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Computational modeling

Flow-injection chemiluminescent immunoassay for α-fetoprotein based on epoxysilane modified glass microbeads

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Abstract

A flow-injection chemiluminescent immunoassay system based on a novel transparent immunoaffinity reactor is proposed for the quantitation of α-fetoprotein. The reactor prepared with α-fetoprotein immobilized epoxysilane modified glass microbeads was used as an immunosensor for chemiluminescent detection. With a non-competitive immunoassay format, the proposed immunosensor system is a low cost, flexible and rapid assay for α-fetoprotein. After an off-line incubation of the analyte α-fetoprotein with horseradish peroxidase-labeled α-fetoprotein antibody as enzyme tracer, the mixture was injected into the reactor, which led to trapping of the free enzyme tracer by the reactor. The trapped enzyme tracer was detected by the *p*-iodophenol–luminol–H_2O_2 chemiluminescence system. Under optimal conditions, the decrease in chemiluminescence intensity was proportional to the α-fetoprotein concentration in the range of 5.0–100 ng/ml with a detection limit of 2.7 ng/ml at a signal/noise ratio of 3. The immunosensor system showed an acceptable reproducibility and stability. Clinical serum samples were assayed with this method and the results were in acceptable agreement with those obtained from immunoradiometric assay.

Keywords: Flow-injection analysis; Chemiluminescence; Immnunoassay; Immunosensor; α-Fetoprotein; Epoxysilane

1. Introduction

α-Fetoprotein (AFP), an oncofetal glycoprotein with a molecule weight of approximately 70,000 Da, is well known as a tumor marker. It is normally excreted by the liver, the yolk sac and the gastrointestinal tract during fetal and neonatal development (Yang et al., 2002b). The serum AFP level decreases rapidly after birth, and by the second year only trace amounts can normally be detected. The concentration of AFP in healthy adults is typically below 25 ng/ml. Increased serum AFP levels have been considered as an early indication of some cancerous diseases including hepatocellular cancer, yolk sac cancer, liver metastasis from gastric cancer, testicular cancer and nasopharyngeal cancer (Li, 2001). Recently, it has been found that AFP is associated with tumor cell growth and may serve as an important target of tumor therapy (Wang and Xu, 1998). Thus, it is very important to explore a rapid detection method for AFP.

Numerous techniques based on immunological methods have been exploited to assay AFP due to the high

Abbreviations: AFP, α-fetoprotein; BSA, bovine serum albumin; CL, chemiluminescence; CPG, controlled pore glass; ELISA, enzyme-linked immunosorbent assay; FIA, flow-injection analysis; FIIA, flow-injection immunoassay; GPMS, γ-glycidoxypropyltrimethoxysilane; HRP, horseradish peroxidase; PIP, *p*-iodophenol; PBS, phosphate buffer solution; PBST, phosphate buffer solution containing Tween-20; PMT, photomultiplier; SEM, scanning electron micrograph.

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specificity of immunoreaction, including fluorometry (Matsuya et al., 2003; Song et al., 2004), electrochemistry (Yu et al., 2004; Honda et al., 2005), inductively coupled plasma mass spectrometry (Zhang et al., 2004), atomic absorption spectrometry (Wang et al., 2001), electrochemiluminescence (Xue et al., 1996; Namba et al., 1999), micellar electrokinetic capillary chromatography (Wang et al., 2002), and liquid-phase binding assay (Nakamura et al., 1998; Kawabata et al., 2005). Since flow-injection analysis (FIA) was introduced in 1975, it has proved to be a powerful analytical tool for a wide variety of analytical problems (Ruzicka and Hansen, 1988). This technique is based on the controlled and reproducible dispersion of a sample zone when it is introduced into a continuously flowing carrier stream, and provides an attractive alternative for automation of the traditional manual method. Flow-injection immunoassay (FIIA), also commonly known as flow through immunosensor, offers many potential advantages, such as acceptable reusability, good reproducibility, less time consuming, and easy automation for high sample throughput. Therefore, it has been widely applied in various areas, including environmental monitoring (Jain et al., 2004), food safety (Nandakumar et al., 2000), pharmaceutical analysis (Eremenko et al., 1998; Yang et al., 2002a), clinical diagnosis (Lin et al., 2004a,b) and identification of bacteria (Abdel-Hamid et al., 1999; Ho et al., 2004), etc.

In FIIA, a packed bed reactor is often used to perform the separation step. The selection of a suitable solid support for the reactor is one of the central decisions when developing a flow immunoassay. An ideal support for FIIA should meet the following requirements: (i) it must provide functional groups to enable appropriate coupling with a sufficient amount of antigen or antibody (Martin-Esteban et al., 1997); (ii) its surface coupling properties should not seriously affect the spatial structure of the antigen or antibody to retain their immunoactivity; (iii) it must have a high surface area to volume ratio to immobilize abundant antigen or antibody; (iv) it should be hydrophilic to avoid non-specific interaction with the analytes and the sample matrix; and (v) it must be a rigid material in order to retain its physical shape during the powerful flow process. Of the different types of materials (nylon, sepharose, silicon material, magnetic beads, etc.) used as solid support in FIIA, activated silicon is of great interest due to its low cost, good covalent bonding ability, high stability over a wide pH range and excellent mechanical intensity. Different silicon materials have been developed to fabricate flow through immunosensors. The most widely used silicon material is capillary (Zhang et al., 1999; Ho et al., 2004; Ho and Huang, 2005). Controlled pore glass (CPG) is another important silicon material with a large surface area (Zhang et al., 1994; Gonzalez-Martinez et al., 1997). However, high non-specific adsorption of CPG is the main drawback due to its tremendous surface area. Here, the glass microbeads were coupled with epoxysilane; AFP was then immobilized onto the surface of the silanized microbeads through an epoxy-amino group reaction to pack a glass column for construction of a transparent immunoaffinity reactor, and a novel flow-injection chemiluminescent immunoassay system based on a non-competitive format was developed.

Glass microbeads are a rigid material of good transparency. The epoxysilanization reaction does not affect the transparency, which is very advantageous for on-column chemiluminescence (CL) detection. Compared with the familiar aminosilane-glutaraldehyde format based on the Schiff base reaction (Zhang et al., 1994; Ho and Huang, 2005), it is more attractive in light detection, since the aminosilane-glutaraldehyde modified material often shows lighttight rufous. Glass microbeads can immobilize much more biomolecules than other membrane materials, such as cross-linked chitosan membrane (Lin et al., 2004b), due to its high surface area to volume ratio; this is of great importance to broaden the linear range of analytes.

2. Materials and methods

2.1. Materials and reagents

An AFP diagnostic kit was purchased from Shanghai Feilong Medical Diagnostic Articles Co. Ltd. (China). The diagnostic kit consisted of a series of AFP standard solutions with different concentrations from 0 to 400 ng/ml and a solution of horseradish peroxidase (HRP)-labeled horse polyclonal AFP antibody used as enzyme tracer. Bovine serum albumin (BSA) was purchased from Roche (Switzerland) and prepared in 0.1 mol/l phosphate buffer solution (PBS, pH 7.0). Luminol stock solution (0.01 mol/l) was prepared by dissolving 177 mg of luminol (Acros, Belgium) in 100 ml of 0.1 mol/l NaOH and kept in the dark. *p*-Iodophenol (PIP) stock solution (0.01 mol/l) was prepared by dissolving 110 mg of PIP (Weihai Newera Chemical, China) in 5 ml of dimethylsulfoxide and then diluted with water to 50 ml and kept in the dark. A 30% H_2O_2 solution was from Sinopharm Chemical Reagent Co., Ltd., China. γ-Glycidoxypropyltrimethoxysilane (GPMS) was purchased from Nanjing Yudeheng Coupling Agent Co., Ltd. (China) and used as received. Glass microbeads (140 mesh) were purchased from Sigma (USA). Clinical serum samples were provided by Jiangsu Institute of Cancer Prevention and Cure,

China. Deionized and distilled water was used throughout the study.

2.2. Modification of glass microbeads and preparation of the immunoaffinity reactor

Five grams of glass microbeads were stirred in 50 ml of boiled HNO_3 solution (5%) for 1 h. After washing thoroughly with water, they were dried at 130 °C for 4 h, and then reacted with 80 ml of 5% GPMS in dry toluene at room temperature with continuous stirring overnight. After the coupling reaction, the modified microbeads were removed from the solution and rinsed thoroughly with toluene and ethanol to remove the physically adsorbed silane. Finally, they were dried under a nitrogen atmosphere at 100 °C for 1 h. Prepared in this way, the surface of the microbeads was exposed to active epoxy groups that could react readily with the amino groups of protein. Three hundred microliters of 400 ng/ml AFP were added to 0.35 g of the epoxysilane coupled microbeads and incubated for 24 h at 4 °C with frequent stirring. To block the residual active epoxy groups on the surface, the AFP immobilized microbeads were treated with 300 μl of 1% BSA for another 24 h. Finally, they were washed with 0.1 mol/l PBS (pH 7.0) and placed into a glass tube of about 200 μl (1.6 mm i.d. and 100 mm length, flexed to a Z shape), which was used as the transparent immunoaffinity reactor and stored in 0.1 mol/l PBS (pH 7.0) at 4 °C when not in use.

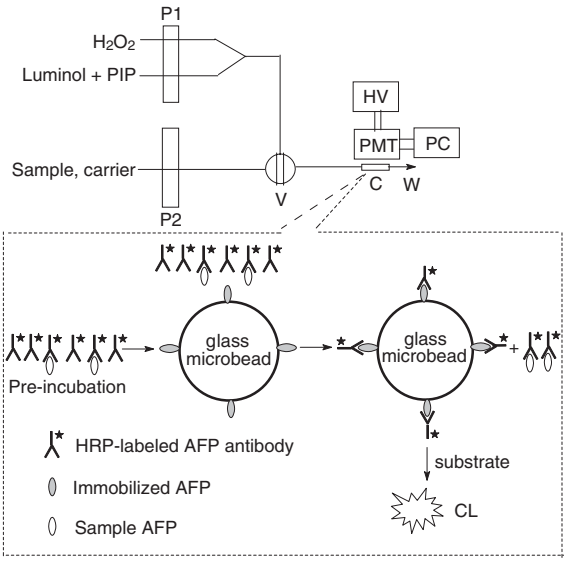

Fig. 1. Scheme of the FIIA system. P1, P2, peristaltic pump; V, injection valve; C, immunoaffinity reactor; W, waste; PMT, photomultiplier; HV, negative high voltage supply; PC, personal computer.

2.3. Apparatus

The immunoaffinity reactor was connected to a flow system as illustrated in Fig. 1. Two peristaltic pumps of the IFFM-D Luminescence Analyzer (Remax, China) were used to deliver all solutions. An injection valve with a 100 μl loop was used to introduce the CL substrates. A Teflon tube (0.8 mm i.d.) was used to connect all the components in the flow system. The immunoaffinity reactor was positioned in front of the photomultiplier (PMT). The CL intensity was determined with the PMT operated at −800 V. The distance from the reactor to the valve was about 25 cm. Control of the instrument and the treatment of the CL signal were performed via IFFM software.

A scanning electron micrograph (SEM) of AFP immobilized support was obtained with a Hitachi X-650 scanning electron microscope (Japan) at an acceleration voltage of 20 kV.

2.4. FIIA procedure for AFP

The detection of AFP was based on a non-competitive flow injection immunoassay method. Fifty microliters of enzyme tracer was first mixed with the same volume of AFP standard or serum sample. After pre-incubation for 30 min at room temperature, the mixture was carried through the transparent immunoaffinity reactor at a rate of 0.05 ml/min. To remove the physically adsorbed enzyme tracer, the reactor was washed with PBS (pH 7.0), containing 0.05% Tween-20 (PBST) for 1 min at a rate of 1.0 ml/min. Then the CL substrates were also carried into the flow system, and 100 μl of substrate were injected with the aid of the valve. The CL signal was captured and recorded by the detector. The AFP immobilized microbeads were regenerated with a flow of 10 mmol/l NaOH for 1 min, and then equilibrated with 0.1 mol/l PBST (pH 7.0) for another 1 min. The whole assay procedure including pre-incubation, CL detection and regeneration of the reactor was performed at room temperature (about 25 °C). The total assay time was 36 min for one sample including 30 min of pre-incubation and 6 min of detection and regeneration of the reactor.

3. Results and discussion

3.1. Preparation and characterization of the immunoaffinity reactor

AFP was immobilized onto the surface of the glass microbeads through the epoxy-amino reaction as shown in Fig. 2. The preparation procedure included cleaning, surface activating, and surface modification. In this

study, the glass microbeads were first treated with hot HNO_3 solution (5%) for 1 h to clean the surface and produce large numbers of hydroxyl groups. Then, the epoxysilane was coupled to the surface hydroxyl groups. According to the previous reports (Luzinov et al., 2000; Ruan et al., 2002), a complete, smooth and homogeneous epoxy-terminated self-assembled monolayer with only a few aggregates can be obtained. The resultant epoxy terminal of the support finally coupled with the amino groups of AFP to form AFP-modified microbeads. SEM (Fig. 3) showed that AFP was successfully linked to the surface of the microbeads.

3.2. Optimization of CL detection conditions

The rate of the CL substrates flowing through the reactor and reacting with the trapped enzyme tracer greatly affected the CL signal. At a high flow rate the recorded signal showed a sharp peak with good reproducibility. However, too high a flow rate can affect the CL reaction and consume more substrate. Consequently, a flow rate of 1.0 ml/min was satisfactory for this FIIA system.

The effects of the concentration of CL substrates including luminal, PIP and H_2O_2 were also studied in detail. The optimal concentrations were 0.5, 0.5 and 3 mmol/l, respectively. All the substrates were prepared in 0.1 mol/l Tris–HCl buffer solution (pH 8.5). The CL emission in Tris–HCl buffer solution was more stable than in other buffers such as PBS, Na_2CO_3–$NaHCO_3$.

3.3. Optimization of the immunoassay procedure

The key factors influencing the immunoassay performance included the working concentration of the enzyme tracer, the pre-incubation time for the immunomixture, and the residence time of the immunomixture in the immunoaffinity reactor.

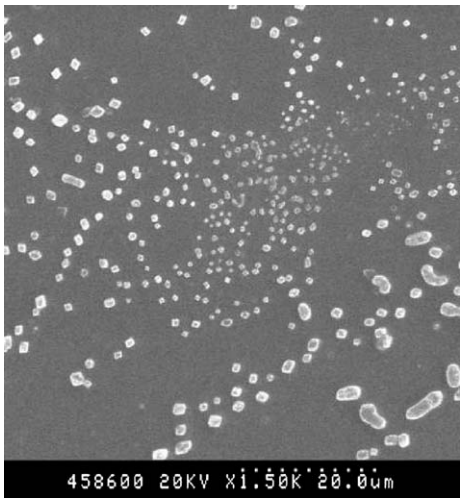

Fig. 3. SEM of the AFP immobilized epoxysilanized glass microbeads.

The working concentration of the enzyme tracer was investigated to give a compromise with good sensitivity and a wide assay range. Different diluted tracer solutions were injected into the reactor and the CL intensity was compared. It was found that the CL intensity decreased with increasing tracer dilution at the tracer flow rate of 0.05 ml/min. Therefore, the enzyme tracer was used as received without any dilution.

A sample containing 30 ng/ml AFP was incubated with the undiluted enzyme tracer at room temperature for different time intervals, and the mixture was then carried into the flow system to obtain an optimal pre-incubation time. With increasing pre-incubation time, the CL signal decreased and tended to a minimum value after a pre-incubation time of 30 min (Fig. 4A), indicating a maximum combination of AFP with its enzyme

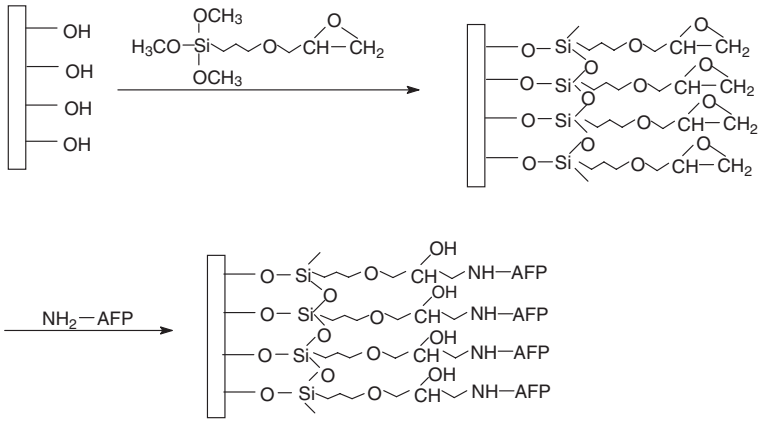

Fig. 2. Immobilization of AFP onto the surface of the glass microbeads.

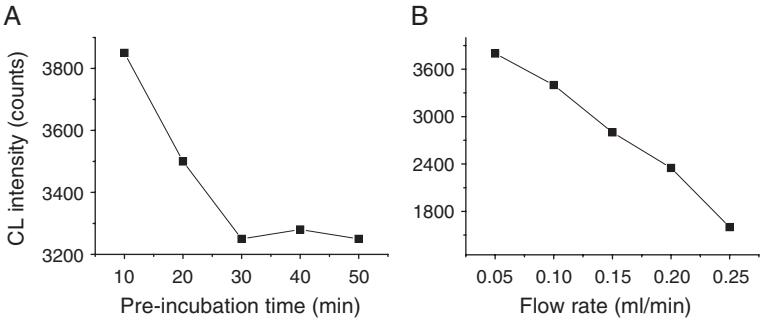

Fig. 4. Effects of pre-incubation time on CL intensity. (A) Flow rate 0.05 ml/min, AFP concentration 30 ng/ml, tracer undiluted; (B) AFP concentration 30 ng/ml, tracer undiluted, pre-incubation time 30 min.

tracer. Thus, a pre-incubation time of 30 min was chosen for AFP detection.

It is well known that the residence time of the immunomixture in the reactor decides the binding efficiency of the free enzyme tracer to the immobilized AFP. The residence time of the immunomixture in the column depends on both the reactor dimensions and the flow rate of the mixture. Here, the reactor dimensions were fixed at 1.6 mm inner diameter and 10 cm length. Therefore, a relatively low flow rate was advantageous in trapping more free enzyme tracer. Thus, the CL intensity was greater at a low flow rate (Fig. 4B). The lowest flow rate that could be obtained on our pump was 0.05 ml/min. Considering the assay performance and the whole assay time, this was selected as the optimal flow rate of the immunomixture. At this rate, the residence time of the immunomixture in the reactor was about 2 min.

Disruption of the antigen–antibody complex, i.e. regeneration of the reactor, is an essential step to render a flow-injection immunoassay system reusable. Different dissociating reagents for the regeneration were tested, including a salt solution of high concentration (5 mol/l NaCl), organic solvent (methanol/H_2O 1/1), buffer with low pH value (0.1 mol/l glycine–HCl, pH 2.0), diluted acid (50 mmol/l H_3PO_4) and diluted alkali (10 mmol/l NaOH). The most efficient dissociating reagent for AFP was found to be 10 mmol/l NaOH, since it allowed fast and complete dissociation of the antigen–antibody complex.

To minimize non-specific adsorption of biochemicals, which is a frequent problem in solid-phase immunoassay, the addition of surfactants or inert proteins is a common practice. In the present work, 0.05% Tween-20 was added to the PBS carrier for this purpose.

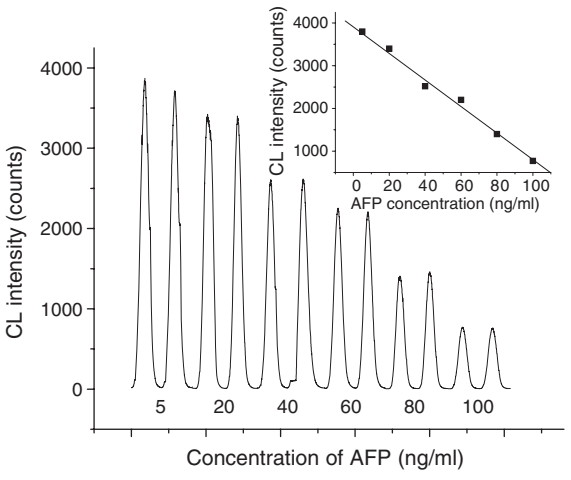

Fig. 5. Dose–response curve for AFP assay.

3.4. Dose–response curve

Under optimal conditions, the CL intensity decreased with increasing AFP concentration. The dose–response curve for AFP showed a linear range from 5.0 to 100 ng/ml with a correlation coefficient of 0.990 (Fig. 5). The regression equation was $I=3908.4-31.075C$ (ng/ml), where I is the CL intensity, and C is the AFP concentration. The detection limit of this FIIA system was 2.7 ng/ml at a signal/noise ratio of 3. When the AFP concentration was more than 100 ng/ml, an appropriate dilution of sample or a higher concentration of enzyme tracer was needed in the pre-incubation step. The latter is

Table 1
AFP concentration in clinical sera

Sample	Proposed method (ng/ml)	Immunoradiometric method (ng/ml)	Relative deviation (%)
1	92	102	9.8
2	260	284	8.4
3	1210	1280	5.7
4	1210	1160	−4.1
5	38	34	−10.5

necessary to obtain a new calibration curve for AFP detection.

3.5. Detection of AFP in a clinical serum sample

Five clinical sera were analyzed using the proposed method as well as the reference immunoradiometric method; the latter was carried out by Jiangsu Institute of Cancer Prevention and Cure. The AFP level in the serum of some patients was beyond the kinetic range of the proposed method, thus appropriate dilution before assay was necessary. The results are shown in Table 1; agreement between the two methods is acceptable.

[Safety precaution: patients with a high serum AFP concentration often suffer from infectious blood liver disease. Thus, all tools in contact with clinical sera were disinfected after use.]

3.6. Reproducibility and stability of the AFP immunoassay system

The reproducibility of the immunoassay system was assessed by intra- and inter-assay coefficients of variation (CVs). The intra-assay CV was the difference between three determinations of one sample on the same reactor. The inter-assay CV was the difference between the measurements of the same sample on three different reactors. The intra- and inter-assay CVs obtained at an AFP concentration of 30 ng/ml were 2.2% and 10.5%, respectively. Sometimes, the microbeads could not be packed uniformly, which affected greatly the performance of the flow immunosensor and resulted in relatively large variation between different immunoaffinity reactors. Considering this factor, an inter-assay CV of 10.5% was acceptable. The low value of intra-assay CV indicated that the proposed immunosensor could be regenerated and used repeatedly. The acceptable inter-assay CV value demonstrated the possibility of batch preparation of immunosensors.

When the immunoaffinity reactor was not in use, it was stored in PBS (pH 7.0) at 4 °C. No obvious change was observed after storing for 10 days. The immunoaffinity reactor could be used up to 20 times. After that, its analytical performance showed an observable decrease. This was because the repeated harsh regeneration process decreased irreversibly the immunoactivity of the immobilized AFP.

4. Conclusion

A novel, simple enzyme immunoassay for AFP based on epoxysilane modified glass microbeads as immobilization support is described. This low cost and flexible chemiluminescent immunosensor, combined with a flow injection system, is a simple, sensitive and rapid non-competitive immunoassay for AFP. The epoxysilanized glass microbeads show an efficient immobilization of AFP. The immunosensor system shows acceptable accuracy, reproducibility and storage stability. In comparison with conventional enzyme-linked immunosorbent assay (ELISA) approaches, the method proposed here is reusable, rapid and easy to automate for high sample throughput.

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