A channel-resolved approach coupled with magnet-captured technique for multianalyte chemiluminescent immunoassay

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Abstract

A concept of channel-resolved multianalyte immunoassay (MAIA) and a semi-automated flow-through chemiluminescent (CL) MAIA system coupled with magnet-captured technique were proposed for rapid quantitation of different analytes in a single run. Using α-fetoprotein (AFP), carcinoembryonic antigen (CEA) and carcinoma antigen 125 (CA 125) as model analytes. They were firstly incubated in the mixtures of capture antibodies-immobilized paramagnetic microspheres (PMs) and corresponding alkaline phosphatase-labeled antibodies under stir and pumped into three parallel detection channels, the PMs were simultaneously captured by magnet, and the CL signals from the three channels were then sequentially collected with the aid of optical shutters to perform quantitative detection. AFP, CEA and CA 125 could be rapidly assayed in the ranges of 1.0–40 μg/l, 0.20–30 μg/l and 1.0–50 kU/l with the detection limits of 0.60 μg/l, 0.080 μg/l and 0.70 kU/l at 3σ, respectively. After manual dispensing of specimen and reagents the whole assay process could be completed in 18 min. The assay results of clinical serum samples with the proposed method were in acceptable agreement with the reference values. This system, based on the designed channel-resolved strategy and magnet-captured technique provides a semi-automated, reusable, simple, sensitive, rapid and low-cost approach for MAIA without using of expensive array detector.

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1. Introduction

In recent years, multianalyte immunoassay (MAIA) has attracted considerable interest due to the growing demand for quantitative analysis of a set of protein biomarkers in biological samples for clinical purpose. Compared with the traditional parallel single-analyte immunoassay, MAIA offers higher sample throughput, less sample consumption, shorter assay time, lower cost per assay and more efficient diagnosis for one disease (Kricka, 1992; Weissenstein et al., 2006). So far two dominant modes have been adopted to realize the goal of MAIA. The first one is multilabel mode performed using different labels (one per analyte) to tag antibodies or antigens corresponding to the analytes. These labels include enzymes (Blake et al., 1982; Choi et al., 1991), fluorescence dyes (Swartzman et al., 1999), lanthanide chelates (Wu et al., 2003; Hu et al., 2007), radioisotopes (Gow et al., 1986; Wlans et al., 1986) and nanocrystals (Goldman et al., 2004; Liu et al., 2004), whose signals can be easily distinguished from others by some analytical parameters such as wavelength, potential and mass/electric charge, etc. However, combination of multiple labels in a single run often leads to loss of assay performance due to their different optimal assay conditions (Kricka, 1992), and the signal overlapping of different labels also results in difficulty in accurate quantitation (Goldman et al., 2004; Liu et al., 2004). To overcome these drawbacks a substrate zone-resolved technique has been designed in a flow-through chemiluminescent (CL) multianalyte immunosensing system for sequential detection of tumor markers (Fu et al., 2006). As other methods based on multilabel mode, the difficult in finding more enzyme labels for this technique greatly restricts the number of analytes can be assayed and limits its application (Mastichiadis et al., 2002).
The second strategy is the spatial resolution of different immunoreaction areas using one universal label, which seems to be well suited for the development of MAIA with high sample and analyte throughput. The most popular spatial-resolved approach is antigen or antibody array with fluorescent (Christodoulides et al., 2002; Taft et al., 2002; Rissin and Walt, 2006), CL (Huang et al., 2001; Roda et al., 2002; Fall et al., 2003; Zhi et al., 2003), colorimetric (Fernández-Sánchez et al., 2005), electrochemical (Jiang et al., 2003; Kojima et al., 2003; Ogasawara et al., 2006; Wilson and Nie, 2006) and piezoelectric (Luo et al., 2006) detection. Some commercially available flow cytometry bead-based microarrays are also used for MAIA (Binder, 2006; Elshal and McCoy, 2006). A common problem in array immunosassay is the requirement of expensive detector, such as charge-coupled device camera or multichannel electrochemical workstation. Furthermore, charge-coupled device camera used in optical assay needs to be cooled for lowering the thermal background. The cross-talk can potentially occur in electrode array due to the diffusion of electroactive product generated at one electrode to a neighboring electrode (Kojima et al., 2003; Wilson and Nie, 2006). Alternatively, some non-array detectors have also been used for spatial-resolved MAIA by moving the affinity microcolumn composed of discrete segments of beads bearing distinct receptors (Pyasena et al., 2004) or multiple-band disposable optical capillary immunosensor (Petrou et al., 2002) with motorized translational stages, which makes the detection devices more complicated.

This work designed a channel-resolved strategy, with which a semi-automated flow-through MAIA system using paramagnetic microspheres (PMs) to capture the alkaline phosphatase (ALP)-labeled immunocomplexes was proposed for parallel and rapid CL detection of multiple tumor markers. Using α-fetoprotein (AFP), carcinoembrionic antigen (CEA) and carcinoma antigen 125 (CA 125) as model analytes that are of great significance in early screening and clinical diagnosis of some tumor diseases (Li, 2001), this system utilized a single photomultiplier (PMT)-based non-array detector to distinguish sequentially CL signals responding to the three analytes with one universal label, thus could detect them in a single run with the aid of three moveable optical shutters.

2. Materials and methods

2.1. Reagents

All mouse monoclonal AFP (M803209), CEA (M111147) and CA 125 (M002201) antibodies, ALP-labeled goat polyclonal antibodies, standard AFP (standardized against Chinese Reference Standard 542-0004), CEA (standardized against Chinese Reference Standard 150540-0210) and CA 125 (standardized against electrochemiluminescence immunoassay (ECLIA) from Roche Diagnostics GmbH) solutions and sample diluent were purchased from Boson Biotech. Co. Ltd. (China). Control 1 and control 2 for the three tumor markers were 5.0 and 20 μg/l (kU/l). If the values outside 4.0–6.0 and 17–23 μg/l (kU/l) were obtained, respectively, the reagents, antibodies immobilized PMs, and detector performance should be checked and the analysis was repeated. CL substrate solution for ALP was composed of 1 g/l Saphire-II™ enhancer (ABI, USA) in 0.1 M Tris–HCl buffer (pH 9.5) and 0.5 M disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7}decan]–4-yl) phenyl phosphate (ABI, USA) (commercially prepared by Boson Biotech. Co. Ltd.). Roche Diagnostics reagent kits for AFP, CEA and CA 125 (Germany) were used for the reference ECLIA. Carboxyl groups modified PMs in an aqueous suspension with the mean diameter of 1.5 μm and concentration of 20 g/l were obtained from Bangs Laboratories Inc., (USA). N-Hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Aldrich and Merck, respectively. All other reagents were of the best grade available and used as received. Activation buffer for carboxyl group was 0.01 M 2-morpholinooethanesulfonic acid, adjusted to pH 5.5 with NaOH. Coupling buffer for antibodies immobilization was 0.01 M phosphate buffer saline at pH 7.4 (PBS). Blocking buffer for blocking the residual reactive site on the antibodies immobilized PMs was composed of 0.01 M Tris, 0.15 M NaCl, 0.1% bovine serum albumin, 0.1 M NaN3 and 0.001 M EDTA, adjusted to pH 7.4 with HCl. Wash buffer was PBS spiked with 0.05% Tween 20. Regeneration buffer was 0.1 M glycine/HCl at pH 2.2.

2.2. Preparation of antibodies immobilized paramagnetic microspheres

0.5 ml of PMs suspension was firstly washed twice with activation buffer and separated with a rare earth magnet to permit removal of the supernatant waste. The obtained PMs were then resuspended in 0.5 ml of activation buffer, in which 10 mg of EDC and 5 mg of NHS were added to activate the carboxyl groups for 30 min under constant stir at room temperature (RT). The activated PMs were washed thrice with coupling buffer and resuspended in 0.5 ml of coupling buffer. AFP, CEA and CA 125 antibodies were then dissolved in the suspensions to a concentration of 10 mg/l and reacted with the activated PMs under gentle stir at RT for 2 h followed with stand overnight in refrigerator at 4°C to obtain AFP, CEA and CA 125 antibodies immobilized PMs, respectively, which were thoroughly washed with blocking buffer, and stored in 0.5 ml of this buffer at 4°C until used.

2.3. Specimen collection and preparation

According to the rules of the local ethical committee, blood samples were collected using the standard venipuncture technique and the sera were centrifugally separated from the cells, without hemolysis. The serum specimens could be directly assayed with the proposed and the reference methods. If necessary, the specimens could be stored at 4°C for less than 48 h or frozen at −20°C for less than 2 months. Prior to use, the samples were allowed to come to RT and mixed by gently swirling.
2.4. Apparatus

The channel-resolved flow-through CL MAIA system was constructed as illustrated in Fig. 1. 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 fluids were delivered with a multichannel peristaltic pump of an IFFM-D Luminescent Analyzer (Remax, China). Three multiposition valves with three inlets and one outlet for each were used to introduce different fluids into the three detection channels. The inlets 1 of the three valves were inserted into three incubation cups that contained AFP, CEA and CA 125 antibodies immobilized PMs, ALP-labeled antibodies and samples, respectively, with mini stir bars to accelerate the immunoreaction. The inlets 2 and 3 were inserted into CL substrate and wash buffer, respectively. A cylindrical neodymium magnet (Ø 10 mm × 10 mm, Magnet Japan, Japan) provided magnetic field to capture PMs. Three quartz tubes of about 30 μl (i.d. = 1.0 mm, length = 4 cm) were parallel positioned beneath the magnet to act as detection channels. The CL signals from the detection channels were collected by a PMT biased at −800 V. Three moveable tubular optical shutters were used to resolve the CL signals produced in different channels to reach the PMT.

The reference ECLIA was performed with a Roche Elecsys 2010 Immunoassay Analyzer (Roche Diagnostics GmbH, Germany). An ESCALAB 250 spectrometer (USA) working in ultra-high vacuum with an Al Kα X-ray source was employed for X-ray photoelectron spectroscopic (XPS) analysis of antibodies immobilized PMs.

2.5. Immunoassay protocol

A detailed description of the events in the assay process for the three tumor markers was given in Table 1, and the whole assay process was performed at RT and controlled automatically by personal computer equipped with IFFM software package instead of manually manipulation. Therefore, the time for each assay cycle could be strictly controlled. Sample (60 μl) containing the three tumor markers was firstly dispensed in three incubation cups (20 μl for each) containing 20 μl of AFP, CEA and CA 125 antibodies immobilized PMs, ALP-labeled antibodies and samples, respectively, with mini stir bars to accelerate the immunoreaction. The inlets 2 and 3 were inserted into CL substrate and wash buffer, respectively. A cylindrical neodymium magnet (Ø 10 mm × 10 mm, Magnet Japan, Japan) provided magnetic field to capture PMs. Three quartz tubes of about 30 μl (i.d. = 1.0 mm, length = 4 cm) were parallel positioned beneath the magnet to act as detection channels. The CL signals from the detection channels were collected by a PMT biased at −800 V. Three moveable tubular optical shutters were used to resolve the CL signals produced in different channels to reach the PMT.

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3. Results and discussion

3.1. Characterization of antibodies immobilized PMs

PMs have been used in immunoassays for the advantages over conventional solid-phase supports (Wijayawardhana et al., 2000; Yu et al., 2000; Li et al., 2003; Zhao and Shippy, 2004; Tudorache et al., 2006): free suspension in the immunoreagents or CL substrate for rapid immunoreaction or CL reaction, large surface area to accelerate reaction and allow binding more capture antibodies for enhancing the sensitivity, easy and rapid isolation in a magnetic field, and effective concentration of trace amount of analyte when the PMs are collected. Therefore, this work used carboxyl group modified PMs as support for immunoreaction. Carboxyl group modified PMs were firstly activated with EDC and NHS, which produced a surface of N-containing intermediate. A N(1s) peak at 399.2 eV in the XPS spectrum of the activated PMs confirmed the presence of this intermediate. After the activated PMs reacted with the antibodies, the N(1s) peak at 399.9 eV increased about 150% compared to that at 399.2 eV, indicating successful covalent binding of the antibodies to the PMs. The slight difference of two peak positions was due to the different N containing groups in the intermediate and the product.

3.2. Kinetic characteristics of CL reaction

The CL reaction occurred immediately after introduction of the substrate into the detection channel, and its intensity increased continuously. Too long triggering time for CL reaction was obviously disadvantageous to the improvement of assay speed. Considering the fact that the sensitivity was enough for clinical application at a triggering time of 5 min, it was chosen as the optimal CL reaction time to give a compromise between high sensitivity and short assay time.

3.3. Effect of incubation time

The incubation time is a bottleneck to the improvement of immunoassay speed and sample throughput, which is controlled by mass transport of immunoreagents and kinetics of immunoreaction. In most cases mass transport process of immunoreagents is believed to be the rate-controlled step (Yu et al., 1999). PMs with mean diameter of 1.5 μm were freely suspended in the mixture of immunoreagents, thus provided a relatively large reaction surface compared to the microwell used in conventional ELISA (Yu et al., 2000). Moreover, the convection achieved with online stir further increased the collision frequency among these immunoreagents, thus accelerated the immunoreaction. With the increasing incubation time the CL intensities examined at 10 μg/l AFP, CEA and 10 kU/l CA 125 at RT increased quickly and reached their maximum values at 15, 15 and 17 min, respectively, indicating the maximum formation of these sandwich immunocomplexes. When the stir was absent, the maximum formation occurred at about 40 min. The times for the maximum formation of these immunocomplexes using this system were also much shorter than that of 1–3 h at 37 °C for the conventional microwell plate ELISA approach. At the incubation time of 10 min the CL signals were 86, 82 and 66% of the maximum values for AFP, CEA and CA 125, respectively. Since the high sensitivity of CL detection provided low-detection limits enough for clinical diagnosis, considering the optimal analytical performance and the further development of this method to high assay speed, the incubation time of 10 min was used in the further study. For obtaining higher sample throughput a large shaking table or stand can be used to replace the magnetic stir bar.

3.4. Evaluation of cross-reactivities

Cross-reactivity and cross-talk are two crucial analytical parameters regarding specificity and reliability of MAIA. In this protocol, the three tumor markers were detected in three 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 avoidable, and the cross-reactivity was the only potential factor influencing the reliability of the proposed method. The cross-reactivities between the three tumor markers and their noncognate antibodies were examined by detecting the change of CL signal at a definite concentration of analyte with the increasing concentrations of coexistent other two analytes. As seen in Fig. 2, upon the addition of CEA and CA 125 the maximum change of the CL signal for AFP was

Table 1

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Valve position</th>
<th>Step</th>
<th>Starting time (min:s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Incubate the mixtures of sample (20 μl), antibody immobilized PMs (20 μl), and tracer antibody (20 μl) in each incubation cup under stir</td>
<td>00:00</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Introduce the PMs suspensions into the detection channels and capture the PMs with the magnet</td>
<td>10:00</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Wash the PMs with wash buffer at a flow rate of 0.4 ml/min</td>
<td>10:10</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Introduce 30 μl of ALP substrate into each detection channel, stop flow and withdraw the magnetic field to trigger CL reaction</td>
<td>12:00</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Collect CL signal from the detection channel for AFP while shadow the other two channels with optical shutters</td>
<td>17:00</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Collect CL signal from the channel for CEA while shadow the other two</td>
<td>17:10</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Collect CL signal from the channel for CA 125 while shadow the other two</td>
<td>17:20</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>Introduce wash buffer to expel PMs at a flow rate of 1.0 ml/min</td>
<td>17:30</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>Ready for the next assay cycle</td>
<td>18:00</td>
</tr>
</tbody>
</table>
5.4% compared to the negative control, while these values for CEA and CA 125 upon the addition of AFP and CA 125, AFP and CEA were 2.1 and 1.8%, respectively, indicating that the cross-reactivity between AFP, CEA and CA 125 antibodies and the other two noncognate antigens was negligible, which suggested that the three tumor markers could be assayed in a single run using the designed system without noticeable interference to each other.

3.5. Regeneration of the antibodies immobilized PMs

The expelled antibodies immobilized PMs were collected and could be treated with some dissociation reagents for regeneration purpose. Of course it was necessary to avoid loss of the activity of the immobilized antibodies or damage of the bonds between the antibodies and the PMs in the regeneration step. Using 10 μg/l AFP, CEA and 10 kU/l CA 125, this work tested different dissociation reagents including buffer with low-pH value, diluted alkali solution and organic solvent. The regeneration efficiencies were calculated following the equation (Botchkareva et al., 2002):

\[
\text{regeneration efficiency} \ (\%) = \left[ 1 - \left( \frac{I}{I_0} \right) \right] \times 100 \quad (1)
\]

where \(I_0\) represents the CL signal obtained before applying any regeneration step and \(I\) is the signal obtained after the regeneration cycle. The best regeneration efficiencies were obtained using 0.1 M glycine/HCl (pH 2.2), which allowed fast and complete dissociation of the immunocomplexes with regeneration efficiencies of 98.0, 98.8 and 99.0% for AFP, CEA and CA 125, respectively. With the regeneration procedure the antibodies immobilized PMs could be used for more than 10 cycles. After 10 assay cycles the signals for 10 μg/l AFP, CEA and 10 kU/l CA 125 decreased by less than 7%; while the decrease were less than 10% for 30 μg/l AFP, CEA and 30 kU/l CA 125, showing acceptable retention of immunoactivity.

3.6. Assay performance

Short assay time with automated testing is a long-cherished goal in the development of both immunoassay and multianalyte test. It is of great significance in early disease screening. The automated flow-through system enables most of manual works to be replaced by programmed manipulations controlled with a computer. After manual dispensing of specimen and reagents the test process including incubation, washing, detection and expelling of PMs could be completed in 18 min, which was much shorter than those of more than 1 h to overnight with other methods (Swartzman et al., 1999; Huang et al., 2001; Fall et al., 2003; Goldman et al., 2004; Liu et al., 2004). It also showed obvious advantage in assay speed over other rapid methods requiring more than 30 min (Petrou et al., 2002; Kojima et al., 2003; Fu et al., 2006) and was comparable with 15 min for a piezoelectric immunosensor array (Luo et al., 2006). In practical application the samples can be incubated under stir to form immunocomplexes in batch. The rest steps including washing, detection and expelling of PMs need only 8 min. If fully automated sampling systems were used to replace the manual sampling for practical clinic application and commercialization of this proposed system, the sample throughput could be further improved.

Fig. 3 shows the dose–response and calibration curves for these analytes. The linear regression equations for AFP, CEA and CA 125 were \(I = 97.0 + 14.7 \times \text{[AFP]} \) (μg/l) \((R^2 = 0.9916)\), \(I = 84.2 + 32.2 \times \text{[CEA]} \) (μg/l) \((R^2 = 0.9908)\) and \(I = 63.4 + 16.3 \times \text{[CA 125]} \) (kU/l) \((R^2 = 0.9906)\) over the concentration ranges of 1.0–40 μg/l, 0.20–30 μg/l and 1.0–50 kU/l, respectively. The limits of detection at 3σ for the three analytes were 0.60 μg/l, 0.08 μg/l and 0.70 kU/l, respectively. The limits of detection at 3σ for the three analytes were 0.60 μg/l, 0.08 μg/l and 0.70 kU/l, respectively. The limits of detection at 3σ for the three analytes were 0.60 μg/l, 0.08 μg/l and 0.70 kU/l, respectively. The system showed slight hook effect when the concentrations were higher than 2500 μg/l, 1500 μg/l and 2000 kU/l for AFP, CEA and CA 125, respectively. Since their cut-off values in clinical diagnosis are 25 μg/l, 5 μg/l and 30 kU/l, the hook effect commonly does not affect the diagnosis purpose. The reproducibility of the proposed system at low, medium, and high concentrations was listed in Table 2, which showed acceptable intra- and inter-assay CVs of 4.2–8.4% and 5.0–11.5%, respectively. Reproducibility of the preparation of the antibodies immobilized PMs was also
examined by detecting 10 μg/l CEA using five batches of CEA antibody immobilized PMs prepared in different days. The results showed a CV of 8.3%, indicating acceptable preparation reproducibility.

3.7. Detection of tumor markers in clinical serum samples

40 clinical serum samples were assayed using the proposed semi-automated channel-resolved MAIA system as well as the reference ECLIA method. When the levels of tumor markers were over the calibration ranges, appropriate dilution of serum samples prior to assay was performed using the sample diluent. All samples were detected for five times, and the results showed the CV values of 3.2–11.1, 3.5–10 and 3.1–7.3% for AFP, CEA and CA 125, respectively. The orthogonal regression analysis of these results obtained with two methods \( (P < 0.0001) \) gave following linear equations and correlation coefficients: \( Y = -1.66 + 1.05X \ (R^2 = 0.9926), Y = -0.216 + 1.02X \ (R^2 = 0.9845) \) and \( Y = -0.428 + 1.05X \ (R^2 = 0.9898) \) for AFP, CEA and CA 125, respectively. The correlation of the proposed method with the reference was acceptable.

When known amounts of analytes were added into two clinical serum samples, the measured results showed satisfied recoveries in the range of 91–112% (Table 3).

### 4. Conclusions

A novel channel-resolved technique and semi-automated flow-through MAIA system were designed for the parallel detection of tumor markers. The MAIA procedure using PMT-based non-array detector is rapid, and the proposed system is simple, convenient, sensitive and programmable. The whole assay system is composed of some low-cost components, such as peristaltic pump, PMT, optical shutter, valve and magnet. The antibodies immobilized PMs are reusable with a regeneration step. Therefore, the proposed technology can be believed to be low-cost. With the aid of on-line stir device the incubation can be completed in a relative short period. This MAIA method can provide more information for cancer diagnosis than other single-analyte immunosensors (Chou et al., 2004; Chung et al., 2006; Salama et al., 2007). Our further work is to prepare the immunoassay system with more detection channels for more analytes.
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