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A disposable amperometric immunosensor for α-1-fetoprotein based on enzyme-labeled antibody/chitosan-membrane-modified screen-printed carbon electrode

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Abstract

A screen-printed three-electrode system is fabricated to prepare a novel disposable screen-printed immunosensor for rapid determination of α-1-fetoprotein (AFP) in human serum. The immunosensor is prepared by entrapping horseradish peroxidase (HRP)-labeled AFP antibody in chitosan membrane to modify the screen-printed carbon electrode. The membrane is characterized with scanning electron microscope and electrochemical methods. After the immunosensor is incubated with AFP at 30 °C for 35 min, the access of the active center of HRP catalyzing the oxidation reaction of thionine by H_2O_2 is partly inhibited. In presence of 1.2 mM thionine and 6 mM H_2O_2, the electrocatalytic current decreases linearly in two concentration ranges of AFP from 0 to 20 and from 20 to 150 ng/mL with a detection limit of 0.74 ng/mL. The immunosensor shows an acceptable accuracy compared with those obtained from immunoradiometric assays. The interassay coefficients of variation are 6.6 and 4.2% at 10 and 100 ng/mL, respectively. The storage stability is acceptable in pH 7.0 phosphate buffer solution at 4 °C for more than 10 days. The proposed method can detect the AFP through one-step immunoassay and would be valuable for clinical immunoassay.

Keywords: Immunosensor; Immunoassay; AFP; Chitosan; Thionine; Screen-printed carbon electrode

Immunoassay techniques, based on highly specific molecular recognition of antigens by antibodies [1], have become the main analytical methods in clinical [2–4] and biochemical [5–7] analyses and in other areas such as environmental control [8,9], food quality control [10,11], etc. Despite the predominance of high sensitivity, the conventional immunoassay methods such as immunoradiometric assays (IRMA),[1] single radial immunodiffusion, immunoturbidimetry, and enzyme-linked immunosorbent assay (ELISA) have some limitations such as the short shelf life of ^{125}I-labeled antibody, the radiation hazards, the complicated wash procedure, and the requirements for a long analysis time, expensive and cumbersome instruments, and/or skillful operators [12–14]. These limit the application of conventional immunoassay techniques in fast, on-line, or fully automated determination of an analyte [13,14]. Thus it is useful to observe the performance of a particular immunosensor in comparison with those of the conventional immunoassays [1].

Immunosensors are miniaturized analytical devices that combine the selectivity of the immunological reaction with the sensitivity and convenience of the various detection techniques and can be effectively applied in untreated samples without requirement for separation

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[1] *Abbreviations used*: AFP, α-1-fetoprotein; BSA, bovine serum albumin; DPV, differential pulse voltammetry; HRP, horseradish peroxidase; HRP-AFP antibody, HRP-labeled monoclonal AFP antibody; IRMA, immunoradiometric assays; PBS, phosphate buffer solution; SEM, scanning electron microscope; SPCE, screen-printed carbon electrode; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay.

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and washing. Many kinds of immunosensors such as electrochemical [15–18], chemiluminescent [19], fluorometric [20,21], piezoelectric [22,23], and surface plasmon resonance [22,24] immunosensors, impedance biochips [25], and even multichannel microchips for multianalyte determination [26,27] have been developed. Electrochemical detection of the label has several advantages, for example, higher sensitivity and lower cost of the resulting sensors and instruments. An electrochemical detector can also be arranged as a microcell that allows miniaturization of the biosensor [1,28]. So electrochemical immunosensors have received considerable attention.

Recently the application of screen-printed electrodes has attracted an increasing interest due to such characteristics as simple and low-cost fabrication and conveniently practical application [29–31]. The biosensors based on screen-printed electrodes have been extensively used for detections of biomolecules [32–34], pesticides [29], antigens [35], and anions [36]. In this work we developed a novel screen-printed three-electrode system as the basis of an immunosensor. This fabrication would make this immunosensor portable for fast clinical diagnosis.

In recent decades, several natural polymers have been used as immobilization matrices for enzymes [37], antigens [38], and antibodies [39] to prepare different biosensors. Chitosan is one of the most promising natural polymers for use as an immobilization matrix, with advantages such as biodegradability, chemical inertness, nontoxicity, biocompatibility, high mechanical strength, good film-forming properties, and low cost [40,41]. The biomolecules can be immobilized in chitosan membrane by adsorption [39,42–45] or cross-linking [38,41,46–49]. This work incorporated HRP-α-1-fetoprotein (AFP) antibody in chitosan matrix to modify screen-printed carbon electrode (SPCE). The active site of enzyme labeled to antibody was shielded and the access of substrate molecules to the enzyme was either partially or completely blocked after the immobilized HRP-AFP antibody reacted with AFP to form immunocomplex during the incubation [50]. The decreased percentage of peak current of the product from the enzymatic reaction was proportional to AFP concentration in two linear ranges from 0 to 20 and 20 to 150 ng/mL. It is known that AFP is an important tumor marker with an average concentration of about 25 ng/mL in healthy human serum [51]. The serum AFP concentration rises greatly in patients with liver cancer. Thus, it is necessary to measure AFP for the clinical diagnosis and even early detection of original liver carcinoma. The design of the immunosensor greatly simplified the assay system and could be used for AFP measurement in a wide concentration range. So this type of immunosensor has an extensive application potential in practical clinical determination of AFP level.

Materials and methods

Reagents

AFP ELISA and IRMA kits were purchased from Everlong (USA). The ELISA kits consisted of a series of AFP standard solutions with different concentrations from 0 to 1000 ng/mL and a solution of HRP-labeled monoclonal AFP antibody. Bovine serum albumin was product of Sigma (USA). Chitosan (MW 1.9×10^5–3.1×10^5; 85–90% deacetylation) was obtained from Aldrich (USA). Thionine and H_2O_2 (analytical reagent grade) were from the Shanghai Biochemical Reagent (China). All other reagents were of analytical reagent grade. Doubly distilled water was used for all experiments; 0.1 M phosphate buffer solutions (PBS) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 and then adjusting the pH with 0.1 M NaOH and H_3PO_4. Serum specimens were obtained from healthy volunteers and clinically diagnosed patients with liver carcinoma were stored at 4 °C. All reagents were brought to room temperature (ca. 20 °C) before use.

Fabrication of SPCE system

A three-electrode SPCE system with graphite working electrode (diameter 4.0 mm), graphite auxiliary electrode, and Ag/AgCl reference electrode was fabricated according to the following steps. First, a layer of silver ink was screen-printed on the surface of a nylon sheet to act as conductive bands according to the shape shown in Tu et al. [52]. Second, layer of graphite was imprinted to cover the silver film except for the area that served as the reference electrode which was oxidized electrochemically in KCl solution to obtain the Ag/AgCl reference. Finally, the conductive bands were insulated by overlaying a silicone rubber layer to expose the conjunction tips and the three-electrode areas.

Preparation of immunosensor

Chitosan solution (1%; wt %) was prepared by ultrasonically dissolving chitosan powder in 1% acetic acid. The HRP-AFP antibody solution and 1% chitosan solution with a volume ratio of 1:1 were mixed thoroughly. After the mixture was placed at 4 °C for 12 h, 10 μL of this mixture was dropped at the working electrode. After drying at room temperature for 5–6 h, the resulting HRP-AFP antibody/SPCE was thoroughly rinsed with doubly distilled water to remove excessively adsorbed antibody. The obtained immunosensor was soaked in pH 7.0 PBS at 4 °C prior to use.

Apparatus

Electrochemical measurements were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems, USA). Electrochemical measurements were run in an unstirred electrochemical cell at 20 ± 0.5 °C without eliminating oxygen. IRMA procedures were carried out with an FMJ-182 Immunoradiometric Gamma Counter (China) according to the instruction and assay procedure in the operator's manual.

Procedure

The analytical procedure for immunoassay was based on detecting the inhibition of the access of the HRP active center to thionine, which resulted from the immunoreaction. After 20 μL of AFP standard solution was dropped at the immunosensor, it was kept at 30 °C for 35 min. The residual was then removed with doubly distilled water. The cyclic voltammetric or differential pulse voltammetric measurement was recorded in PBS solution containing 1.2 mM thionine and 6.0 mM H_2O_2. The determination of AFP was performed by detecting the decrease of the reduction peak current of the enzymatic reaction product between thionine and H_2O_2 by the immobilized HRP. The differential pulse voltammetric measurements were performed from −150 to −500 mV with a pulse amplitude of 50 mV and a pulse width of 60 ms.

Results and discussion

Morphologies of antibody-doped chitosan film on SPCE

The response of an immunosensor is related to its morphology. Thus, the surface morphology of the chitosan film is a vital factor affecting the performance of an immunosensor. Fig. 1 shows the morphologies of bare SPCE, chitosan membrane, and HRP-AFP-antibody-doped chitosan-membrane-modified SPCEs characterized respectively by scanning electron microscope (SEM). The surface of bare SPCE (Fig. 1A) was inhomogeneous and uneven, while chitosan membrane (Fig. 1B) showed a homogeneous porous structure with a narrow particle size distribution. After bare SPCE was immersed in HRP-AFP antibody solution for 12 h, its SEM did not show any obvious change (not shown here), indicating that no protein was absorbed. However, the HRP-AFP-antibody-incorporated chitosan membrane (Fig. 1C) showed a uniform porous structure with little change of pore size compared with chitosan membrane and aggregates of trapped biomolecules with a regular distribution. Thus, the presence of chitosan played an important role in immobilization of HRP-AFP antibody. This porous structure facilitated immobilized HRP to expose enzymatic activity sites and made substrate more easily access to the enzyme, which resulted in a good amperometric response of an immunosensor.

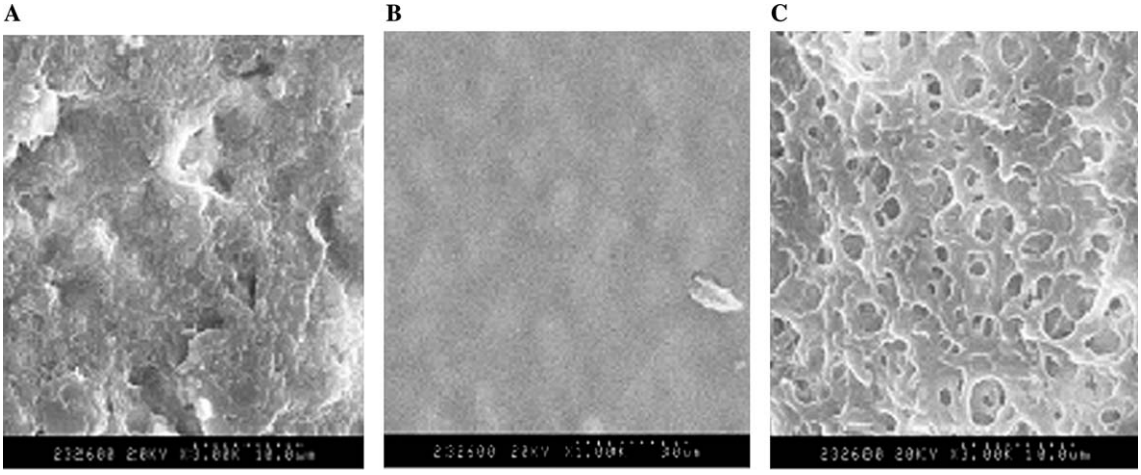

Fig. 1. SEM images of (A) bare SPCE, (B) chitosan film, and (C) HRP-AFP antibody/chitosan-modified SPCE.

$$\mathrm{HRP} + \mathrm{H_2O_2} \longrightarrow \mathrm{Compound\ I} + \mathrm{H_2O}$$

$$\mathrm{Compound\ I} + \mathrm{Thionine\ (Red)} \longrightarrow \mathrm{Compound\ II} + \mathrm{Thionine\ (Ox)^*}$$

$$\mathrm{Compound\ II} + \mathrm{Thionine\ (Ox)^*} + 2\mathrm{H^+} \longrightarrow \mathrm{HRP} + \mathrm{Thionine\ (Ox)} + \mathrm{H_2O}$$

$$\mathrm{Thionine\ (Ox)} + 2\mathrm{e^-} + 2\mathrm{H^+} \longrightarrow \mathrm{Thionine\ (Red)}$$

Scheme 1. Mechanisms of enzymatic and electrode reactions.

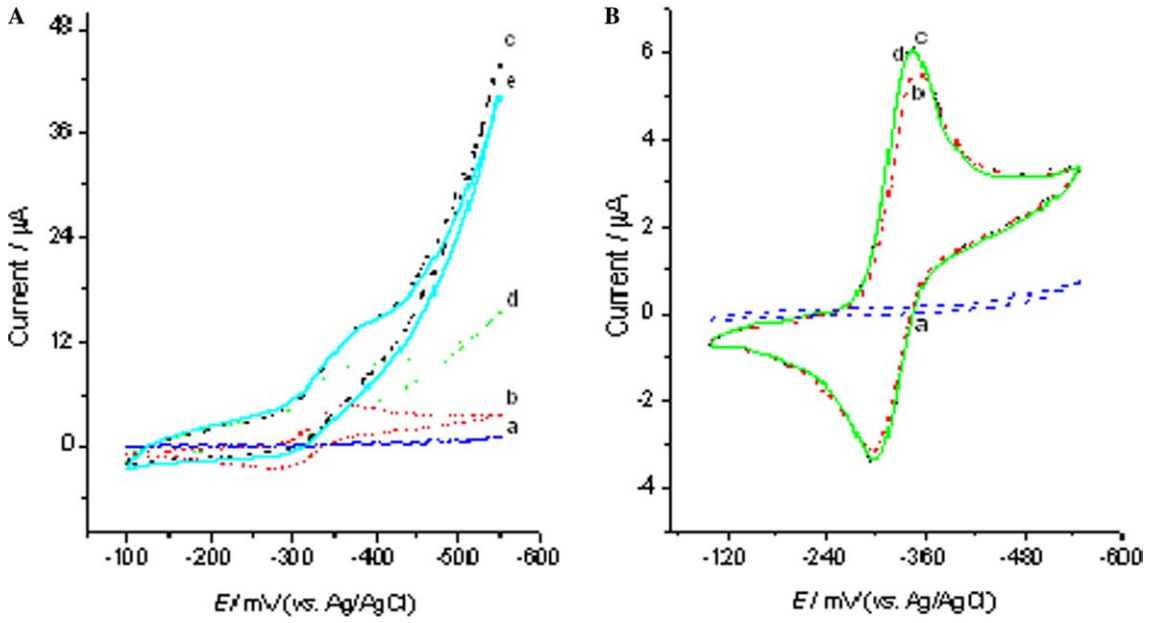

Fig. 2. Cyclic voltammetric response of (A) immunosensor and (B) chitosan-membrane-modified SPCE at 50 mV/s in (a) pH 7.0 PBS, (b) (a) + 1.2 mM thionine, (c) (b) + 6.0 mM H_2O_2, (d) pH 7.0 PBS containing 1.2 mM thionine and 6.0 mM H_2O_2 after the immunosensor was incubated with 300 ng/mL AFP for 35 min, and (e) pH 7.0 PBS containing 1.2 mM thionine and 6.0 mM H_2O_2 after the immunosensor was incubated with 0.04% BSA for 35 min.

Cyclic voltammetric response of enzymatic reaction product

It is well known that HRP can catalyze the oxidation reaction of thionine by H_2O_2, and the mechanisms of enzymatic catalysis and oxidation reaction are shown in Scheme 1 [53]. Fig. 2 shows the cyclic voltammograms of the immunosensor and chitosan-membrane-modified SPCE in different solutions. Neither electrode showed obvious electrochemical response in 0.1 M pH 7.0 PBS (curve a in Fig. 2A and B). When thionine was added to the solution, the cyclic voltammograms of both HRP-AFP-antibody-doped chitosan membrane and chitosan-membrane-modified SPCEs showed pairs of stable and

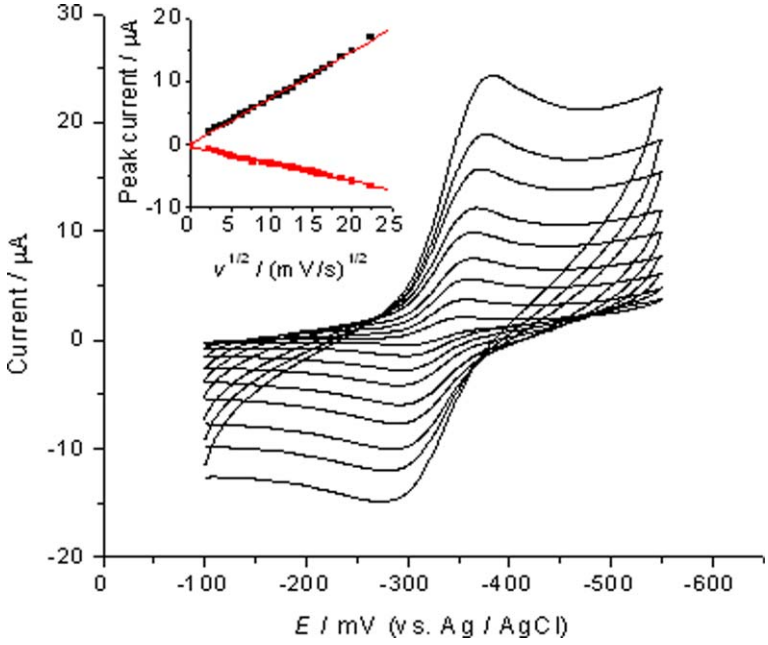

Fig. 3. Cyclic voltammograms of immunosensor in pH 7.0 PBS containing 1.2 mM thionine at 5, 20, 60, 100, 150, 200, 300, 400, and 500 mV/s (from lowest to highest peak currents). Inset: plots of peak currents vs $v^{1/2}$.

well-defined redox peaks at −289 and −354 mV and at −300 and −348 mV at 50 mV/s (curve b in Figs. 2A and B). Obviously, the presence of HRP-AFP antibody decreased the reversibility of the electrode process. Fig. 3 shows the cyclic voltammograms of the immunosensor in pH 7.0 PBS containing thionine at scan rates ranging from 5 to 500 mV/s. With an increasing scan rate the anodic peak potential shifted to a more positive value and the cathodic peak potential shifted in a negative direction; the redox peak currents are proportional to the square root of scan rate, $v^{1/2}$ (inset in Fig. 3), indicating a typical quasireversible process. Upon addition of H_2O_2 to the solution containing thionine, the oxidation peak current at immunosensor decreased, the reduction peak current increased greatly, and the reduction peak potential shifted slightly (curve c in Fig. 2A), while chitosan-membrane-modified SPCE showed a light change, indicating an obvious enzymatic catalysis for the oxidation of thionine by the H_2O_2 process at the immunosensor.

After incubation in 300 ng/mL AFP solution under the optimal conditions, the cyclic voltammogram of the immunosensor exhibited a great decrease in reduction peak current (curve d in Fig. 2A), while no change was observed at chitosan-membrane-modified SPCE (curve d in Fig. 2B). In contrast, no obvious peak change was observed when the immunosensor was incubated in the equivalent amount of BSA solution (curve e in Fig. 2A), which supported the specific recognition between AFP in solution and HRP-AFP antibody immobilized in chitosan membrane on electrode surface. During the incubation process, immobilized HRP-AFP antibody and AFP in solution reacted to produce an immunocomplex, which partly shielded the active center of immobilized HRP and decreased its catalytic capability or enzymatic activity; as a result, the peak current at the immunosensor decreased.

Optimization conditions for immunoassay

The analytical performance of the immunosensor was related to the concentrations of H_2O_2 and thionine in the detection solution, the pH of the solution, and the incubation time and the temperature. When thionine concentration increased in pH 7.0 PBS containing 6.0 mM H_2O_2, the current response increased correspondingly to reached a plateau at 1.2 mM (Fig. 4A). So this value was the optimal thionine concentration in this work.

The effect of H_2O_2 concentration in enzymatic reaction was also examined as shown in Fig. 4B. With increasing H_2O_2 concentration, the DPV peak current in pH 7.0 PBS containing 1.2 mM thionine increased and reached a maximum response at the H_2O_2 concentration of 6.0 mM. When H_2O_2 concentration was greater than 6.0 mM, the response decreased slightly, which was the result of irreversible transition of the immobilized HRP to its higher oxidized and inactive form at higher H_2O_2 concentration [12]. Therefore, the optimal H_2O_2 concentration was 6.0 mM.

The acidity of the solution greatly affects the enzyme activity. Most enzymes display good activity in only a limited range of pH. Therefore, the response of the product of enzymatic catalysis is related to the solution pH for catalytic reaction. The immunosensor in 0.1 M PBS containing 1.2 mM thionine and 6.0 mM H_2O_2 showed the maximum current response over the pH range of 6.5–7.0, exhibiting a maximum activity of enzymatic catalysis. Thus, the optimal pH value of the enzymatic reaction was pH 7.0, which was chosen for the whole work.

The formation of immunocomplex on electrode surface depends on the time and temperature of incubation. Temperatures from 20 to 40 °C were chosen to perform the immunoreaction. Due to the inhibition of immunocomplex to the access of HRP active center to thionine,

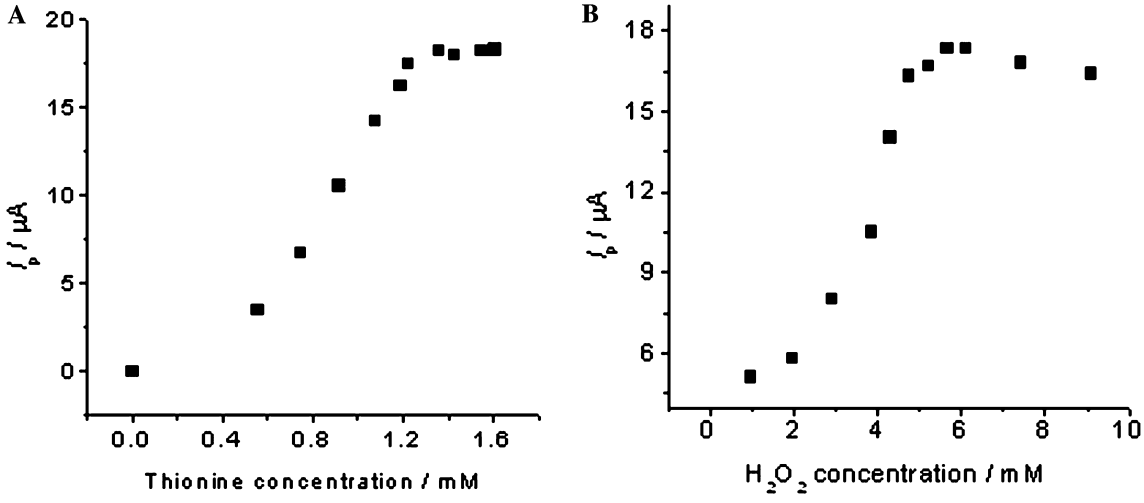

Fig. 4. Dependence of DPV response of immunosensor on thionine (A) and H_2O_2 (B) concentrations.

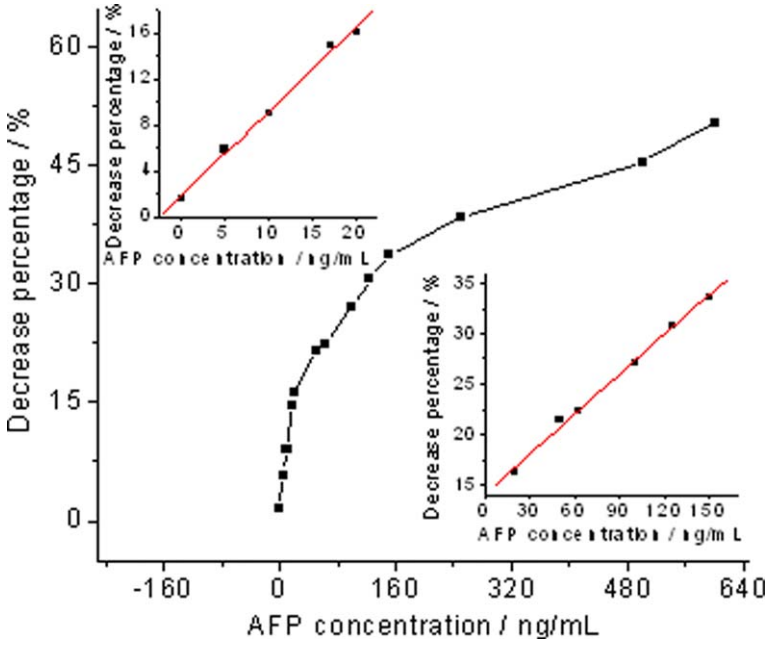

Fig. 5. Calibration for AFP determination under optimal conditions.

the current response of the immunosensor after incubation decreased. The maximum decrease percentage occurred at a temperature of 30 °C. At this temperature, the decrease percentage increased with increasing incubation time and reached a maximum value at 35 min. When the incubation time was longer than 35 min the decrease percentage maintained a constant value, indicating a saturated binding of the immobilized AFP antibody by AFP. Thus the incubation temperature of 30 °C and the incubation time of 35 min were chosen as the optimal incubation conditions for the immunoassay of AFP using the immunosensor.

Electrochemical immunoassay

Under the optimal conditions, the calibration graph for the determination of AFP was obtained using HRP-AFP antibody/chitosan-modified SPCE as an immunosensor. The catalytic capability of HRP labeled on AFP antibody to the oxidation reaction of thionine by H_2O_2 decreased proportionally to the AFP concentration in two ranges, from 0 to 20 ng/mL and from 20 to 150 ng/mL, with linear slopes of 0.74 and 0.13%/ng/mL and correlation coefficients of 0.9973 and 0.9977, respectively (Fig. 5). The detection limit was 0.74 ng/mL (S/N = 3), which was almost the same as that of traditional spectrophotometric ELISA. Thus, the present method could be used for immunoassays of AFP in clinical diagnosis.

Precision, reproducibility, and stability

The interassay precision of AFP was estimated using six disposable immunosensors for every AFP concentration. The coefficient of variation (CV) for interassay on this method was 6.6% at 10 ng/mL and 4.2% at 100 ng/mL, indicating good detection precision and fabrication reproducibility of the immunosensors. The sensor could be fabricated in batches; thus it was economic, disposable, and portable for users. The immunosensor had acceptable storage stability with 93 and 85% of initial responses remaining after the storage periods of 1 week and 10 days in pH 7.0 PBS at 4 °C, respectively.

Accuracy and clinic application

The accuracy of AFP determination was examined by comparing the results obtained using this method with those using IRMA. With both techniques, AFP contents in two sera were quantified. The AFP concentrations determined with this method were 17.5 and 112.0 ng/mL, while the values obtained with IRMA were 12.9 and 121.0 ng/mL. These results were in good agreement. Thus, the proposed method could be satisfactorily applied to the clinical determination of AFP in human serum for clinical diagnosis.

Conclusions

A novel disposable immunosensor for rapid determination of AFP is prepared by incorporating HRP-AFP antibody in chitosan matrix to modify screen-printed carbon electrode. AFP concentration in sample solution is detected based on the inhibition of the enzymatic activity to the oxidation reaction of thionine by H_2O_2 due

to the formation of immunocomplex on immunosensor surface. Under optimized conditions for immunoreaction and electrochemical determination, the immunosensor shows a detection limit of 0.74 ng/mL and can be used for AFP detection up to the level of 150 ng/mL. It possesses good precision and reproducibility for fabrication in batch and acceptable accuracy and storage stability. The proposed method is valuable for clinical immunoassay and could be extended readily to preparation of other amperometric immunosensors and detection of other clinically important antigens.

Acknowledgments

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References

[1] L.G. Andrey, A. Plamen, W. Michael, W. Ebtisam, Immunosensor: electrochemical sensing and other engineering approaches, Biosens. Bioelectron. 13 (1998) 113–131.
[2] M. Worwood, Serum transferrin receptor assays and their application, Ann. Clin. Biochem. 39 (2002) 221–230.
[3] A.K. Trull, The clinical validation of novel strategies for monitoring transplant recipients, Clin. Biochem. 34 (2001) 3–7.
[4] Y. Itoh, K. Ichihara, Standardization of immunoassay for CRM-related proteins in Japan: from evaluating CRM 470 to setting reference intervals, Clin. Chem. Lab. Med. 39 (2001) 1154–1161.
[5] K. Sato, A. Hibara, M. Tokeshi, H. Hisamoto, T. Kitamori, Microchip-based chemical and biochemical analysis systems, Adv. Drug Deliv. Rev. 55 (2003) 379–391.
[6] J. Rossier, F. Reymond, P.E. Michel, Polymer microfluidic chips for electrochemical and biochemical analyses, Electrophoresis 23 (2002) 858–867.
[7] M. Panteghini, Present issues in the determination of troponins and other markers of cardiac damage, Clin. Biochem. 33 (2000) 161–166.
[8] Knopp Dietmar, Application of immunological methods for the determination of environmental pollutants in human biomonitoring. A review, Anal. Chim. Acta 311 (1995) 383–392.
[9] J.M. Van Emon, C.L. Gerlach, K. Bowman, Bioseparation and bioanalytical techniques in environmental monitoring, J. Chromatogr. B 715 (1998) 211–228.
[10] J. Ring, K. Brockow, H. Behrendt, Adverse reactions to foods, J. Chromatogr. B 756 (2001) 3–10.
[11] J. Belloque, M.C. Garcia, M. Torre, M.L. Marina, Analysis of soyabean proteins in meat products. A review, Crit. Rev. Food Sci. 42 (2002) 507–532.
[12] H.X Ju, G.F. Yan, F. Chen, H.Y. Chen, Enzyme-linked immunoassay of α-1-fetoprotein in serum by differential pulse voltammetry, Electroanalysis 11 (1999) 124–128.
[13] D.A. Palmer, J.N. Miller, Thiophilic gels: applications in flow-injection immunoassay for macromolecules and haptens, Anal. Chim. Acta 303 (1995) 223–230.
[14] M. Nilsson, H. Hakason, B. Mattiasson, Process monitoring by flow-injection immunoassay evaluation of a sequential competitive binding assay, J. Chromatogr. 597 (1992) 383–389.
[15] P. Sarkar, P.S. Pal, D. Ghosh, S.J. Setford, I.E. Tothill, Amperometric biosensors for detection of the prostate cancer marker (PSA), Int. J. Pharm. 238 (2002) 1–9.
[16] C.H. Liu, K.T. Liao, H.J. Huang, Amperometric immunosensors based on protein-A coupled polyaniline-perfluorosalfonated lonomer composited electrodes, Anal. Chem. 72 (2000) 2925–2929.
[17] G. Cui, S.J. Kim, S.H. Choi, H. Nam, G.S. Cha, K.J. Paeng, A disposable amperometric sensor screen-printed on a nitrocellulose strip-A glucose biosensor employing lead-oxide as an interference-removing agent, Anal. Chem. 72 (2000) 1925–1929.
[18] J. Li, L.T. Xiao, G.M. Zeng, G.H. Huang, G.L. Shen, R.Q. Yu, Amperometric immunosensor based on polypyrrole/poly (m-pheylenediamine) multiplayer on glassy carbon electrode for cytokinin N^6- (Δ^2-isopentenyl) adenosine assay, Anal. Biochem. 321 (2003) 89–95.
[19] M.Y. Rubtsova, G.V. Kovba, A.M. Egorov, Chemiluminescent biosensors based on porous supports with immobilized peroxidase, Biosens. Bioelectron. 13 (1998) 75–85.
[20] J. Penalva, R. Puchades, A. Maquieira, Analytical properties of immunosensors working in organic media, Anal. Chem. 71 (1999) 3862–3872.
[21] M.A GonzalezMartinez, S. Morais, R. Puchades, A. Maquieira, A. Abad, A. Montoya, Monoclonal antibody-based flow-through immunosensor for analysis of carburyl, Anal. Chem. 69 (1997) 2812–2818.
[22] T. Kaiser, P. Gudat, W. Stock, G. Pappert, M. Grol, D. Neumeier, P.B. Luppa, Biotinylated steroid derivatives as ligands for biospecific interaction analysis with monoclonal-antibodies using immunosensor devices, Anal. Biochem. 282 (2000) 173–185.
[23] Z.Y. Wu, Y.H. Yan, G.L. Shen, R.Q. Yu, A novel-approach of antibody immobilization based in N-butyl amine plasma-polymerized films for immunosensors, Anal. Chim. Acta 412 (2000) 29–35.
[24] L.A. Lyon, M.D. Musick, M.J. Natn, Colloidal Au-enhanced surface-plasmon resonance immunosensing, Anal. Chem. 70 (1998) 5177–5183.
[25] C.M. Ruan, L.J. Yang, Y.B. Li, Immunosensor clips for detection of *Escherichia coli* 0157:H7 using electrochemical impedance spectroscopy, Anal. Chem. 74 (2002) 4814–4820.
[26] T.C. Tang, A.P. Deng, H.J. Huang, Immunoassay with a microtiter plate incorporated multichannel electrochemical detection system, Anal. Chem. 74 (2002) 2617–2621.
[27] J. Yakovleva, R. Davidsson, A. Lobanova, M. Bengtsson, S. Eremin, T. Laurell, J. Emneus, Microfluidic enzyme immunoassay using silicon microchip with immobilized antibodies and chemiluminescence detection, Anal. Chem. 74 (2002) 2994–3004.
[28] P. Skladal, T. Kalab, A multichannel immunochemical sensor for determination of 2,4-dichlorophenoxyacetic acid, Anal. Chim. Acta 316 (1995) 73–78.
[29] I. Palchetti, A. Cagnini, M.D. Carlo, C. Coppi, M. Mascini, A.P.F. Turner, Determination of anticholinesterase pesticides in real samples using s disposable biosensor, Anal. Chim. Acta 337 (1997) 315–321.
[30] J.P. Hart, S.A. Wring, Recent developments in the design and application of screen-printed electrochemical sensors for biomedical, environmental and industrial analyses, Trends Anal. Chem. 16 (1997) 89–103.
[31] M.P. O' Halloran, M. Pravda, G.G. Guilbault, Prussian Blue bulk modi.ed screen-printed electrodes for H_2O_2 detection and for biosensors, Talanta 55 (2001) 605–611.
[32] N.A. Morris, M.F. Cardosi, B.J. Birch, A.P.F. Turner, An electrochemical capillary. ll device for the analysis of glucose incorporating glucose oxidase and ruthenium(III) hexamine as mediator, Electroanalysis 4 (1992) 1–9.

[33] M.A.T. Gilmartin, J.P. Hart, D.T. Patton, Prototype, solid-phase, glucose biosensor, Analyst 120 (1995) 1973–1981.
[34] J. Wang, P.V.A. Pamidi, D.S. Park, Screen-printable sol-gel enzyme-containing carbon inks, Anal. Chem. 68 (1996) 2705–2708.
[35] J. Wang, P.V.A. Pamidi, K.R. Rogers, Sol-gel-derived thick-film amperometric immunosensors, Anal. Chem. 70 (1998) 1171–1175.
[36] G.G. Neuhold, J. Wang, X. Cai, K. Kalcher, Screen-printed electrodes for nitrite based on anion-exchanger-doped carbon inks, Analyst 120 (1995) 2377–2380.
[37] L.T. Ng, Y.J. Yuan, H.J. Zhao, Natural polymer-based sulfite biosensor, Electroanalysis 10 (1998) 1119–1124.
[38] Y.H. Yu, B.L. He, The preparation of immunoadsorbents and their adsorption properties for anti-DNA antibodies in SLE serum, React. Funct. Polym. 41 (1999) 191–195.
[39] J.P.M. Sardinha, M.H. Gil, J.V. Mercader, Angel Montoya enzyme-linked immunofiltration assay used in the screening of solid supports and immunoreagents for the development of an azinphos-methyl flow immunosensor, J. Immunol. Methods 260 (2002) 173–182.
[40] R.Y.M. Huang, G.Y. Moon, R. Pal, Chitosan/anionic surfactant complex membranes for the pervaporation separation of methanol/MTBE and characterization of the polymer/surfactant system, J. Membr. Sci. 184 (2001) 1–15.
[41] G. Wang, J.J. Xu, H.Y. Chen, Z.H. Lu, Amperometric hydrogen peroxide biosensor with sol-gel/chitosan network-like film as immobilization matrix, Biosens. Bioelectron. 18 (2003) 335–343.
[42] F.L. Mi, S.S. Shyu, C.T. Chen, J.Y. Schoung, Porous chitosan microsphere for controlling the antigen reliase of Newcastle disease vaccine: preparation of antigen-adsorbed microsphere and in vitro release, Biomaterials 20 (1999) 1603–1612.
[43] J. Benesch, P. Tengvall, Blood protein adsorption onto chitosan, Biomaterials 23 (2002) 2561–2568.
[44] H. Huang, N.F. Hu, Y.H. Zeng, G. Zhou, Electrochemistry and electrocatalysis with heme protein in chitosan biopolymer films, Anal. Biochem. 308 (2002) 141–151.
[45] C.X. Lei, S.Q. Hu, G.L. Shen, R.Q. Yu, Immobilization of horseradish peroxidase to a nano-Au monolayer modified chitosan entrapped carbon paste electrode for the detection of hydrogen peroxide, Talanta 59 (2003) 981–988.
[46] Y. Miao, S.N. Tan, Amperometric hydrogen peroxide biosensor with silica sol-gel/chitosan film as immobilization matrix, Anal. Chim. Acta 437 (2001) 87–93.
[47] X.F. Zeng, E. Ruckenstein, Cross-linked macroporous chitosan anion-exchange membranes for protein separations, J. Membr. Sci. 148 (1998) 195–205.
[48] S. Hikima, T.K. Zkizaki, M. Taga, K. Hasebe, Enzyme sensor for L-lactate with a chitosan-mercury film electrode, Fresenius J. Anal. Chem. 345 (1993) 607–609.
[49] J. Cruz, M. Kawasaki, W. Gorski, Electrode coatings based on chitosan scoffolds, Anal. Chem. 72 (2000) 680–686.
[50] P. Skládal, Advances in electrochemical immunosensors, Electroanalysis 9 (1997) 737–745.
[51] Z.Z. Zhu, Modern Medical Labelled immunology (in Chinese), People's Military Doctor Press (2000) 56.
[52] Y.F. Tu, Z.Q. Fu, H.Y. Chen, The fabrication and optimization of the disposable amperometric biosensor, Sensor. Actuator. B 80 (2001) 101–105.
[53] C.M. Ruan, F. Yang, C.H. Lei, J.Q. Deng, Thionine covalently tethered to multilayer horseradish peroxidase in a self-assembled monolayer as an electron-transfer mediator, Anal. Chem. 70 (1998) 1721–1725.