

Channel and Substrate Zone Two-Dimensional Resolution for Chemiluminescent Multiplex Immunoassay

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A two-dimensional resolution system of channels and substrate zones was proposed for multiplex immunoassay performed with a designed multichannel chemiluminescent (CL) detection device coupled with a single photomultiplier. Using carcinoma antigen 125 (CA 125), carcinoma antigen 153 (CA 153), carcinoma antigen 199 (CA 199), and carcinoembryonic antigen (CEA) as two couples of model analytes, two couples of capture antibodies were immobilized in two channels, respectively. With a sandwich format, the CL substrates for alkaline phosphatase and horseradish peroxidase were delivered into the channels sequentially to perform a multiplex immunoassay after the sample and tracer antibodies were introduced into the channels for on-line incubation. CA 125, CA 153, CA 199, and CEA could be assayed in the ranges of 0.50–80, 2.0–100, and 5.0–150 U/mL and 1.0–70 ng/mL with limits of detection of 0.15, 0.80, and 2.0 U/mL and 0.65 ng/mL at 3σ , respectively. The whole assay process including regeneration of the device could be completed in 37 min. The proposed system showed acceptable detection and fabrication reproducibility, and the results obtained were in acceptable agreement with those from parallel single-analyte test of practical clinical sera. This technique provides a new strategy for a simple, automated, and near-simultaneous multianalyte immunoassay.

The measurement of disease markers has been showing its significance in early screening of disease, evaluating the extent of disease, and monitoring the response of disease to therapy. However, a single marker is often not sufficient for diagnosis purpose due to its limited specificity and sensitivity. The assay of markers panel can improve their diagnostic value.¹ Thus, multiplex immunoassay has attracted considerable interest to meet the growing demand for diagnostic application. Furthermore, multiplex immunoassay can offer higher sample throughput, less sample consumption, shorter assay time and lower cost than the traditional parallel single-analyte immunoassay.²

So far, two dominant modes have been adopted to realize the goal of a multiplex immunoassay. The first mode is the spatial resolution of different immunoreaction areas using a universal label for fluorescent,^{3,4} chemiluminescent (CL),^{5,6} spectrophotometric,^{7,8} electrochemical,^{9,10} and piezoelectric¹¹ detection of antigen or antibody array. These methods are commonly performed with expensive array detectors, such as a charge-coupled device (CCD) camera or multichannel electrochemical workstation, and an electrochemical sensor array often suffers from crosstalk potentially occurring due to the diffusion of electroactive product generated at one electrode to a neighboring electrode.^{1,10} A CL multichannel immunosensor has been designed for simultaneous immunoassay of three analytes by using three parallel photodetectors.¹² Some nonarray detector-based immunoassay systems have also been proposed for spatial-resolved multiplex detection, including an immunosensor with three channels for the spectrophotometric detection of biological warfare agents,¹³ an affinity microcolumn composed of discrete segments of beads bearing distinct receptors for the simultaneous detection of two analytes,¹⁴ and two multiple-band disposable optical capillary immunosensors for pesticides¹⁵ and hormones.¹⁶ The second mode

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is the multilabel mode performed using different labels (one per analyte) to tag the antibodies or antigens corresponding to the analytes, in which the fluorescent,^{17,18} spectrophotometric,¹⁹ time-resolved fluorescent,^{20,21} electrochemical,^{22,23} mass spectroscopic,^{24,25} and radiometric²⁶ signals from the labels can be resolved. The simultaneous detection of the signals from two or more labels inevitably involves a compromise in the optimal assay conditions,² and the signal overlapping of different labels also results in difficulty in accurate quantitation.¹⁸ To overcome these drawbacks, a substrate zone-resolved technique has been proposed in a flow-through multiplex CL immunosensing system by using alkaline phosphatase (ALP) and horseradish peroxidase (HRP) to label the antibodies for sequential CL detection of two tumor markers.²⁷ However, as with other multilabel modes,¹⁵ this technique is limited in finding more available enzyme labels and, therefore, limits the number of analytes that can be assayed in a single run. This work combined multilabel mode with spatial resolution to design a novel two-dimensional resolution technique coupled with flow-through CL immunoassay; thus, the number of detectable analytes were doubled when two channels were used for this system.

The two-dimensional resolution device included channel- and substrate zone-resolved techniques. The use of parallel channels coupled with an optical shutter for channel resolution avoided the inconvenience of moving the microcolumn or capillary immunosensor with a motor^{14–16} and the need for several photodetectors¹² and thus simplified the detection device. After the ALP- and HRP-labeled immunocomplexes were formed in two channels, this proposed technique and device could be used for multiplex immunoassay of tumor markers panel composed of carcinoma antigen 125 (CA 125), carcinoma antigen 199 (CA 199), carcinoma antigen 153 (CA 153), and carcinoembryonic antigen (CEA), which shows great significance in early screening and clinical diagnosis of some tumor diseases including colorectal cancer, gastric cancer, and lung cancer.²⁸ The results obtained were in acceptable agreement with those from the parallel single-analyte test of practical clinical sera. This strategy possesses attractive characteristics such as low cost, simple manipulation, and easy automation and has the potential to assay more analytes when more channels with the two enzyme labels were applied.

EXPERIMENTAL SECTION

Apparatus. The flow-through CL detection device was composed of a double-channel flow cell and two double-antibodies immobilized polyethersulfone membranes. As shown in Figure 1a, the flow cell consisted 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 thickness. Two antibodies immobilized polyethersulfone membranes (2.5 cm × 0.4 cm) were attached on the inner side of the Teflon cover of the channels with double-side glue tape. The total thickness of the membrane and glue tape was ~0.6 mm. The Teflon cover, silicon rubber spacer, plexiglass slice, and membranes produced two channels of 40 μL (2.5 cm × 0.4 cm × 0.4 mm).

The flow-through multiplex immunoassay system based on a two-dimensional resolution technique was constructed as shown in Figure 1b. All solutions were driven with a multichannel peristaltic pump of an IFFM-D Luminescent Analyzer (Remax). Sequential introduction of different solutions into the multichannel detection device was performed by two multiposition valves with five inlets and one outlet for each. 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. The CL signals were collected by a photomultiplier (PMT) biased at -800 V, which was sensitive to photons with a wavelength range of 200–800 nm. An optical shutter was set between the device and PMT to resolve the CL signals produced in different channels for sequentially recording (Figure 1c,d).

A Roche Elecsys 2010 Immunoassay Analyzer (Roche Diagnostics GmbH) was used to perform the reference electrochemiluminescent immunoassay (ECLIA) in single-analyte test mode.

Reagents. Standard solutions of CA 125 and CA 199 (standardized against ECLIA of Roche Diagnostics GmbH), mouse monoclonal antibodies for CA 125 (M002201) and CEA (M111147), ALP-labeled goat polyclonal antibodies for CA 125 and CA 199, sample diluent, and disodium 3-(4-methoxy Spiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate solution used as ALP CL substrate were all purchased from Boson Biotech. Co. Ltd. Mouse monoclonal antibody for CA 153 (Ma695) was provided by Santa Cruz Biotech. Inc. (Santa Cruz, CA). Mouse monoclonal antibody for CA 199 (C192,103-01), standard solution of CA 153 (standardized against Reference Standard T20012-16 of CanAg Diagnostics AB), and HRP-labeled mouse monoclonal antibody (Ma552) for CA 153 were obtained from CanAg Diagnostics AB. Standard solution of CEA (standardized against Chinese Reference Standard 150540-0210) and HRP-labeled horse polyclonal antibody for CEA were purchased from Feilong Medical Diagnostic Articles Co. Ltd. The activities of HRP and ALP labeled to the antibodies were ~70–80% of their original activities, i.e., 175–200 U/mg for HRP and 1750–2000 U/mg for ALP, respectively. Luminol-*p*-iodophenol-H₂O₂ solution used as HRP CL substrate was supplied by Autobio Diagnostics Co. Ltd. ECLIA reagent kits for CA 125, CA 199, CA 153, and CEA used for the reference ECLIA were provided by Roche Diagnostics GmbH. Aldehyde-activated polyethersulfone membrane was obtained from Pall Gelman Science Corp. (Ann Arbor, MI). All other reagents were of the best grade available and used as received.

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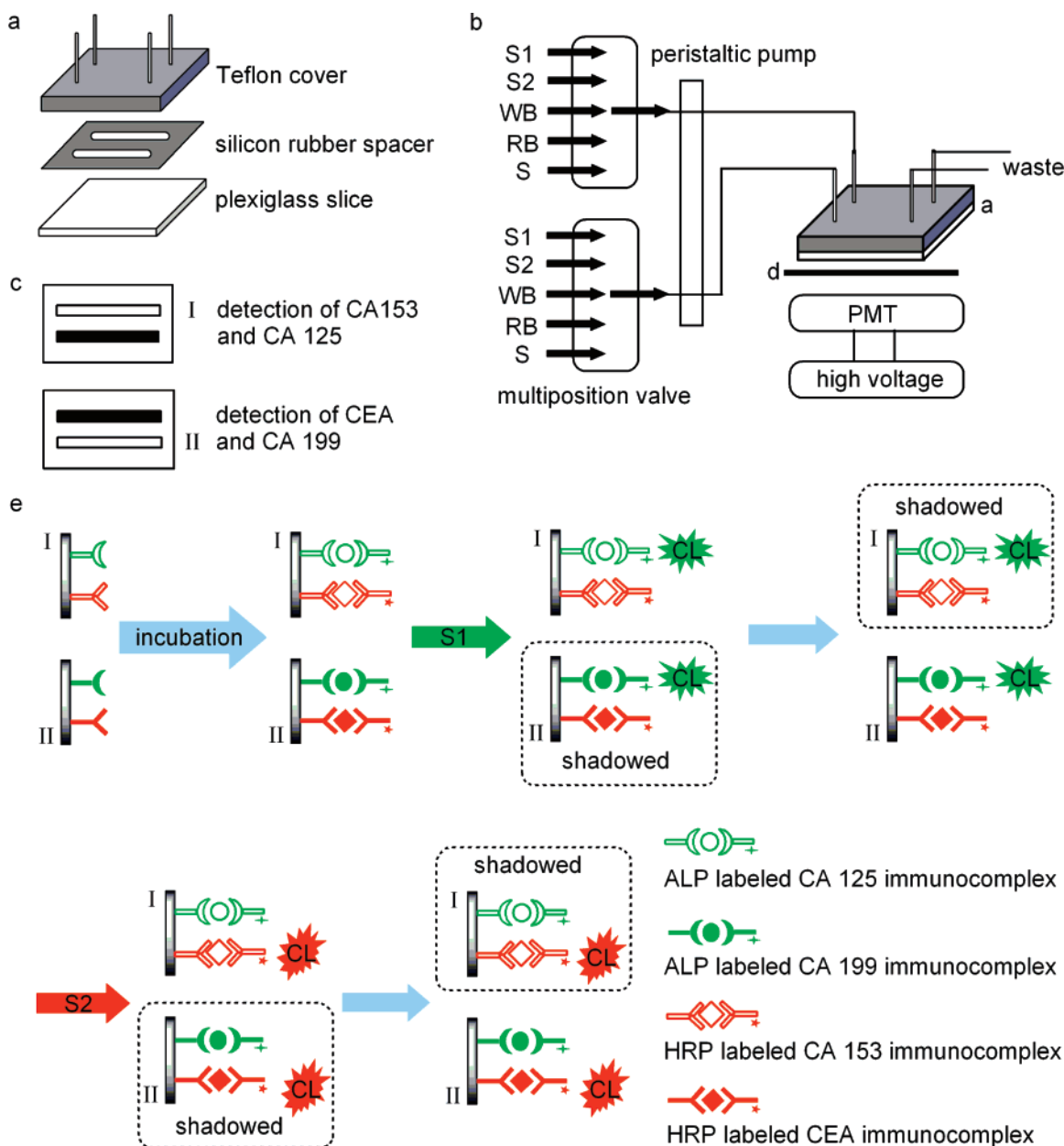


Figure 1. Scheme of the channel and substrate zone two-dimensional resolution system for multiplex immunoassay of tumor markers panel: (a) flow cell; (b) flow-through system; (c) transect of flow cell for immunoassay; (d) optical shutter; and (e) near-simultaneous multianalyte immunoassay procedure. (S1) ALP substrate, (S2) HRP substrate, (WB) wash buffer, (RB) regeneration buffer, and (S) mixture of sample and tracer antibodies.

Buffers. All buffers were prepared with water purified in a Milli-Q system (Millipore, Bedford, MA). Coupling buffer for antibodies immobilization was 0.01 M phosphate buffer saline (PBS) at pH 7.4. Blocking buffer was PBS containing 0.5% bovine serum albumin and 0.5% casein and was used to block the residual reactive sites on the antibodies immobilized membranes. Wash buffer was PBS spiked with 0.05% Tween-20. Regeneration buffer was 0.1 M glycine-HCl at pH 2.0. PBS containing 0.01% NaN₃ was used as storing buffer for antibodies immobilized membranes.

Antibodies Immobilization. Two mixed solutions of CA 125 and CA 153 antibodies and CA 199 and CEA antibodies (50 μ g/mL each in coupling buffer) were deposited on two aldehyde-activated polyethersulfone membranes (20 μ L for each), respectively, and reacted at room temperature for 30 min and then 4 $^{\circ}$ C overnight. The antibodies immobilized membranes were blocked

with blocking buffer for 6 h. Afterward, the membranes were treated with 1 mg/mL NaCNBH₃ solution for 40 min at 4 $^{\circ}$ C to perform a reductive alkylation process for obtaining stable antibodies immobilized membranes. After the resulting membranes were thoroughly washed with PBS and dried in air, they were attached on the inner side of the Teflon cover and assembled with the silicon rubber spacer and plexiglass slice to form a flow-through detection device with two channels. Membrane I coupled with CA 153 and CA 125 antibodies was in channel I, and membrane II coupled with CA 199 and CEA antibodies was in channel II.

Multiplex Detection of Tumor Markers Panel. A detailed description of the multiplex detection of tumor markers panel composed of CA 125, CA 199, CA 153, and CEA is illustrated in Figure 1e (see Table S1 in Supporting Information). The whole

assay process was performed at room temperature and controlled automatically by a personal computer equipped with IFFM software package run under Windows 2000. Different solutions were introduced into the flow-through detection device by switching the two multiposition valves.

After sample containing the four tumor markers was mixed with the tracer antibodies and introduced into the device to perform on-line incubation, ALP-labeled CA 125 and HRP-labeled CA 153 immunocomplexes were formed in channel I, and ALP-labeled CA 199 and HRP-labeled CEA immunocomplexes were formed in channel II. Following wash with wash buffer, ALP CL substrate zone was injected into the two channels to trigger the CL reaction. With the aid of the optical shutter, the CL signals from the two channels were sequentially collected to detect the levels of CA 125 and CA 199 in the ALP substrate zone. After a zone of wash buffer was introduced for separating two substrate zones, the HRP substrate zone was introduced to detect the levels of CA 153 and CEA with the same protocol. Afterward, the antibodies immobilized membranes were regenerated by the introduction of regeneration buffer and wash buffer into the flow cell for two cycles. At a time of 37 min, the immunoassay system was ready for the next assay cycle.

RESULTS AND DISCUSSION

Design of Multiplex Detection Protocol. The restriction of available labels is a bottleneck for increasing the number of analytes in the substrate zone-resolved technique.²⁷ Therefore, a flow-through device with multiple detection channels composed of different capture antibodies was designed to improve the analyte throughput by combining with substrate zone resolution in each channel. In this work, the ALP and HRP labeled to antibodies were used for catalyzing the CL reactions of their corresponding substrates. The CL signals could respectively be detected by introducing the separate substrate zones into each channel. Thus, compared to the multichannel immunosensor with one universal label,^{12,13} this design doubled the number of analytes. To reduce the cost of analytical instrument or multiplex immunoassay, a PMT was used instead of a CCD or parallel detector array to perform a channel-resolved immunoassay by using an optical shutter to shadow other channels when the CL signal from one channel was collected. This work used two channels, channel I assembled with CA 153 and CA 125 antibodies and channel II assembled with CA 199 and CEA antibodies, to perform this design. Thus, the two-dimensional resolution could detect at least four analytes in a single run with a single PMT, which is commonly believed to be more sensitive than CCD and other silicon detectors, and advantageous to quantitation of biomarkers at low levels.

Optimization of Signal Collection Times. After the substrates were introduced, the enzyme-catalyzed CL reactions produced detectable CL emission in a static format. The emission intensities were the functions of reaction time. Therefore, it was necessary to optimize the times for CL signal collection. As seen in Figure 2, the kinetic curves of HRP- and ALP-catalyzed CL reactions showed different tendencies. The intensity of CL emission catalyzed by the HRP-labeled immunocomplex increased with increasing time and reached a plateau within 4 min. After 6 min, it decreased slowly. Therefore, the optimal CL signal could be collected at 4 min. The CL intensity catalyzed by the ALP-labeled immunocomplex increased continuously with increasing

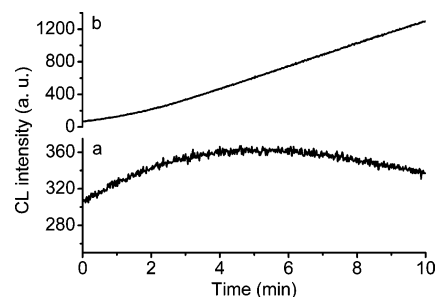


Figure 2. Kinetic curves of the CL reactions catalyzed by HRP (a) and ALP (b) labeled to CEA and CA 199 sandwich immunocomplexes formed on the polyethersulfone membrane at 50 ng/mL CEA and 50 U/mL CA 199 concentrations.

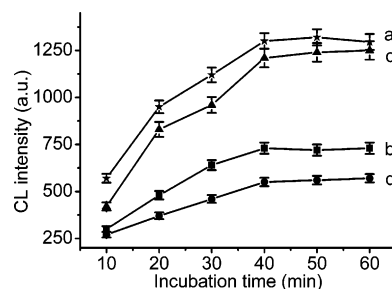


Figure 3. Effects of incubation time on CL intensities for 50 U/mL CA 125 (a), CA 199 (b), and CA 153 (c) and 50 ng/mL CEA (d), $n = 5$ for each point.

time, implying that the improved sensitivity could be obtained with a long CL reaction time. However, too long a time for CL signal collection was obviously disadvantageous to the improvement of assay speed. Considering the fact that the CL detection sensitivity at a reaction time of 4 min was enough for clinical application, it was also chosen as the optimal time for signal collection. The same collection time was also favorable to a simple control of the automated multiplex immunoassay. The signals observed at the moment zero resulted from the introduction process of CL substrates. Before the stop-flow and signal collection, the fronts of substrate zones contacted the immobilized enzymes, which triggered the enzymatic reactions to produce CL emission. On the other hand, the CL reaction of the luminol-*p*-iodophenol- H_2O_2 -HRP system was very fast at the initial stage, leading to a larger signal at the moment zero.

Choice of Incubation Time. The incubation process was performed on-line at room temperature. The successful development of the multiplex immunoassay system required that the common incubation time must be suitable for all analytes. With an increasing incubation time, all the CL intensities for 50 U/mL CA 125, CA 199, CA 153, and 50 ng/mL CEA increased quickly and reached their maximum values at 40 min (Figure 3), indicating the maximum formation of these sandwich immunocomplexes. The optimal incubation times for forming these sandwich immunocomplexes at room temperature were much shorter than those of 1–3 h at 37 °C for the conventional microwell plate ELISA approach. Incubation time generally depends on the kinetic characteristics of immunoreaction and mass transfer of immunoreagents. The use of the flow cell with a thickness of 0.4 mm produced a short diffusion distance for the immunoreagents and thus accelerated their mass transport and increased the immunoreaction rate.²⁹ At the incubation time of 20 min, the CL signals

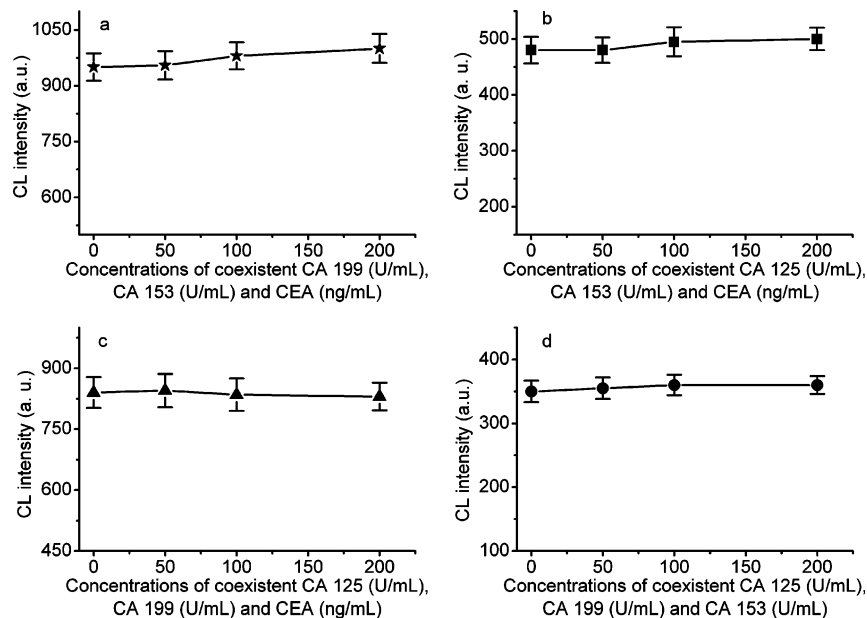


Figure 4. Cross-reactivity to (a) CA 125, (b) CA 199, (c) CA 153, and (d) CEA antibodies in the presence of 50 U/mL CA 125, CA 199, and CA 153 and 50 ng/mL CEA, respectively, $n = 5$ for each point.

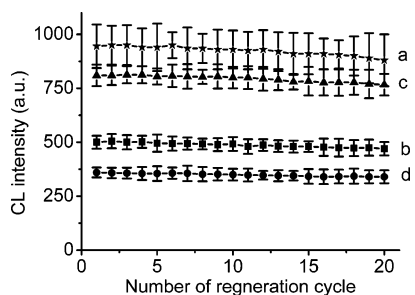


Figure 5. Plots of CL intensity for 50 U/mL CA 125 (a), CA 199 (b), and CA 153 (c) and 50 ng/mL CEA (d) in 20 regeneration cycles vs the cycle number. Data were obtained with five couples of antibodies immobilized membranes.

were 73, 66, 68, and 66% of the maximum values for CA 125, CA 199, CA 153, and CEA, respectively. Since the high sensitivity of CL detection provided enough low detection limits for clinical application, considering the analytical time and the throughput of this system, the incubation time of 20 min was used in the further study.

Cross-Reactivity. Cross-reactivity is a crucial analytical parameter regarding specificity and, thus, the reliability of multiplex immunoassay. The cross-reactivity among the four tumor markers and their noncognate antibodies was examined by comparing the CL signals at a definite concentration of specific analyte with increasing levels of other three coexistent analytes, respectively. The effects of the coexistent analytes on the signal for each tumor marker at a concentration of 50 U/mL or ng/mL are shown in Figure 4. Even when the concentrations of the interferences reached 200 U/mL or 200 ng/mL for each, the maximum changes of the CL signals for 50 U/mL CA 125, CA 199, and CA 153 and 50 ng/mL CEA did not go beyond 5.3, 4.2, 1.2, and 2.8%, respectively, indicating that the cross-reactivity between CA 125, CA 199, CA

153, and CEA antibodies and their noncognate antigens was negligible.

Furthermore, the HRP-labeled immunocomplexes were not found to affect the CL signal from the ALP CL substrate, and vice versa. These results indicated the performance of the multiplex immunoassay system for each specific analyte was independent of the presence or absence of the other analytes.

Regeneration of Antibodies Immobilized Membranes.

Disruption of the immunocomplexes, i.e., regeneration of the antibodies immobilized membranes, is very important for the further development of this strategy in low-cost application and automated continuous detection. Of course it is necessary to avoid loss of the activity of the immobilized antibodies or damage of the bonds between the antibodies and the support in the regeneration step.³⁰ Different dissociating reagents were tested using 50 U/mL CA 125, CA 199, and CA 153 and 50 ng/mL CEA for regeneration purposes, including organic solvent (CH₃OH–H₂O 1:1), buffer with low pH value (0.1 M glycine-HCl, pH 2.0) and diluted alkali (50 mM NaOH). The regeneration efficiencies (REs) were calculated according to the following equation.³¹

$$RE = [1 - (RT - B)/T] \times 100\% \quad (1)$$

Here, RT represents the CL signal obtained after the regeneration cycle, B is the signal for blank, and T is the signal before applying any regeneration step. The most efficient dissociating reagent was found to be 0.1 M glycine-HCl (pH 2.0), which allowed a fast and complete dissociation of the immunocomplexes with REs of 98.2, 98.0, 97.4, and 98.0% for CA 125, CA 199, CA 153, and CEA, respectively. As a mild regeneration reagent, glycine-HCl buffer showed good activity retention to the immobilized antibodies, and the antibodies immobilized polyethersulfone membranes could

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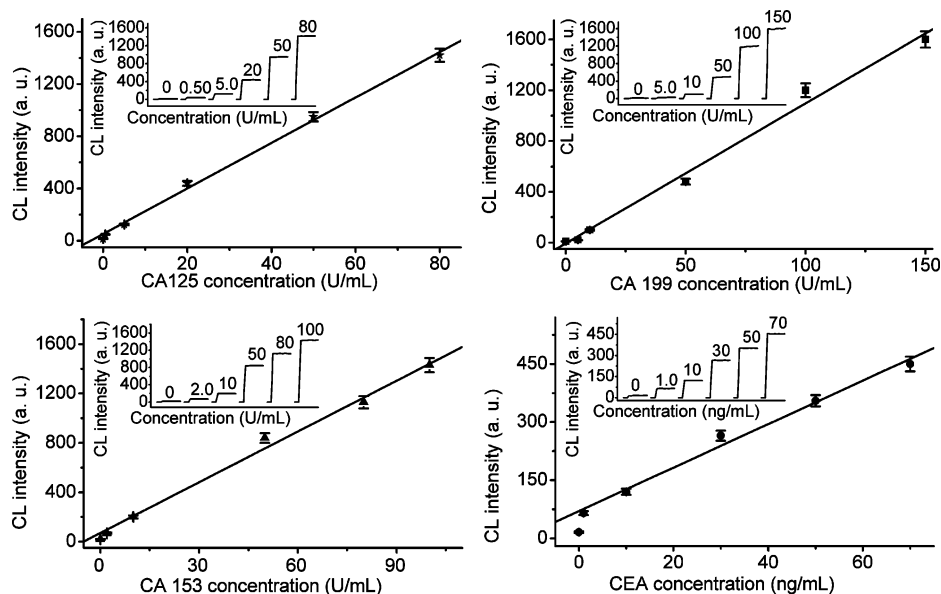


Figure 6. Calibration curves for CA 125, CA 199, CA 153, and CEA (five measurements for each point). Inset: dose–response curves.

Table 1. Assay Results of Clinical Serum Samples Using the Proposed and Reference Methods ($n = 5$)

sample no.	CA 125 (U/mL)			CA 199 (U/mL)		
	proposed method	reference method	relative error (%)	proposed method	reference method	relative error (%)
1	6.2 ± 0.3	6.7 ± 0.3	−7.5	209.1 ± 9.8	199.1 ± 5.6	5.0
2	98.5 ± 3.4	106.7 ± 3.2	−7.7	19.9 ± 1.0	21.2 ± 0.8	−6.1
3	16.4 ± 0.7	16.9 ± 0.7	−3.0	66.8 ± 4.0	65.5 ± 2.0	2.0
4	37.5 ± 2.7	33.9 ± 1.7	10.6	85.4 ± 3.3	84.3 ± 3.0	1.3

sample no.	CA 153 (U/mL)			CEA (ng/mL)		
	proposed method	reference method	relative error (%)	proposed method	reference method	relative error (%)
1	14.1 ± 0.7	15.0 ± 0.6	−6.0	237.7 ± 10.4	220.1 ± 7.1	8.0
2	15.0 ± 0.8	16.3 ± 0.5	−8.0	5.2 ± 0.3	4.8 ± 0.2	8.3
3	8.4 ± 0.5	8.1 ± 0.4	3.7	31.2 ± 1.8	33.7 ± 1.2	−7.4
4	27.9 ± 1.2	24.6 ± 1.0	13.4	4.8 ± 0.3	4.7 ± 0.4	2.1

be used for more than 20 cycles with an acceptable reproducibility (Figure 5). After that, the CL signals showed observable decrease.

Analytical Performance. Figure 6 shows the dose–response and calibration curves for these analytes. The linear regression equations for CA 125, CA 199, CA 153, and CEA were $I = 17.4$ [CA 125] (U/mL) + 53.7 ($R = 0.999$), $I = 11.0$ [CA 199] (U/mL) − 1.1 ($R = 0.995$), $I = 13.8$ [CA 153] (U/mL) + 69.3 ($R = 0.997$), and $I = 5.6$ [CEA] (ng/mL) + 70.3 ($R = 0.995$) over the concentration ranges of 0.50–80, 5.0–150, and 2.0–100 U/mL and 1.0–70 ng/mL, respectively, where I was the CL intensity. The limits of detection at a signal-to-noise ratio of 3 (3σ) for the four tumor markers were 0.15, 2.0, and 0.80 U/mL and 0.65 ng/mL, respectively. The cutoff values of the four tumor markers in clinical diagnosis are 35, 35, and 35 U/mL and 5 ng/mL, respectively.²⁸ Therefore, the sensitivity and linear ranges of the described system were enough to practical application.

A mixed sample composed of 50 U/mL CA 125, CA 199, and CA 153 and 50 ng/mL CEA was used to assess the reproducibility of the immunoassay system by intra- and interassay coefficients of variation (CVs). The intra-assay CV was the difference among five determinations of the sample on the same detection device.

The interassay CV was the difference among the measurements of the sample on five detection devices prepared in batch. The intra- and interassay CVs obtained were 3.6 and 9.6% for CA 125, 4.7 and 6.4% for CA 199, 4.5 and 7.0% for CA153, and 4.7 and 8.6% for CEA, respectively, which showed acceptable detection and fabrication reproducibility of the proposed immunoassay system.

The total assay process including incubation, wash, detection, and regeneration of antibodies immobilized membranes could be completed in 37 min, which was much shorter than those of more than 1 h to overnight with other methods.^{1,18,19,21,22} When the device was not in use, it was filled with storing buffer and stored at 4 °C. No obvious change was observed after storage of more than 20 days.

Method Comparison. To assess the analytical reliability and application potential of the developed two-dimensional resolution system for a multiplex immunoassay, it was compared with the commercially proven ECLIA method; the latter was carried out with parallel single-analyte test as a reference. Four clinical serum samples drawn using the standard venipuncture technique followed with centrifugation from the blood cells were assayed for this purpose. When the levels of tumor markers were over the

calibration ranges, serum samples were appropriately diluted with sample diluent prior to assay. The results are shown in Table 1, which showed an acceptable agreement with relative errors less than 13.4%.

CONCLUSION

A two-dimensional resolution system including channel- and substrate zone-resolved techniques has been designed for a flow-through CL multiplex immunoassay. This system combines multilabel mode with spatial resolution; thus, the number of detectable analytes can be doubly increased when two substrate zones are used for channel resolution. It can detect at least four analytes in a single run with a single PMT. The multiplex immunoassay procedure is rapid, simple, and convenient and can be programmed. The whole assay system is composed of low-cost components, such as peristaltic pump, PMT, multiposition valve, and optical shutter, and the antibodies immobilized membranes are reusable with a regeneration step; thus, the proposed technology can be low-cost. The immunoassay results for CA 125, CA 199, CA 153, and CEA show acceptable accuracy, detection

and fabrication reproducibility, and storage stability. The sensitivity and linear ranges are also sufficient for practical application. The proposed system and method are especially suitable for detection of a tumor marker panel for diagnosis and screening purpose.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Supporting Information

Channel and Substrate Zone Two-Dimensional Resolution for Chemiluminescent Multiplex Immunoassay

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Table S1. Details of Flow-Through Multiplex Immunoassay Protocol for Tumor Markers Panel Based on Channel and Substrate Zone Two-Dimensional Resolution

step No.	valve position	event	starting time (min:second)
1	S	introduce mixture of sample (20 μ L) and tracer antibodies for CA 125, CA 153, CA 199 and CEA (5 μ L for each) into each channel of the flow cell	00:00
2		stop flow and incubate at RT	00:30
3	WB	wash the flow cell with wash buffer at a flow rate of 1.0 mL/min	20:30
4	S1	introduce ALP substrate into the flow cell (40 μ L per channel) and stop flow to trigger the ALP catalyzed CL reaction	22:00
5		collect the CL signal from channel I for CA 125 detection while shadow channel II with the optical shutter	26:30
6		collect the CL signal from channel II for CA 199 detection while shadow channel I with the optical shutter	26:40
7	WB	wash the flow cell with wash buffer at a flow rate of 1.0 mL/min	26:50
8	S2	introduce HRP substrate into the flow cell (40 μ L per channel) and stop flow to trigger the HRP catalyzed CL reaction	27:20
9		collect the CL signal from channel I for CA 153 detection while shadow channel II with the optical shutter	31:50
10		collect the CL signal from channel II for CEA detection while shadow channel I with the optical shutter	32:00
11	RB	introduce regeneration buffer to regenerate the device at a flow rate of 1.0 mL/min	32:10
12	WB	introduce wash buffer to recondition the channels at a flow rate of 0.5 mL/min	34:00
13	RB	regenerate the device for the 2nd cycle	34:30
14	WB	recondition the channels for the 2nd cycle	36:30
15	S	ready for the next assay cycle	37:00