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Layer-by-layer hydroxymethyl ferrocene modified sensor for one-step flow/stop-flow injection amperometric immunoassay of α-fetoprotein

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Abstract

A rapid one-step flow/stop-flow injection amperometric immunoassay for α-fetoprotein (AFP) using a novel home-produced electrochemical sensor was proposed. The sensor was prepared using layer-by-layer adsorption of positively charged poly(allylamine) (PAA) and negatively charged hydroxymethyl ferrocene on a screen-printed electrode (SPE). The electrochemistry of the immobilized ferrocene moieties showed a surface-controlled electrode process. Based on an electrochemical enzyme-linked immunoassay with the immobilized ferrocene moieties as an electron transfer mediator between the electrode and the horseradish peroxidase (HRP)-labeled anti-AFP antibody, a calibration curve with two linear ranges from 5 to 20 and 20 to 150 ng ml^{-1} and a detection limit of 2 ng ml^{-1} for AFP determination was obtained under the optimized conditions of 0.891 ml min^{-1} flow rate, 20 μl injection volume and +25 mV applied potential. The sensor showed good repeatability and reproducibility and retained more than 95% of its original signal after 15 days of storage. The proposed method eliminated the need for washing and addition of any substrate or mediator. The complete assay could be handled in less than 25 min with a one-step injection of a 40 μl sample solution. The proposed method would be valuable for the diagnosis and monitoring of carcinoma and its metastasis.

Keywords: α-Fetoprotein; Hydroxymethyl ferrocene; FIA; Immunoreactor; SPE; Poly(allylamine)

1. Introduction

α-Fetoprotein (AFP) is a glycoprotein with a molecular weight of approximately 70,000 Da. It is normally excreted during fetal and neonatal development by the liver, yolk sac, and in small concentrations by the gastrointestinal tract (Yang et al., 2002). AFP levels decrease rapidly after birth and normally only trace amounts are detected in serum after the second year. For normal adults, the production of AFP is almost completely switched off. Serum AFP elevated to abnormally high values usually occurs in several malignant diseases or non-cancerous diseases (Javadpour, 1980; Sell, 1990). In some cases, the AFP level is related to the stage of disease. Thus, it is very important to explore a rapid detection method for AFP.

Enzyme-linked immunosorbent assay (ELISA) using spectrophotometry to detect the products of an enzymic reaction is the traditional method for AFP immunoassay detection (Christiansen et al., 2001; Sun et al., 2001). However, this method is usually time and reagents consuming. Numerous immunoassay techniques have been developed for the detection of AFP, such as surface plasmon resonance (Kato et al., 1997), fluorescence measurement (Ci et al., 1995; Matsuya et al., 2003; Song et al., 2004), chemiluminescence assay (Xue et al., 1996; Kokado et al., 1997; Sutter et al., 2000), phosphorescene measurement (O'Riordan et al., 2002) and atomic absorption spectrometry (Wang et al., 2001). Electrochemical sensors for immunoassays, being safe, economical, simple, easy to handle, and highly sensitive, have also attracted wide interest in recent years (Leopoldo et al., 1996; Guan et al., 2004; Yu et al., 2004; Nobuo et al., 2002; Wilson, 2005).

Flow injection immunoassays (FIIA) have been shown to be useful for improving cumbersome, time-consuming, and labor-intensive traditional immunoassays (Xu et al., 1989; Gunaratna and Wilson, 1993). FIIA has been applied to many fields including the food, pharmaceutical, environmental, and clinical fields (Bjarnason et al., 1997; Burestedt et al., 2000; Nandakumar et

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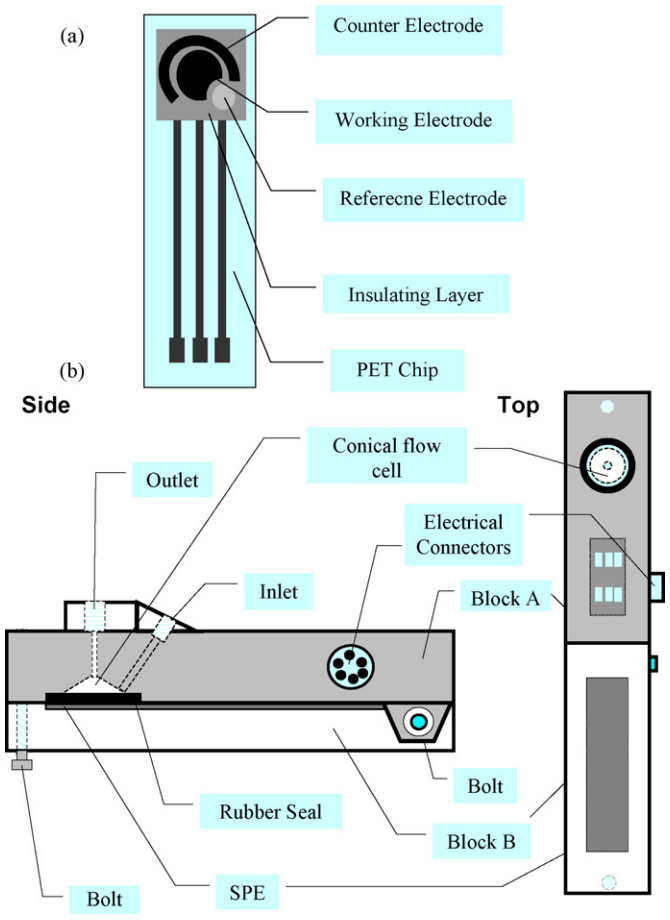
al., 2000) due to the small volume required, the reduced sample handling, good reproducibility, and easy automation for high sample throughput. This technique has been combined with electrochemical sensors and soluble electron transfer mediators for FIIA determination of various analytes (Es et al., 2001; Deng et al., 2002; Gao et al., 2003; Guan et al., 2004). The present work developed a FIIA for AFP by using a novel electrochemical sensor based on a multilayered hydroxymethyl ferrocene modified screen-printed electrode (SPE) prepared with a layer-by-layer (LbL) technique.

LbL technique, a promising and environmental preparation method, has been used extensively to fabricate multilayer films (Lvov et al., 2001; Gao et al., 2002). The advantages of this method are its precise control of thickness, simplicity, versatility (Ai et al., 2003; Shi et al., 2003) and in particular, its unique tendency for self-healing (Eis et al., 1998). The simplicity and universality of the LbL technique combined with a uniform distribution of nanoparticles in the film make its potential particularly attractive both in research and in industry. In this paper, the electrochemical sensor prepared with this technique showed good repeatability, reproducibility and stability for a one-step amperometric flow/stop-flow injection immunoassay of AFP. Based on a sandwich immunoassay, the AFP sample and HRP-labeled anti-AFP antibodies were first pumped into the immunoreactor for incubation. The AFP concentration was obtained by monitoring the decrease of current caused by the sandwich immunocomplex in the immunoreactor. In comparison with traditional immunoassays, the proposed method presents the advantages of being simple, flexible, time and sample conserving, and suitable for automated sample handling. This method could further be developed for practical clinical detection of serum AFP levels.



Scheme 1. Structure of screen-printed electrode (a), electrochemical flow cell, side and top (open cell) views (b).

2. Experimental

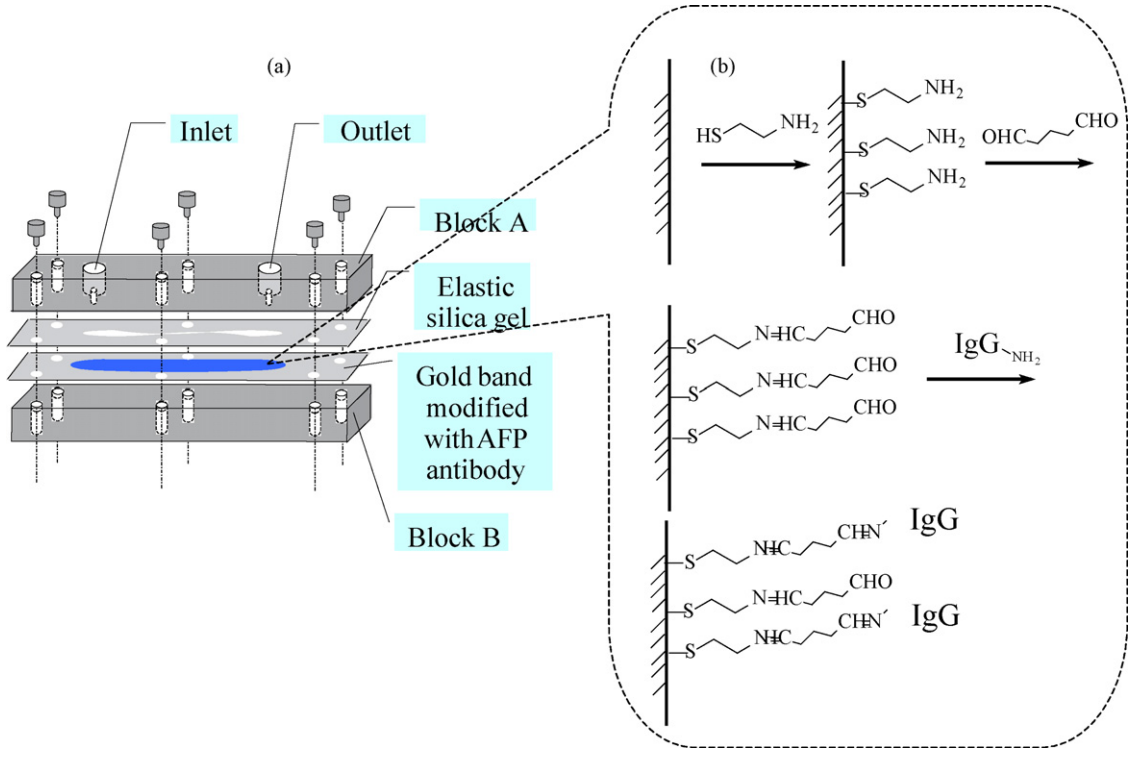
2.1. Materials

An AFP ELISA kit, containing a series of AFP standard solutions and anti-human-AFP monoclonal IgG, was purchased from Panomics Inc. Polyclonal anti-human-AFP IgG labeled with horseradish peroxidase (HRP-labeled anti-AFP antibody) was purchased from Serotic Ltd. Horseradish peroxidase was obtained from Biozyme. Bovine serum albumin BSA-Fraction V, glutaraldehyde, cystamine, Tween-20, and hydrogen peroxide were obtained from Sigma. Hydroxymethyl ferrocene and poly(allylamine) were obtained from Aldrich. The serum samples were kind gifts from Hotel Dieu (Nantes, France). The supporting electrolyte solution was 0.1 M phosphate buffer (PBS, pH 7.0) containing 0.1 M KCl. All other reagents used were of analytical grade and deionised water was used throughout.

2.2. Apparatus, screen-printed electrode and flow-through electrochemical cell

The disposable SPEs, consisting of carbon as the working and counter electrodes and Ag/AgCl as the reference electrode, were kindly offered by GEM Ltd. (Pontypool, Gwent U.K.). They were prepared by screen-printing conductive inks onto a polyester substrate (0.5 mm thickness). The SPEs were then covered by a dielectric insulator, leaving the reactive parts of the electrodes and the electrical connectors exposed. The total diameter of the planar electrochemical cell is 10 mm and the surface of the working electrode is 0.2 cm^2 (Scheme 1a).

Scheme 1b illustrates the schematic of the electrochemical flow cell with the side and top (open cell) views. The flow cell is composed of two plastic blocks. On block A the electrolysis micro-chamber (0.8 cm diameter and 0.5 cm height) is carved in a conical shape. The flow outlet is located on top of the cone and the flow inlet is near its base. A 1 cm diameter rubber ring is placed as a seal between the two plastic blocks and on top of the SPE thus positioning the three electrodes facing the flow, limiting, in a reproducible way, the volume of the electrochemical cell and preventing any leakage of the flowing solutions. The electrical connectors of the SPE were used to connect the electrochemical flow cell to the potentiostat. Cyclic voltammetry was performed with a VMP Multichannel Potentiostat from Perkin-Elmer Instrument using the multilayered hydroxymethyl ferrocene modified SPE.



Scheme 2. Structure of immunoreactor (a) and immobilization procedure of anti-AFP antibody IgG onto gold band (b).

2.3. Immobilization of mediator on electrode surface

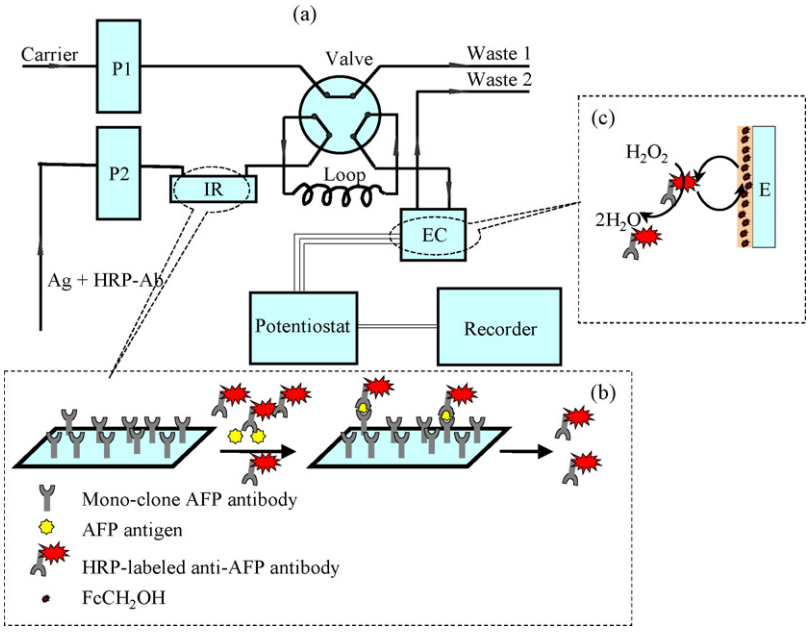
The SPEs were modified with positively charged PAA and negatively charged hydroxymethyl ferrocene. The PAA aqueous solution (9.69 mg ml^{-1}) containing 1 M NaCl was adjusted to pH 4.0 with HCl. The hydroxymethyl ferrocene aqueous solution (0.05 mM) was adjusted to pH 8.0 with NaOH. Multilayer films were then grown by alternately dipping the PAA aqueous solution and the hydroxymethyl ferrocene aqueous solution onto the surface of the SPEs. The films were carefully washed with distilled water after each dipping step and then dried with N_2 gas.

2.4. Fabrication of immunoreactor

Scheme 2 illustrates the schematic of the immunoreactor. The immunoreactor is composed of two resin blocks (block A and block B), an elastic silica gel (0.2 mm thickness) and a polyester substrate (0.5 mm thickness) on which a gold band was screen-printed (the polyester gold band screen printed was purchased from GEM Ltd. Company; U.K.). The elastic silica gel was adhered onto the surface of the polyester leaving the gold band uncovered. Resin block A contains pathways (the inlet and outlet) for the carrier stream. All the components are held securely together by six sets of bolts (Scheme 2a). The gold band was treated by flowing a 10 mM cystamine solution in 50 mM PBS (pH 7.0) through the cell under a flow rate of 0.110 ml min^{-1} for 1 h, and then washed by flowing 50 mM PBS (pH 7.0) and distilled water for 10 min each. Then a 0.1 M PBS containing 0.1 M KCl and 10% (v/v) glutaraldehyde was flowed through the cell for 1 h (pump rate: 0.110 ml min^{-1}) and then washed as described above. After that, a 0.1 M PBS containing 0.1 M KCl and 50 μg ml^{-1} anti-human-AFP monoclonal IgG was flowed slowly through for 15 h (0.110 ml min^{-1}), and the gold band was washed and blocked by flowing through a 1% BSA solution in 50 mM PBS (pH 7.0) containing 0.1% Tween-20 for 2 h (0.110 ml min^{-1}). The procedure for the preparation of the immunoreactor is shown in Scheme 2b. The final immunoreactor was washed for 30 min as described before. When not in use, the prepared immunoreactor was dried by flowing air through it to remove all liquid and then stored at 4 °C.

2.5. Flow injection analysis

Amperometric flow injection measurements were performed using a single-channel FIA manifold (Scheme 3a). The flow-injection system consisted of one flow carrier, two sample pumps (Ismatec) and an electrical six-port rotary injection valve (Rheodyne) for the injections of the samples by means of a 20 μl injection loop. The flow carrier, 0.1 M phosphate buffer (pH 7.0) containing 0.1 M KCl and 0.01 mM H_2O_2, was pumped continuously by pump 1 at a flow rate of 0.891 ml min^{-1} through the six-port rotary injection valve and then the electrochemical cell. The sample solution (AFP antigen and HRP-labeled anti-AFP antibody) was controlled to flow and stop-flow through the immunoreactor by pump 2 at a flow rate of 0.184 ml min^{-1} and introduced into the carrier stream via the sample loop. Electrochemical measurements were detected in the home-made electrochemical cell with a Potentiostat (BAS model Petit Ampere LC-3D) connected to an x-t recorder (LINSEIS, model L200



Scheme 3. Amperometric flow-injection system (a), immunoreaction procedure (b) and enzymatic reaction on electrode surface (c). P1: pump 1; P2: pump 2; IR: immunoreactor; EC: electrochemical flow cell.

E) to monitor the amperometric response of the multilayered hydroxymethyl ferrocene modified SPE to the enzyme.

The detection of AFP was based on a one-step sandwich flow/stop-flow injection amperometric immunoassay. The schematic diagram of the immunoassay procedure is shown in Scheme 3b and c. Forty microliters of AFP standard solution or serum sample, mixed with HRP-labeled anti-AFP antibody and the supporting electrolyte buffer to the volume ratios of 1:1:1, was pumped into the anti-human-AFP monoclonal IgG modified immunoreactor at a flow rate of 0.184 ml min^{-1}, and then stopped for 20 min at room temperature ($25 \pm 2\,^{\circ}C$). After the immunoreactions were finished, the formed sandwich immunocomplex was retained in the immunoreactor and the non-reacted HRP-labeled anti-AFP antibodies were pumped out and injected into the carrier (0.1 M PBS 7.0 containing 0.1 M KCl and 0.01 mM H_2O_2) by turning the valve. At the flow rate of 0.891 ml min^{-1}, the non-reacted HRP-labeled anti-AFP antibodies were carried by the carrier to the electrochemical flow cell to be detected on the multilayered hydroxymethyl ferrocene modified SPE at a potential of +25 mV *versus* Ag/AgCl.

The immunoreactor was regenerated by pass flowing 0.1 M glycine–HCl (pH 2.2) through the column for 30 min, then washing with the carrier for another 10 min.

3. Results and discussion

3.1. Electrochemistry of mediator immobilized on electrode surface

The carbon surface of the SPE usually contains many –OH and –COOH groups, thus PAA, which is positively charged at pH 4.0, can easily adsorb onto the surface to form a positively charged polymer film. After the PAA film was formed on the SPE, the hydroxymethyl ferrocene aqueous solution (pH 8.0) was dropped onto the PAA film. At basic pH hydroxymethyl ferrocene is negatively charged and further adsorbs onto the PAA film to form an electroactive multilayer film. Fig. 1 shows the cyclic voltammograms of blank SPE, PAA modified SPE and $\{PAA/FcCH_2OH\}_n$ multilayer film modified SPE with the number of layers n of 2, 4 and 6 in pH 7.0 PBS containing 0.1 M KCl at a scan rate of 5 mV s^{-1}. No response was observed at both the blank and PAA modified SPEs. However, after $FcCH_2OH$ was adsorbed onto the PAA film, a pair of stable and well-defined redox peaks were observed at +176 and +132 mV (curve c),

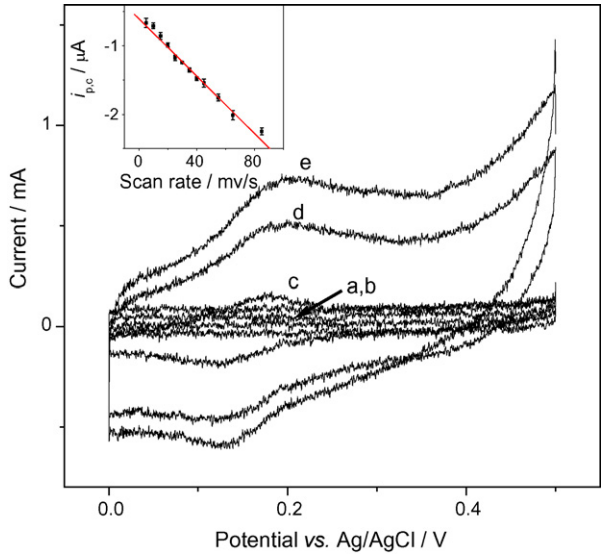


Fig. 1. Cyclic voltammograms of blank SPE (a), PAA modified SPE (b) and $\{PAA/FcCH_2OH\}_n$ multilayer film modified SPE with the layer numbers n of 2 (c), 4 (d) and 6 (e) in pH 7.0 PBS containing 0.1 M KCl at a scan rate of 5 mV s^{-1}. Inset: plot of cathodic peak current of $\{PAA/FcCH_2O^-\}_6$ modified SPE with layer number of 6 *vs.* scan rate.

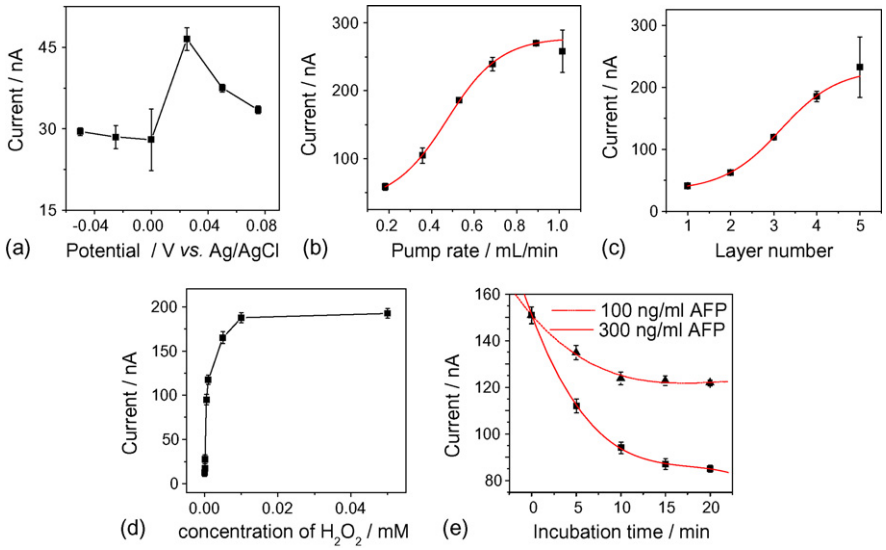


Fig. 2. Effects of applied potential (a), flow rate of carrier and immunoconjugate (b), number of layers of $\{PAA/FcCH_2O^-\}_n$ multilayer film modified SPE (c) and concentration of H_2O_2 (d) on detection current, and effect of incubation time on AFP detection (e) under optimized conditions of other parameters.

which correspond to the oxidation and reduction of the immobilized ferrocene moieties, respectively. With the increasing number of $\{PAA/FcCH_2OH\}_n$ layers, the peak currents increased linearly.

At different scan rates from 5 to 85 mV s^{-1}, the cyclic voltammograms of $\{PAA/FcCH_2O^-\}_6$ with multiplayer number of 6 showed an average formal potential of +150 mV. The peak currents were proportional to the scan rate (inset in Fig. 1), and the peak potentials remained at almost constant values, indicating that electron transport in the film was fast enough to oxidize or reduce the whole ferrocene moieties in the film.

3.2. Blocking time for preparation of immunoreactor

A 1% BSA solution in 50 mM PBS pH 7.0 containing 0.1% Tween-20 was chosen as the blocking reagent. With increasing blocking time the blocking percentage increased. When the blocking solution passed through the immunoreactor for only 0.5 h, the blocking percentage was only about 55%. After 2-h blocking, the blocking percentage reached almost 100%, which indicated that no adsorption occurred after HRP molecules passed through the immunoreactor, and the gold band surface was totally blocked by BSA.

3.3. Optimal detection conditions for FIIA

The factors affecting the immunoassay procedure include the working potential, the flow rates of the carrier and the immunoconjugate, the number of layers of the $\{PAA/FcCH_2OH\}_n$ multilayer film, the concentrations of H_2O_2 and the incubation time.

The potential applied to the working electrode affected directly the sensitivity of the amperometric detection and the stability of the method. Therefore, flow-injection hydrodynamic voltammetric experiments were conducted to obtain optimum detection. As shown in Fig. 2a, as the potential decreased from +75 mV, the current response increased and reached a maximum at +25 mV, the current response then decreased and maintained a constant value. The baseline noise and the background current also increased, which are disadvantages for sensitive and stable detection. Furthermore, no obvious current response of H_2O_2 was observed from 0 to +150 mV according to the hydrodynamic voltammogram of H_2O_2. To avoid interferences and obtain the best signal-to-noise ratio, a working potential of +25 mV was chosen as the applied potential for the immunoassay.

The effect of the flow rate of the carrier (0.1 M pH 7.0 PBS containing 0.1 M KCl) and the immunoconjugate solutions on the determination of AFP was investigated by measuring the current responses of a mixture of HRP-labeled anti-AFP antibody, standard serum without AFP and the supporting electrolyte buffer with the volume ratios of 1:1:1 at different flow rates. Fig. 2b shows the change in the response upon the increasing flow rate. The current response increased significantly with the increasing flow rate in the range from 0.184 to 0.891 ml min^{-1} and then leveled off and even decreased slightly at flow rates higher than 0.891 ml min^{-1}. At lower flow rates, the diffusion layer was thick and the concentration of HRP-labeled anti-AFP antibody on the surface of the electrode was low, which led to a small current response. An increase in flow rate led to a thinner diffusion layer, thus higher concentrations of HRP-labeled anti-AFP antibody on the surface of the electrode could be found. At very high flow rates, the residence time of HRP-labeled anti-AFP antibody in the carrier became shorter, leading to a deficiency of mass transfer. As a result, the current response decreased. Considering the high sensitivity and fast analysis time, a flow rate of 0.891 ml min^{-1} was selected for subsequent experiments.

With increasing in the number of layers, the current increased, however the margin of error increased as well (Fig. 2c; see error bars). In order to obtain a high quality response, a $\{PAA/FcCH_2OH\}_n$ multilayer film modified SPE with 5 layers was used for FIIA of AFP in the following experiments.

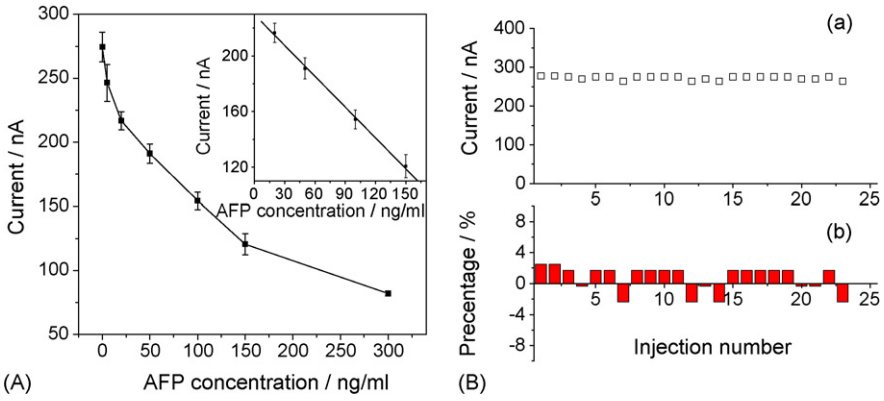


Fig. 3. (A) Calibration curve for one-step amperometric flow/stop-flow injection immunoassay of AFP and (B) operational repeatability (a) and detection accuracy (b) with 23 successive injections of 40 μl commercial HRP-labeled anti-AFP antibody solution. Inset in (A): plot of current response *vs*. AFP concentration under optimized conditions.

The amount of substrate (H_2O_2) was a very important parameter for the enzymatic catalytic reaction. The current response depended on the concentration of H_2O_2 in the carrier. The effect of H_2O_2 concentration on the enzymatic reaction is shown in Fig. 2d. The current response to increasing concentrations of H_2O_2 displayed a Michaelis–Menten-shaped curve. At H_2O_2 concentrations less than 0.01 mM, the peak current increased linearly with the increasing H_2O_2 concentration. Once the H_2O_2 concentration exceeded 0.01 mM, the reduction peak current approached a constant value. Therefore, 0.01 mM was chosen as the optimal H_2O_2 concentration.

3.4. Incubation time of AFP sample in immunoreactor

A serum sample with an AFP concentration of 100 or 300 ng ml^{-1} was first mixed with commercial HRP-labeled anti-AFP antibody solution and the supporting electrolyte buffer with the volume ratios of 1:1:1 and was injected into the immunoreactor at room temperature for different time intervals to select an optimal incubation time. With increasing incubation time, the current response decreased and approached a constant value. This was due to the sandwich immunoreactions of the immobilized monoclonal anti-AFP with the analyte AFP and HRP-labeled anti-AFP, approaching the maximum completion (Fig. 2e). Considering the complete analytical time and the practical operations of this method, an incubation time of 20 min was used for the detection of the AFP level in practical samples.

3.5. Calibration

A series of AFP standard solutions with the concentrations of 0, 5, 20, 50, 100, 150 and 300 ng ml^{-1} were mixed with HRP-labeled anti-AFP and the supporting electrolyte buffer at the volume ratios of 1:1:1 and separately injected into the immunoreactor. Under the optimized conditions, the current responses were measured for the flow-injection sandwich immunoassay of AFP. The plot of the response *versus* the AFP concentration is shown in Fig. 3A. It can be seen that with increasing AFP concentration in the incubation solution, the current decreased and approached a minimum value. The current decrease was proportional to the AFP concentration in two linear ranges from 5 to 20 and from 20 to 150 ng ml^{-1}. The linear slopes were −2.0 and −0.74 nA (ng ml^{-1})$^{-1}$, and the correlation coefficients were 1.000 and 0.9986, respectively. The coefficients of variation for three determinations of AFP at 5, 50 and 150 ng ml^{-1} were 14%, 7.5% and 8.3%, respectively. The complete assay time was less than 25 min for one sample. The detection limit was calculated to be 2 ng ml^{-1} with a signal-to-noise ratio of 3:1.

3.6. Repeatability, fabrication reproducibility and stability

Compared to traditional methods, the proposed method eliminates the washing step and mediator addition. It has the advantages of simple and flexible operations as well as reduced sample time and size. The immobilized mediator by LbL technique on the electrode surface was stable at high flow rates and assured high sensitivity and reproducibility. Fig. 3B shows the responses to 23 successive injections of the mixture of HRP-labeled anti-AFP antibody, standard serum without AFP and the supporting electrolyte buffer with the volume ratios of 1:1:1. No significant decrease of the current response was observed. The relative standard deviations were less than 3.0%. This indicates that the operational stability of the sensor and the proposed FIIA method were good and that the hydroxymethyl ferrocene was stable at the electrode surface even at the high flow rate of 0.891 ml min^{-1}.

The reproducibility of the multilayered hydroxymethyl ferrocene modified SPE was investigated with inter-assay precision, which was estimated by determining, in duplicate, the current response to standard serum without AFP with three multilayered hydroxymethyl ferrocene modified SPEs prepared independently. The coefficient of variation for the inter-assay was 2.8%, showing a good reproducibility. Thus, the LbL technique could provide a simple method for reproducible preparation of the hydroxymethyl ferrocene modified SPE in batches.

When the multilayered hydroxymethyl ferrocene modified SPE was not in use, it was stored in air at room temperature.

The electrode retained more than 95% of its original signal after 15 days of storage, showing good stability.

3.7. Real serum sample testing

The serum AFP levels in two samples were detected using the proposed amperometric flow/stop-flow injection immunoassay. The serum samples were mixed with HRP-labeled anti-AFP and supporting electrolyte buffer at the volume ratios of 1:1:1, respectively, and were injected into the immunoreactor separately. The average concentrations of the serum AFP samples were determined to be 45 and 142 ng ml^{-1}, respectively, while the values obtained from IRMA were 50 and 150 ng ml^{-1}, respectively. The results were in acceptable agreement and the relative errors obtained with the two methods were 10 and 5.3%, respectively. Thus, the proposed method could be satisfactorily applied to the clinical determination of AFP levels in human serum.

4. Conclusions

A one-step amperometric flow/stop-flow injection immunoassay for rapid and automatic detection of AFP in human serum was proposed using mediator modified screen-printed electrodes. Based on a sandwich immunoassay, the mixture of AFP sample and HRP-labeled anti-AFP antibody was pumped into the immunoreactor and stopped for incubation. The AFP concentration could be obtained on the multilayered hydroxymethyl ferrocene modified SPE by the current decrease caused by the sandwich immunocomplex in the immunoreactor. In comparison with the traditional method, the proposed method proved to be fast, simple and efficient using small amounts of sample. The sensor and the proposed FIIA method showed good repeatability, storage stability and reproducibility. The method is also flexible and well suited for automated sample handling. By combining an automation system with multiple-injections, high throughput or multi-sample detection could be achieved. This method could be further developed for practical clinical detection of serum AFP levels.

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