Channel-resolved multianalyte immunosensing system for flow-through chemiluminescent detection of α-fetoprotein and carcinoembryonic antigen

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Received 5 July 2007; received in revised form 19 September 2007; accepted 23 October 2007
Available online 30 October 2007

Abstract

A novel flow-through immunosensing system for chemiluminescent (CL) multianalyte immunoassay was designed based on channel-resolved technique. Using α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) as model analytes, two polyethersulfone membranes modified with the corresponding capture antibodies were set in two channels of a flow cell, and then two mixtures of the sample and corresponding alkaline phosphatase labeled antibodies were introduced into the channels for on-line incubation, respectively. Upon injection of CL substrate the catalyzed CL signals from the two channels were sequentially collected with the aid of an optical shutter for CL detection of two analytes. The antibodies immobilized membranes could be regenerated for reuse. Under optimal conditions AFP and CEA could be assayed in the ranges of 5.0–150 and 0.50–80 ng/ml with detection limits of 1.5 and 0.25 ng/ml, respectively. The assay results of clinical serum samples with the proposed system were in acceptable agreement with those with the reference method in single-analyte test mode. This novel immunosensing system based on the designed channel-resolved technique provided an automated, reusable, simple, sensitive and low-cost approach for multianalyte immunoassay without using of expensive array detector.

Keywords: Channel-resolved technique; Chemiluminescence; Multianalyte immunoassay; Immunosensors; α-Fetoprotein; Carcinoembryonic antigen

1. Introduction

Immunosensor of biomarkers has been extensively applied in early screen of disease, evaluating the extent of disease, monitoring the response of disease to therapy, and predicting recurrence. The measurement of a single marker is often insufficient to diagnosis purpose due to its limited specificity and sensitivity, thus multianalyte immunoassay of biomarkers panel has attracted considerable interest in recent years (Wilson and Nie, 2006). Since simultaneous radioimmunoassay of human insulin and growth hormone in serum sample using I-131 and I-125 as labels was reported in 1966 (Morgan, 1966), some attempts based on multilabel mode have been made towards multianalyte immunoassay. Various labels including enzymes (Blake et al., 1982; Choi et al., 1991), organic fluorescent dyes (Swartzman et al., 1999), lanthanide chelates (Bookout et al., 2000; Wu et al., 2003; Zhang et al., 2004; Hu et al., 2007) and nanoparticles (Goldman et al., 2004; Liu et al., 2004; Ihara et al., 2006; Hu et al., 2007) have been adopted to tag the antibodies or antigens of the corresponding analytes (one per analyte) to realize multianalyte immunoassay. However, combination of multiple labels in a single run often leads to loss of assay performance due to their incompatible optimal assay conditions (Kricka, 1992), and the signal overlapping of different labels results in difficulty in accurate quantitation (Goldman et al., 2004). In our previous work (Fu et al., 2006) a substrate zone-resolved technique has been proposed to overcome these drawbacks. However, as other multilabel mode based multianalyte immunoassay, the restriction in number of available labels greatly limits the number of analytes that can be assayed in a single run (Mastichiadis et al., 2002).
Another dominant mode for multianalyte immunoassay is the spatial resolution of different immunoreaction areas with a universal label. The most familiar spatial-resolved approach is antigen or antibody array combined with optical (Taitt et al., 2002; Fall et al., 2003; Strasser et al., 2003; Knecht et al., 2004; Golden et al., 2005; Chen and Durst, 2006) and electrochemical (Kojima et al., 2003; Ogasawara et al., 2006; Shi et al., 2006; Wilson and Nie, 2006) detection using expensive array detector such as charge-coupled device camera or multi-channel electrochemical workstation. Alternatively, non-array detector has also been used for spatial-resolved detection by moving the affinity microcolumn (Piyasena et al., 2004) or capillary immunosensor (Petrou et al., 2002) with a motorized translational stage, which makes the detection device more complicated. This work designed a two-channel flow-through immunosensing system based on channel-resolved technique for multianalyte immunoassay of tumor markers panel composed of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA).

AFP is a tumor marker related to hepatocellular cancer, yolk sac cancer, liver metastasis from gastric cancer, testicular cancer and nasopharyngeal cancer (Li, 2001). Numerous methods such as fluorescent (Matsuya et al., 2003), electrochemical (Qiang et al., 2006), inductively coupled plasma mass spectrometric (ICPMS, Zhang et al., 2004; Hu et al., 2007), atomic absorption spectrometric (Wang et al., 2001), electrochemiluminescent (Zhuang et al., 1999), micellar electrokinetic capillary chromatographic (Wang et al., 2002), and liquid-phase binding (Kawabata et al., 2005) immunoassays have been exploited to detect AFP level. CEA is a cell surface glycoprotein indicative of lung, liver, pancreas, breast, ovarian, cervix and prostate cancer (Li, 2001). In recent years chemiluminescent (CL, Dungchai et al., 2007), time resolved fluorescent (Yuan et al., 2002), electrochemical (Tang et al., 2006), ICPMS (Hu et al., 2007), piezoelectric (Shen et al., 2005), and thermal lens microscopic (Sato et al., 2001) methods have been proposed for immunoassay of CEA level. Here a flow-through two-channel immunosensing system was fabricated using polyethersulfone membrane as support for immobilization of AFP and CEA capture antibodies. CL signals catalyzed by alkaline phosphatase (ALP) labeled AFP and CEA sandwich immunocomplexes were sequentially collected with a non-array detector in channel-resolved mode to quantitate the two tumor markers. This method avoided the inconvenience to move the microcolumn or capillary immunosensor with a motor (Petrou et al., 2002; Piyasena et al., 2004) and the need of multiple detection units (Yacoub-George et al., 2002), thus simplified the detection device and lowered the instrument cost. This strategy possesses attractive characteristics such as low cost, simple manipulation and easy automation.

2. Materials and methods

2.1. Reagents

Mouse monoclonal anti-AFP and anti-CEA used as capture antibodies, ALP labeled goat polyclonal anti-AFP and anti-CEA used as tracer antibodies, standard AFP (standardized against Chinese Reference Standard 542-0004) and CEA (standardized against Chinese Reference Standard 150540-0210) solutions were all purchased from Boson Biotech. Co. Ltd. (China). Prior to use the tracer antibodies solutions were 1:1 diluted with 0.01 M phosphate buffer saline (PBS, pH 7.4). CL substrate for ALP was composed of 1 mg/ml enhancer commercially named Sapphire-ITM and 0.5 M disodium 3-(4-methoxy)-spiro-[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan]-4-y] phenyl phosphate (ABI, USA) in 0.1 M Tris–HCl buffer at pH 9.5 (commercially prepared by Boson Biotech. Co. Ltd.). Roche diagnostics kits for AFP and CEA (Roche Diagnostics GmbH, Germany) were used for the reference electrochemiluminescent immunoassay (ECLIA). Aldehyde-activated polyethersulfone membrane used for antibody immobilization was obtained from Pall Galman Science Corporation (USA). The clinical serum samples were provided by Jiangsu Institute of Cancer Research (China). All other chemicals were reagent grade and used as received. All buffers were prepared using ultra-pure water treated by Milli-Q system (Millipore, USA). 0.01 M PBS (pH 7.4) was used as coupling buffer for antibody immobilization. PBS containing 0.5% bovine serum albumin and 0.5% casein was used as blocking buffer to block the residual reactive sites on the antibody immobilized membranes. Wash buffer was PBS spiked with 0.05% Tween-20. Regeneration buffer was 0.1 M glycine/HCl at pH 2.0 and used to regenerate the membranes. PBS containing 0.01% NaN3 was used for the storage of the membranes.

2.2. Antibody immobilization

20 μl of capture antibody solution (50 μg/ml anti-AFP or anti-CEA in coupling buffer) was deposited on the aldehyde-activated polyethersulfone membrane (2.5 cm × 0.4 cm), reacted at room temperature (RT) for 30 min and then 4 °C overnight. The antibody immobilized membrane was then blocked with blocking buffer for 6 h and treated with 1 mg/ml NaCNBH3 solution for 40 min at 4 °C. The resultant membrane was thoroughly washed with PBS and dried in air.

2.3. Apparatus

The detection device consisted of a two-channel flow cell (Fig. 1a) and two antibodies immobilized polyethersulfone membranes. The flow cell was composed of a Teflon cover (4.0 cm × 3.5 cm × 0.8 cm) with two inlets and two outlets, a silicon rubber spacer (1.0 mm thickness) with two channels (2.5 cm × 0.4 cm) and a transparent Plexiglass slice of 0.5 cm thick. Anti-AFP immobilized membrane I and anti-CEA coupled membrane II were attached on the inner side of the Teflon cover with double-side glue tape and assembled with the silicon rubber spacer and Plexiglass slice to form channels I and II with the volume of 40 μl (2.5 cm × 0.4 cm × 0.4 mm), respectively. The total thickness of the membrane and glue tape was about 0.6 mm.

The flow-through multianalyte immunosensing system was constructed as Fig. 1b–d. Teflon tubes (0.8 mm i.d.) and silicon rubber tubes (1.0 mm i.d.) were used to connect all components in the system. All solutions were driven with a
multi-channel peristaltic pump equipped with two multiposition valves. Sequential introduction of different solutions into the flow cell was performed by switching the valves with four inlets and one outlet. The CL signals emitted from the channels were collected by a photomultiplier (PMT) biased at $-800$ V, equipped in an IFFM-D Luminescent Analyzer (Remax, China), and positioned beneath the flow cell. The PMT was sensitive to photos with a wavelength range of 200–800 nm. An optical shutter was set between the flow cell and PMT to resolve the signals produced in different channels for sequentially recording.

An Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH) was used for the reference ECLIA method in single-analyte test mode.

2.4. Multianalyte immunoassay procedure

A detailed description of the events in the assay process for tumor markers panel composed of AFP and CEA is given in Table 1 and illustrated in Fig. 1b. The whole assay process was performed at RT and controlled automatically by personal com-

![Diagram](image_url)

Fig. 1. Scheme of the flow-through channel-resolved immunosensing system for the determination of AFP and CEA: (a) flow cell; (b) flow-through CL immunosensing system and process of immunoassay; (c) transect of flow cell for immunoassay; (d) optical shutter. (WB) Wash buffer, (RB) regeneration buffer, (CS) ALP CL substrate, (S) mixture of sample and tracer antibody, (M I) anti-AFP immobilized membrane, (M II) anti-CEA immobilized membrane.
Table 1
Details of flow-through channel-resolved multianalyte immunoassay protocol for detection of tumor markers panel composed of AFP and CEA

<table>
<thead>
<tr>
<th>Step number</th>
<th>Valve position</th>
<th>Step</th>
<th>Starting time (min:s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>Introduce mixtures of sample (20 μl) and ALP labeled AFP or CEA antibody (20 μl) into each channel of the flow cell</td>
<td>00:00</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>Stop flow and incubate at RT</td>
<td>00:30</td>
</tr>
<tr>
<td>3</td>
<td>WB</td>
<td>Wash the flow cell with wash buffer at a flow rate of 1.0 ml/min</td>
<td>02:30</td>
</tr>
<tr>
<td>4</td>
<td>CS</td>
<td>Introduce ALP substrate into the flow cell (40 μl per channel)</td>
<td>02:40</td>
</tr>
<tr>
<td>5</td>
<td>S</td>
<td>Stop flow to trigger the ALP-catalyzed CL reaction</td>
<td>02:50</td>
</tr>
<tr>
<td>6</td>
<td>S</td>
<td>Collect CL signal from channel I for AFP detection while shadow channel II with the optical shutter</td>
<td>03:10</td>
</tr>
<tr>
<td>7</td>
<td>S</td>
<td>Collect CL signal from channel II for CEA detection while shadow channel I with the optical shutter</td>
<td>03:30</td>
</tr>
<tr>
<td>8</td>
<td>RB</td>
<td>Introduce regeneration buffer to regenerate the membranes at a flow rate of 1.0 ml/min</td>
<td>03:50</td>
</tr>
<tr>
<td>9</td>
<td>WB</td>
<td>Introduce wash buffer to recondition the membranes at a flow rate of 0.5 ml/min</td>
<td>04:20</td>
</tr>
<tr>
<td>10</td>
<td>RB</td>
<td>Regenerate the membranes for the second cycle</td>
<td>04:50</td>
</tr>
<tr>
<td>11</td>
<td>WB</td>
<td>Recondition the membranes for the second cycle</td>
<td>05:20</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>Ready for the next assay cycle</td>
<td>05:50</td>
</tr>
</tbody>
</table>

A computer equipped with IFFM software run under Windows 2000 instead of manually manipulation.

After the sample was mixed with the diluted ALP labeled anti-AFP or anti-CEA solution, the resulting mixtures were introduced into the two channels of the flow cell to perform on-line incubation in a static format for 20 min, respectively. The ALP labeled sandwich immunocomplexes of AFP and CEA were formed in channels I and II, respectively. Wash buffer then passed through the flow cell to remove unbound immunoreagents, and ALP CL substrate was injected into the two channels to trigger CL reaction. With the aid of the optical shutter the CL signals from the two channels were sequentially collected to detect the levels of AFP and CEA. Afterwards, the antibody immobilized membranes were regenerated by the introduction of regeneration buffer and wash buffer into the flow cell for two cycles. At a time of 32 min the whole assay procedure was completed and the immunosensing system was ready for the next assay cycle.

3. Results and discussion

3.1. Optimization of signal collection time

After the CL substrate was introduced into the channels, the ALP-catalyzed CL reaction produced detectable CL emission in a static format. Therefore, the flow rate of CL substrate did not influence the CL signal and the sandwich complex. As seen in Fig. 2a, the emission intensity was the function of reaction time, and increased continuously with time, revealing that the improved sensitivity could be obtained with a long CL reaction time. However, too long time for CL signal collection was obviously disadvantageous to the improvement of assay speed. Therefore, CL signal was collected at 4 min to compromise between high sensitivity and short assay time, at which the CL emission was stable for each repetitious measurement.

3.2. Choice of incubation time

Incubation time is a restriction factor to the improvement of immunoassay speed and sample throughput, which is controlled by mass transport of immunoreagents and kinetics characteristics of immunoreaction. As shown in Fig. 2b, with the increasing incubation time the CL intensities for 50 ng/ml AFP and 50 ng/ml CEA increased quickly and reached their maximum values at 50 and 40 min, respectively, implying the maximum formation of these sandwich immunocomplexes. The times for obtaining the maximum responses at RT were obviously shorter than that of 1–3 h at 37 °C for the conventional microwell plate ELISA method. It resulted from the short diffusion distance of the immunoreagents in the flow cell with thickness of 0.4 mm, which accelerated the process of mass transport of the immunoreagents and thus improved the immunoreaction rate.

Fig. 2. Effects of CL reaction time (a) and incubation time (b, n = 5 for each point) on CL intensity in the presence of 50 ng/ml AFP (a and curve 1 in b) and 50 ng/ml CEA (curve 2 in b).
At the incubation time of 20 min the CL signals were 70 and 67% of the maximum values for AFP and CEA, respectively. Since the high sensitivity of CL detection provided low limits of detection enough for clinical diagnosis, the incubation time of 20 min was used in the further study to improve assay speed.

3.3. Evaluation of cross-reactivity

Cross-reactivity and cross-talk are two crucial parameters regarding specificity and reliability of multianalyte immunoassay. In this protocol, AFP and CEA were detected in two separated close detection channels, therefore, the cross-talk resulted from the diffusion of the reaction product (Kojima et al., 2003; Wilson and Nie, 2006) was unavoidable, and cross-reactivity potentially occurred between the tumor markers and their noncognate antibodies was the only factor influencing the reliability of the proposed multianalyte immunosensing system. To examine the cross-reactivity between AFP antibodies and CEA, increasing level of CEA was spiked into 50 ng/ml AFP standard solution and incubated with AFP antibodies in channel I. Even when the concentration of coexistent CEA reached 200 ng/ml, the maximum change of the resultant CL signal from channel I did not go beyond 5.9% (Fig. 3a). With the same protocol, the presence of variant amount of AFP in 50 ng/ml CEA led to a change less than 3.9% of the CL signal from channel II (Fig. 3b). These results indicated that the cross-reactivity was negligible and the two tumor markers could be detected in a single run using the designed system without noticeable interference to each other.

3.4. Reusability of antibody immobilized membranes

Regeneration of the antibodies immobilized membranes, i.e. disruption of the immunocomplex without obviously damaging the activity of the immobilized antibody and antibody-support bond, is an essential step to further develop this strategy to low-cost repeatable application and automated continuous assay. This process could easily be achieved by a simple and short-period treatment with the regeneration reagent. Using 50 ng/ml AFP and 50 ng/ml CEA, this work compared the performance of several most commonly employed regeneration reagents inclusive of 1.0 M NaCl solution, CH3OH–H2O (1:1), 0.1 M glycine/HCl buffer (pH 2.0) and 50 mM NaOH solution. The regeneration efficiencies (REs) were calculated according to the following formula (Yakovleva et al., 2002):

\[
RE = \left[ 1 - \frac{(RT - B)}{T} \right] \times 100\% \tag{1}
\]

where RT is the residual tracer signal detected after the regeneration step, B is the signal for blank, and T is the signal before applying any regeneration step. The highest REs were obtained using 0.1 M glycine/HCl (pH 2.0), which allowed fast and complete dissociation of the immunocomplexes in two regeneration cycles with REs of 98.1 and 98.3% for AFP and CEA, respectively. As a mild regeneration reagent, glycine/HCl buffer also exhibited good activity retention to the immobilized antibodies, and the antibody immobilized polyethersulfone membranes could be used for more than 20 cycles. After 20 assay cycles for 50 ng/ml AFP and CEA the signals showed a decrease of less than 6.0%.

3.5. Analytical performance

Fig. 4 shows the dose–responses and calibration curves for multianalyte immunoassay of AFP and CEA. The linear regression equations for AFP and CEA are:

\[
\begin{align*}
I_{\text{AFP}} &= 12.9 [\text{AFP}] (\text{ng/ml}) + 3.2 \quad (R^2 = 0.9974) \\
I_{\text{CEA}} &= 16.2 [\text{CEA}] (\text{ng/ml}) + 38.6 \quad (R^2 = 0.9937)
\end{align*}
\]

over the concentration ranges of 5.0–150 and 0.50–80 ng/ml, respectively, where \( I \) is CL intensity. The limits of detection for AFP and CEA are 1.5 and 0.25 ng/ml at a signal/noise ratio of 3 (3σ), respectively. Since their cut-off values in clinical diagnosis are 25 and 5 ng/ml, respectively, the sensitivity of the proposed multianalyte immunosensing system was enough to practical application.

Reproductibility of the proposed immunosensing system was assessed with a standard sample composed of 50 ng/ml AFP and 50 ng/ml CEA for five consecutive measurements. The coefficient of variation was 6.1 and 3.8% for AFP and CEA, respectively. Reproducibility was also examined at the cut-off concentrations, which showed acceptable precision of 6.8 and 4.2% for AFP and CEA, respectively. Fabrication reproducibility of the antibodies immobilized membranes was also assessed by measuring this sample on five couples of membranes prepared in batch. The acceptable coefficient of variation of 8.1 and 5.6% for AFP and CEA demonstrated the possibility of batch preparation of the antibodies immobilized membranes. Detection of serum samples near the cut-off concentrations showed relative errors of less than 8.8% against the reference ECLIA.
method performed in single-analyte test mode for the two tumor markers.

After mixing the sample and the tracer antibodies, the whole assay process including incubation, wash, signal collection and membranes regeneration could be completed in 32 min. When this system was not in use, the channels were filled with 0.01 M PBS containing 0.01% NaN₃ (pH 7.4) and stored at 4°C. No obvious change was observed after storage of more than 20 days.

4. Conclusions

A novel flow-through multianalyte immunosensing system has been proposed with a designed channel-resolved technique for CL detection of tumor markers panel with a non-array detector. The channels can be prepared with the antibodies immobilized membranes. The proposed system is simple, convenient, sensitive, and programmable. The whole assay system is composed of some low-cost components, such as peristaltic pump, multiposition valve and PMT, and the membranes are reusable with a convenient regeneration step. This method exhibits acceptable accuracy, detection and fabrication reproducibility, and the membranes show good storage stability. The sensitivity and linear ranges are also sufficient for practical application. This work presents a proof of concept of a multianalyte immunosensing device, which will be further developed for assay of markers panel to improve their diagnostic value. The further work will improve the markers channel by using more channels or combining this concept with other techniques (Fu et al., 2007) and sample throughput by using more flow cells and incubating multiple samples in batch.

Acknowledgements

We gratefully acknowledge to the financial support from the National Science Funds for Distinguished Young Scholars (20325518) and Creative Research Groups (20521503), the Key Program (20535010) from the National Natural Science Foundation of China and the Science Foundation of Jiangsu (BS2006006, BS2006074).

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