Electrochemical sensor for immunoassay of carcinoembryonic antigen based on thionine monolayer modified gold electrode

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Abstract

A sensor based on thionine monolayer modified gold electrode for determination of carcinoembryonic antigen (CEA) in human serum is proposed. The sensor is prepared by covalently binding thionine to a cysteamine self-assembled monolayer with $p$-phthaloyl chloride as a linkage, which gives a surface coverage of $8.97 \pm 3.28 \times 10^{-12}$ mol/cm$^2$ for thionine. The electrochemistry of the immobilized thionine displays a surface-controlled electrode process with an average electron transfer rate constant of $1.47 \pm 0.84 \text{ s}^{-1}$. Based on an electrochemical enzyme-linked immunoassay by using the immobilized thionine as an electron transfer mediator between the electrode and the horseradish peroxidase (HRP) labeled anti-CEA antibody, a calibration curve with two linear ranges from 0.6 to 17 and 17 to 200 ng/mL and a detection limit of 0.2 ng/mL for CEA determination is obtained in pH 4.2 PBS containing 2.0 mmol/L H$_2$O$_2$ and 0.5 mol/L NaCl. The sensor shows a good accuracy. The precision and reproducibility are acceptable with the intra-assay CV of 4.9% and 5.9% at 10 and 100 ng/mL CEA concentrations, respectively, and the inter-assays CV of 7.8% at 100 ng/mL CEA. The response of thionine modified electrode shows only 1.6% decrease after 100 replicate measurements and the storage stability is acceptable in a pH 7.0 PBS at 4°C for 1 week. The method avoids the addition of electron transfer mediator to the solution, thus is much simpler. The proposed method would be valuable for the diagnosis and monitoring of carcinoma and its metastasis.

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1. Introduction

Carcinoembryonic antigen (CEA), an acidic glycoprotein with a molecular weight of about 200 kDa, is one of the most widely used tumor markers. It was first described in 1965 as a marker of colon cancer. Up to now, it has been found to exist in the serum of most tumor patients. The CEA level in serum is related to lung cancer [1–3], ovarian carcinoma [4,5], colon cancer [6–8], breast cancer [9–11], cystadenocarcinoma [5] and others [12–15]. Thus, the determination of CEA level is very helpful to clinical tumor diagnoses. On the other hand, the CEA level in serum is also related to the state of tumor, so it can be directly used for evaluating curative effect, judging recrudescence or metastasis as a marker.

The techniques usually used for quantitative determination of tumor markers are immunological methods, which have become the predominant analytical techniques in the fields of clinical diagnoses and biochemical studies [16]. Immunoassay methods possess extremely high selectivity and sensitivity [17]. The selectivity results from the capacity of antibody recognizing the corresponding antigen. A number of immunoassay methods for the determination of CEA level in serum have been reported [18–28]. These methods with commercially available assay kits include radioimmunoassay [18], chemiluminescence immunoassay [19,20], enzyme-linked immunosorbent assay [21,22], fluoroimmunoassay [23] and liposome immunoassay [24]. Several years ago, radioimmunoassay was the most commonly used method for antigen detection. However, radioactive labels are harmful to the operators. Thus, test kits of enzyme-linked immunosorbent assay and chemilumines-
cence immunoassay have currently been used extensively for CEA determination in clinical diagnoses. Although these traditional methods have detection limits of 0.1–0.5 ng/mL for CEA [25,26] and are suitable enough for the determination of CEA in sera, they are usually time and sample consuming because of several incubation and washing steps needed in detection procedure. Moreover, implement point-of-care (POC) testing is difficult with conventional immunoassay because rather large equipment is necessary. In order to overcome these drawbacks, some works on microchip-based immunoassays have been reported [27–31]. These systems were based on the separation of the free form of antigen and the complex of antigen and antibody. They were not suitable for every analyte contained in biological samples due to the precipitation of the antigen–antibody complex [17,28]. Sato et al. structured a bead-bed immunoassay system on a microchip for the determination of CEA [17]. Anti-CEA molecules were firstly precoated on polystyrene beads, which were introduced into a microchannel. The serum sample containing CEA, the primary antibody and the secondary antibody conjugated with colloid gold were then reacted successively. The resulting complex was finally detected using a laser-induced thermal lens microscope (TLM) and gave a detection limit of 0.03 ng/mL that was lower than those of the conventional ELISA. But the TLM is obviously more expensive than the equipment required usually for ELISA.

In this paper, we prepared a thionine monolayer modified gold electrode as a sensor to develop a novel fast and cheap thionine–H2O2–horseradish peroxidase (HRP) solid-phase immunoassay for CEA determination. The immobilized thionine was used as an electron transfer mediator between the electrode and horseradish peroxidase labeled anti-CEA antibody. This method avoids the addition of electron transfer mediator to the solution; thus, it needs less incubation and washing steps and is simpler than traditional ELISA methods. Moreover, the equipment required for this method is very cheap. In comparison with other immuno-sensors prepared with graphite [32–36], the presented sensor is steady, avoids being contaminated by the addition of mediator and can be repeatedly used without further pretreatment. At the same time, it is easily miniaturized and does not contain the sample. This method is more sensitive than traditional RIA and almost has the same detection limit as that of chemiluminescence immunoassay and fluoroimmunoassay, and thus, has a good application perspective for continuous determination of CEA.

2. Materials and methods

2.1. Reagents

CEA-ELISA and IRMA kits, including 96 assay tubes precoated with murine monoclonal anti-CEA, were purchased from Diagnostic Products (DPC, USA). The ELISA kits consisted of a series of CEA standard solutions with different concentrations from 0 to 500 ng/mL, and a solution of horseradish peroxidase conjugated monoclonal anti-CEA antibody. CNBr-activated Sepharose 6 MB was purchased from Amersham Pharmacia Biotech. Thionine, cysteamine, p-phthaloyl chloride and H2O2 with analytical grade were from the Shanghai Biochemical Reagent Company (China). All other reagents were of analytical grade. Double distilled water was used for all experiments. The eluent buffer was a solution of 0.1 mol/L pH 4.2 PBS containing 0.5 mol/L NaCl. All reagents were brought to room temperature (ca. 20 °C) before use.

2.2. Preparation of thionine modified gold electrode

Gold electrodes, made by sealing gold wire (0.5 mm diameter) in a glass tube of 3 mm exterior diameter, were polished to a mirror-like surface with 0.3 and 0.05 μm alumina slurry on microcloth pads (Buehler Co., USA), followed by being rinsed with water and ethanol and sonicated in double distilled water for 2 min. After cleaning, the electrode was immersed in mercury for about 30 min for amalgamation, the amalgamated gold electrode was immersed in 1.0 mol/L HNO3 till the amalgam was dissolved and the gold surface was exposed. The roughness factor of the electrode surface was 6.59, which was obtained from the geometric area of 1.05 × 10−2 cm² and the real area of 6.90 × 10−2 cm². The real area was obtained by measuring the cathodic peak area of cyclic voltammogram formed during the re-reduction of superficial gold oxide in 1.0 mol/L H2SO4 [37].

Modification of the gold electrode was performed by soaking a previously pretreated electrode in a deoxygenated 20 mmol/L cysteamine solution for more than 20 h. The resulting monolayer-modified electrode was thoroughly rinsed with water to remove physically adsorbed cysteamine and dried in a jet of nitrogen. The cysteamine-modified electrode was then immersed into a p-phthaloyl chloride saturated solution of toluene for 5 h and rinsed with toluene. Finally, the modified electrode was transferred into the acetonitrile solution of 2 mmol/L thionine for 10 h and rinsed with acetonitrile thoroughly to yield a thionine monolayer modified electrode, which was washed with double distilled water and soaked in pH 7.0 PBS at 4 °C for prior to use [38]. Scheme 1 shows the procedure for the preparation of thionine modified gold electrode.

2.3. Apparatus

Electrochemical measurements were performed with a three-electrode system. A platinum wire with 1 mm diameter was used as auxiliary electrode, a “homemade” Ag/AgCl electrode with 2 mm exterior diameter acted as reference and a thionine monolayer modified gold electrode performed as working electrode. The three electrodes were connected with a BAS-100B electrochemical analyzer.
IRMA procedure was carried out with a FMJ-182 Immunoradiometric Gamma Counter (China) according to the instruction and assay procedure in the operator’s manual. The chemiluminescent detections were carried out with an IFFM-D Luminescence Analyzer (Remex Electronic Instrument Limited Co., Xi’an, China). Polytetrafluoroethylene (PTFE) tubing (0.25 mm i.d., 0.8 mm o.d.) was used to connect all components in the flow system.

2.4. Procedure

The analytical procedure for immunoassay is schematically depicted in Scheme 2. The principle of the sandwich assay was based on one-step immunoreactions. Each microtiter well precoated with primary anti-CEA antibody had 50 μL of CEA sample solution and 100 μL of HRP labeled anti-CEA antibody added. The incubation mixture was thoroughly mixed for 10 s and incubated at 23 °C for 60 min. After the HRP labeled anti-CEA antibody molecules were adsorbed on the microtiter plate wells through immune complex formation, the incubation mixture was removed by emptying the plate contents into a waste container, the microtiter wells were rinsed and emptied five times with double distilled water. The residual water droplets were removed by striking the plate sharply onto absorbent paper. A 200 μL deoxygenated eluent buffer was then added into the wells and left at 25 °C for 10 min to elute the HRP labeled anti-CEA antibody from the wells. In order to increase the elution efficiency, the eluent buffer contained 0.5 mol/L NaCl. After dispensing 2 μL 0.2 mol/L H2O2 into the well, the electrochemical detection was performed by inserting the three-electrode system into the microtiter well. Differential pulse voltammetric (DPV) measurements were performed from 150 to −250 mV with a pulse amplitude of

Scheme 1. The procedure of thionine covalently binding to a cysteamine self-assembled monolayer with p-phthaloyl chloride for preparation of thionine modified gold electrode.

Scheme 2. Schematic diagram of FIA system: (P) peristaltic pump, (V) eight-way valve, (C) affinity column, (PMT) photo multiplier, (D) detector and (PC) computer.
50 mV, a pulse width of 60 ms, a scan rate of 20 mV/s, a pulse period of 200 ms and a sampling width of 4 mV.

3. Results

3.1. Electrochemical behavior of thionine modified gold electrode

Fig. 1 shows the cyclic voltammograms of different electrodes in 0.1 mol/L pH 4.2 PBS. No response was observed with bare, amalgamated and cysteamine modified gold electrodes in the working potential window (curves a–c). After binding thionine to the cysteamine self-assembled monolayer with p-phthaloyl chloride, a pair of stable and well-defined redox peaks was observed. The anodic and cathodic peak potentials of thionine monolayer were −32 and +28 mV at 50 mV/s, respectively (curve d). Both the anodic and cathodic peak currents increased with the increasing scan rate and were proportional to the scan rate in the range from 5 to 50 mV/s (Fig. 2), displaying a surface-controlled electrode process. The surface coverage of thionine was calculated to be 8.97 × 10⁻¹² mol/cm². When the scan rate was higher than 50 mV/s the electrode reaction showed a diffusion-controlled process due to the kinetic limitation.

3.2. Cyclic voltammetric response of enzymatic reaction product

It is known that HRP can catalyze the oxidation reaction of thionine by H₂O₂. In the absence of CEA antigen in the incubation solution, the thionine monolayer modified gold electrode showed a pair of symmetrical redox peaks in pH 4.2 PBS containing 2.0 mmol/L H₂O₂ (Fig. 3a). When the incubation solution contained 120 ng/mL CEA the oxidation peak current decreased and the reduction peak current increased greatly and the reduction peak potential shifted slightly to more negative value (Fig. 3b), showing an obvious enzymatic reaction process. Because DPV method can eliminate the background current of voltammetric response, the following experiments were done by DPV method for getting more sensitive results.

When thionine modified electrode was scanned in pH 4.2 PBS using DPV, a reduction peak of the immobilized thionine was found at −46 mV (curve a, inset in Fig. 3). The reduction peak current slightly increased upon addition of HRP.
2.0 mmol/L H₂O₂ (curve b, inset in Fig. 3). After 40 ng/mL HRP was added into above solution, the reduction peak current increased greatly with a slight negative shift of the reduction peak potential (curve c, inset in Fig. 3), indicating an obvious enzymatic reaction process.

3.3. Optimization of CEA determination conditions

The important factors for the elution of HRP labeled anti-CEA antibody from the primary anti-CEA antibody/CEA immunocomplex included pH value of eluent buffer and elution time. The effect of the pH of the eluent buffer on the DPV peak current was studied in the pH range from 2.5 to 6.9 at a 500 ng/mL CEA concentration. From the results shown in Fig. 4A, the maximum response occurred at pH 4.2.

The effect of elution time on the elution efficiency was studied using flow injection chemiluminescence (CL) immunoassay (Scheme 2). CEA molecules were immobilized on CNBr-activated Sepharose 6 MB and introduced into an affinity column according to the manufacturer’s instructions. After the affinity, column was incubated with HRP labeled anti-CEA antibody and the uncomplexed HRP labeled anti-CEA antibody was washed out, the eluent buffer was pumped through the column for 25 s and then the luminol and H₂O₂ were added each 60 s. The elution process was evaluated by the decrease of CL intensity of luminol. With increasing elution time, the CL intensity of luminol decreased greatly (Fig. 5), indicating a decrease in the amount of HRP in the affinity column. In Fig. 5 the elution efficiency was calculated by 100 × ΔI/(Iᵢ − I₀), in which Iᵢ was the initial luminol CL intensity without being eluted, I₀ was the blank value and ΔI was the decrease of the CL intensity of luminol relative to Iᵢ(Iᵢ − Iᵢ). After elution for about 400 s, the CL intensity was as low as that of the blank, and the elution efficiency tended to be 100%, indicating all the HRP labeled anti-CEA antibodies completed with CEA in the affinity column had been eluted. Thus, the optimal elution time of 10 min in the well was selected for CEA determination.

The effect of H₂O₂ concentration on the enzymatic reaction was examined using DPV. As shown in Fig. 4B, with increasing H₂O₂ concentration, the DPV response displayed a Michaelis–Menten’s shaped curve. At H₂O₂ concentrations less than 2.0 mmol/L, the peak current increased linearly with increasing H₂O₂ concentration. When H₂O₂ concentration was more than 2.0 mmol/L, the reduction peak current trended to a constant value. Therefore, the optimal H₂O₂ concentration was 2.0 mmol/L.

3.4. Calibration for CEA determination

With thionine monolayer modified electrode as a sensor, the calibration graph for the determination of CEA was obtained under optimal conditions (Fig. 6). Following the standard immunometric sandwich and the enzymatic reaction procedures, the DPV peak current increased with increasing CEA concentration in two linear ranges from 0.6 to 17 and 17 to 200 ng/mL, the linear slopes were 9.5 and 0.55 nA/(ng mL), and the correlation coefficients were 0.9995 and 0.9880, respectively. The detection limit was 0.2 ng/mL at 3σ.

3.5. Precision, reproducibility and stability

The typical intra-assay and inter-assay precision for CEA assay were also estimated. The variation coefficients (CV) of

![Fig. 4. Effects of pH value of eluent buffer (A) and H₂O₂ concentration (B) on DPV peak current for CEA determination.](image)

![Fig. 5. Effect of elution time on luminol CL intensity (a) for a flow elution process and (b) an affinity column without incubation with HRP labeled CEA antibody. Inset: effect of elution time on the elution efficiency.](image)
intra-assay, obtained by three repeated determinations with the same modified electrode, were 4.9% and 5.9% at CEA concentrations of 10 and 100 ng/mL, respectively, and the CV value for inter-assay variation was 7.8% at 100 ng/mL. Moreover, when the peak currents of the thionine modified gold electrode in pH 4.2 PBS were determined 100 times, the response showed only a 1.6% decrease, indicating a good reproducibility of this sensor. The stability of this sensor was acceptable in pH 7.0 PBS at 4°C for 1 week.

3.6. Accuracy and clinic application

The CEA levels in two sera were quantified by using standard addition method. The CEA concentrations determined with this method and the IRMA method are shown in Table 1. These results by both methods were in good agreement. Thus, the proposed method could be satisfactorily applied to the clinical determination of CEA in human serum.

4. Discussion

The amalgamated bare gold electrode shows a charging current much larger than that of bare gold electrode (curves a and b in Fig. 1), indicating a rougher surface is obtained by amalgamation. The formation of a cysteamine monolayer results in a smaller charging current (curve c in Fig. 1) than that of amalgamated bare gold electrode. The surface is clean and smooth. This is also an effective way to provide the gold electrode with abundant amino groups for covalent immobilization of thionine to form thionine modified gold electrode. From the peak-to-peak separation of the thionine modified gold electrode at different scan rates, an average electron transfer rate constant of $1.47 \pm 0.84 \text{s}^{-1}$ was obtained for the surface-controlled redox process.

When the incubation solution contains CEA, the HRP labeled anti-CEA antibody molecules are eluted into the wells after a deoxygenated eluent buffer is added into the wells. The presence of HRP results in an enzymatic reaction process upon addition of H$_2$O$_2$. The oxidation reaction of thionine by H$_2$O$_2$ under the enzymatic catalysis of HRP increases the concentration of thionine in its oxidized state at the electrode surface, thus, increasing greatly the reduction peak current of thionine modified gold electrode. The immunoassay procedure and the enzymatic reaction mechanism can be expressed as shown in Schemes 3 and 4 [39], respectively.

Both the elution efficiency and the enzyme activity are related to the pH value of eluent buffer. High elution efficiency is usually obtained at low pH. However, most enzymes display their activity in a limited pH range. The maximum activity of HRP is in the range from pH 6.5 to 7.0. With increasing solution acidity its activity decreases greatly. The integrated action of solution acidity results in that the maximum response occurs at pH 4.2, which is selected for elution of HRP labeled anti-CEA antibody and immunoassay of CEA. The optimal pH is higher than those reported in flow immunoassay [40–42].

The elution efficiency also depends on the elution time, which affects the amount of HRP introduced into the detection solution. The decrease of the CL intensity of luminol is due to the reducing amount of HRP in the affinity column, which is a mark of elution efficiency. From the analysis shown in Fig. 5 the optimal elution time in the well is 10 min. With the optimal elution time and pH value of eluent buffer the amperometric immunoassay of CEA in serum samples can be performed at the proposed thionine self-assembled gold electrode at a H$_2$O$_2$ concentration of 2.0 mmol/L. The detection limit of CEA is 0.2 ng/mL, which is more sensitive than traditional RIA.

Table 1

<table>
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<tr>
<th>Serum samples</th>
<th>This method (ng/mL)</th>
<th>IRMA (ng/mL)</th>
<th>Relative deviation (%)</th>
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<td>3.9</td>
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<td></td>
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and is almost the same as that of chemiluminescence immunoassay and fluoroimmunoassay. Considering its acceptable intra-assay and inter-assay precision and the good reproducibility and storage stability of the sensor, the presented method could be used in CEA determination for clinical diagnosis.

5. Conclusions

A sensor prepared by immobilizing thionine monolayer on gold electrode can be used for determination of serum CEA levels. Under the optimal conditions, a detection limit of 0.2 ng/mL CEA and two linear ranges from 0.6 to 17 and 17 to 200 ng/mL for CEA determination were obtained. The sensor shows good accuracy and acceptable precision, reproducibility and storage stability. It can be repeatedly used without further pretreatment and seems to be rather suitable for clinic immunoassay. The method avoids the addition of electron transfer mediator to the solution, thus is much simpler and cheaper than traditional assays. Furthermore, both electrochemical analyzer and the sensor can be easily miniaturized; thus, this method is particularly well suited for point-of-care testing and application in emergency rooms, ambulances, bedside, or doctor’s office. At the same time, it can be readily extended toward detection of other clinically important antigens.

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