CVs of (a) polymer (TCAP) coated electrode and (b) TCAP|HRP modified electrode in 0.2 M PBS, pH 7.0 at scan rate 20 mV/s.
trode in a PBS (pH 7.0). Two pairs of redox peaks were observed for the TCAP|HRP|avidin modified electrode in the CV recorded in PBS at around 150/75 mV and —150/—200 mV. The first redox peak might have appeared for a shift in the peak potential towards more positive direction for HRP. The more negative redox peaks at —150 mV might be corresponded to the redox couple of streptavidin. In contrast, these redox peaks completely disappeared after treated with biotinylated anti-RlgG and hence only the redox peaks for HRP was observed in a shifted potential. The exact mechanism, by which the CV was influenced, is not known exactly. Possibly, the adsorptive behavior between avidin and biotin-antibody changes the shape and peak position of the CV. This antibody was used for the competitive reaction between antigen (RlgG) and GOx labeled antigen. Finally, the detection was achieved measuring glucose concentration as an enzyme substrate.

3.2. Amperometric measurement of glucose oxidase activity bound to the sensor surface

The sequence of steps and principle of the sensing part of the separation-free competitive immunosensor technique used in this present work can be shown schematically in Fig. 6. Initially, the TTCA conducting polymer was coated on the SPCE, then HRP and streptavidin were covalently bonded on the polymer-coated electrode. Finally, biotinylated antibody was immobilized on the streptavidin-modified surface. Then, it involved an incubation of the antibody-modified electrode in a solution containing free antigen and GOx labeled antigen. In presence of glucose, H₂O₂ formed by the GOx was reduced by the enzyme-channeling via the HRP present in the electrode surface. Eventually, in the format designed for immunosensing, the enzymatic activity can be assessed by amperometric or voltammetric detection of the reduction current generated by the catalytic product. The TCAP|HRP|av:bio-anti-

![Fig. 5. CVs of (a) TCAP|HRP|avidin modified electrode and (b) TCAP|HRP|av:biotinlated-anti-RlgG modified electrode in 0.2 M PBS, pH 7.0 at scan rate 20 mVs.](image)

![Fig. 6. Schematic representation of principle and sequence of steps for the proposed separation-free competitive immunoassay format for RlgG.](image)

RlgG electrode would be required to measure H₂O₂ produced by the GOx bound specifically in the proximity to the HRP electrode surface. The catalytic cycle can be described by the scheme in Fig. 6, where GOx corresponds to the enzyme used as label. In order to maximize the sensitivity of the sensor, it would be necessary to use the lowest concentration of the analyte. Therefore, the first evaluation was done with this TCAP|HRP|av:bio-anti-RlgG electrode after immersion in a 50 μl aliquot of 5 μg/ml free RlgG and 50 μl labeled RlgG containing solution. The activity of the GOx label in surface was measured using amperometric technique and steady-state responses were obtained after adding various aliquots of glucose solution as a substrate at a potential —0.35 V vs. Ag/AgCl. Fig. 7 illustrates the amperometric current responses and calibration plot of the immunosensor as a function of glucose concentration. As can be seen in Fig. 7, the electrode retains its ability to exhibit linear amperometric responses with the glucose concentration up to 12

![Fig. 7. Amperometric responses and calibration plot using TCAP|HRP|av:bio-anti-RlgG modified electrode toward different concentrations of glucose at —0.35 V vs. Ag/AgCl.](image)
mM with the regression coefficient of 0.989, and the standard deviation of ±0.0057 (n = 4).

3.3. Optimization of analytical conditions

An increase of temperature had a favorable effect on the immunoreaction using constant concentrations of RlgG-GOx and free RlgG. The effect of temperature on the competitive immunoreaction was examined at the range from 10 to 50 °C as shown in Fig. 8a. It was found that the current response increased with the increasing temperature up to 35 °C. However, temperatures over 40 °C caused irreversible behavior (denaturation of proteins) involved in the process. Different incubation temperature was reported in the literature ranging from 25 to 37 °C (Santandreu et al., 1997). As is well known, an optimal temperature of immunoreaction would be 37 °C, which supports this observation at this temperature, however, a long incubation time might decrease the activity of enzyme, antigen, and antibody, leading to the deterioration of response signals. Thus, the temperature of 25 °C (room temperature) was selected as a compromise.

The pH of catalytic medium shows a strong effect on the activity of the enzyme label attached to the immunosensor surface. The trends of the dependence of pH of the sensor on the competitive immunoreaction are shown in Fig. 8b. The influence of pH was studied between 5.5 and 8.0 using constant concentrations of free RlgG and GOx-RlgG at 25 °C. The immunosensor response increases with increasing pH value from 6.5 to 7.4 and then decreases as the pH increases further. It is well known that at relatively high pH, the activity of the enzyme is inhibited. The experimental results show that the maximum response occurs at pH 7.4 of the physiological pH and most immunoreactions exhibit optimal binding at this pH (Ishikawa et al., 1981). Therefore, a pH 7.4 of PBS was used as the medium for the immuno-competitive reaction.

The influence of the immunochemical incubation (i.e. when the antigen–antibody reaction occurs) time on response signals was also investigated. The antibody-modified electrode was used to study the effect on the sensitivity of the immunosensor to RlgG. In the incubating solution, when the analyte antigens reach the antibodies at the electrode surface of the immunosensor, it takes some times for the contacting species to form immunocomplexes. Fig. 8c displays the effect of incubation time on the competitive immunoassay. The incubation times were 3, 5, 10, 15, 20 and 30 min, using the same analyte and analyte-conjugate concentrations. The current responses obtained in this study increase with the incubation time rapidly up to 25 min and after that the variation slowed. An equal response was found suggesting that an equilibrium state was reached very fast, and 15 min was chosen for the subsequent study to evaluate the analytical performance of the immunosensor, because in an attempt to employ shorter incubation times, a reduction in sensitivity was observed. The $i_p$ values obtained for a 15 min incubation time, PBS, pH 7.4 and at a temperature of 25 °C were not exactly the same, might be due to the surface roughness of screen-printed electrode.

It was also attempted to optimize the concentration of RlgG-GOx conjugate at a fixed concentration of RlgG, because the response signal of the immunosensor depends on the amount of conjugate bound on the surface of the sensor, which in turn corresponds to the amount of conjugate in the incubation solution. In order to obtain a maximum response with a minimum amount of conjugate was determined by incubating the immu-
nosensor with increasing amount of RIgG-GOx and the best results have so far been obtained using 20 µl/ml RIgG-GOx solution, and this amount was routinely used for the competitive reaction. The concentration of substrate was set about 10 mM in PBS (pH 7.4) and this concentration was applied to all the subsequent experiments.

3.4. Immunosensor response to rabbit IgG

Since the TCAP|HRP|av:bio-anti-RIgG electrode was finally employed as an immunosensing device, the feasibility of this new design for a separation-free homogeneous competitive immunoassay format was evaluated for RIgG (Fig. 6). The electrode response observed, when no incubation in an analyte (RIgG) containing solution was performed would be the baseline signal, since there was no competition between analyte and conjugate for antibody binding sites. Then from the baseline signal, a progressive decrease in the current responses would be obtained if the incubation in RIgG solution were carried out. A first experiment was made employing a solution containing a fixed amount of analyte-GOx and variable concentrations of analyte, when the enzyme substrate was present in the concentration of 10 mM. A significant decrease in the current response could be attributed to the formation of the anti-RIgG and RIgG (antibody–antigen) immunocomplex. The data indicates that the immunosensor is capable of distinguishing RIgG concentrations ranging from 0.5 to 2 µg/ml. The highest the concentration of free analyte increased, the lowest the amount of analyte-GOx conjugated to the antibody immobilized on the electrode surface. Eventually, a calibration plot for the current vs. the RIgG concentration can be obtained under the optimal conditions described above. It shows that the response decreased as less RIgG-GOx binds to the antibody sites on the electrode as the concentration of free RIgG in solution increased. Fig. 9 shows that the maximum current represents a zero analyte of free RIgG and, the response to the injection of RIgG was fully linear in the range of the amperometric response for a competitive analysis. The calculated limit of detection for RIgG is 0.33 µg/ml, \( r^2 = -0.995 \), S.D. = ±0.0145 (n = 4). This followed the linear equation: \( I (\mu A) = 0.06249 - 0.17526 \times (\text{RIgG}) \) (µg/ml). The resultant overall time for the analysis under these optimized conditions was 35 min.

4. Conclusions

The main attractive feature of the present research is that the potential use of a conducting polymer having a carboxyl group was reported. In this work, it is possible to design a mediatorless immunosensor system by covalent immobilization of HRP and streptavidin (i.e. avidin) based on the conducting polymer coated SPCE. The need of using labels in voltammetric or amperometric devices leads us to choose an enzymatic label such as GOx and glucose as a substrate. The resulting TCAP|HRP|av:bio-anti-RIgG immunosensor offers a relatively sensitive response with a RIgG-GOx conjugate after a relatively short incubation time. The linear range was determined for RIgG concentration from 0.5 to 2 µg/ml with the regression coefficient of −0.995, and the standard deviation of ±0.0145. The linear range obtained agreed favorably with those already obtained reported in the literatures. The advantage of using SPCE for measurement of RIgG lies in disposability and the fact that it is a cost-effective method. The feasibility of this methodology has been demonstrated with RIgG as a model analyte in the format of a separation-free competitive analysis, in which the binding step occurs directly in the measurement solution, and therefore, it could be applied to the detection of different compounds of interest. Such a conducting polymer based immunosensor concept is currently undertaking investigation in our laboratory.

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