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Flow-Through Multianalyte Chemiluminescent Immunosensing System with Designed Substrate Zone-Resolved Technique for Sequential Detection of Tumor Markers

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A novel flow-through immunosensing system for performing a multianalyte chemiluminescent determination in a single run was designed. A new analytical strategy of substrate zone-resolved technique was proposed. Using carcinoma antigen 125 (CA 125) and carcinoembryonic antigen (CEA) as model analytes, the capture antibodies for CA 125 and CEA were immobilized on an UltraBind aldehyde-actived membrane to act as an immunoreactor, to which the mixture of CA 125, CEA, and their corresponding tracers, horseradish peroxidase (HRP)-labeled anti-CA 125 and alkaline phosphatase (ALP)-labeled anti-CEA, was introduced for on-line incubation. The substrates for HRP and ALP were then delivered into the detection cell sequentially to perform substrate zone-resolved immunoassay by a sandwich format. Under optimal conditions, CA 125 and CEA could be assayed in the ranges of 5.0−100 units/mL and 1.0−120 ng/mL, respectively. The whole assay process including incubation, wash, detection, and regeneration could be completed in 35 min. The serum samples from the clinic were assayed with the proposed method, and the results were in acceptable agreement with the reference values. This method and the strategy of substrate zone-resolved technique could be further developed for high-throughput multianalyte immunoassay.

Immunoassay, as a promising approach for selective and sensitive analysis, has recently gained increasing attention in different fields including environmental monitoring, food safety, and clinical diagnosis. Multianalyte immunoassay in a single run is often necessary to monitor or quantitate several components in a complex system for diagnostic purposes. Compared with parallel single-analyte immunoassay methods, a multianalyte immunoassay offers some remarkable advantages, such as high sample throughput, improved assay efficiency, low sample consumption, and reduced overall cost per assay.[1] Thus, considerable effort has been paid to the development of a multianalyte assay. The developed multianalyte immunoassay can be classified into two main modes. The first mode is based on spatially separated immunoreaction areas, i.e., array immunoassay, coupled with electrochemical,[2,3] fluorescent,[4,5] and chemiluminescent[6] determination. This mode includes antibody arrays for achieving quantitative protein profiling in a multiplex format.[7] Similarly, two arrays of flow-through multichannel immunosensors have also been proposed for multianalyte detection.[8,9] Although this mode can screen large numbers of analytes, accurate quantitative data in these arrays are usually limited or difficult to obtain.[10] The array immunoassay system often needs an expensive array detector such as a charge-coupled device (CCD) camera for optical detection[4−7] and multichannel potentiostat for electrochemical measurement.[3,10]

The second mode is performed using antibodies or antigens with different labels (one per analyte), including radioisotopes,[11,12] enzymes,[13,14] fluorescence compounds,[15−17] and metal compounds.[18−20] Usually, the detectors used in this mode include radiometric,

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fluorometric, spectrophotometric, electrochemical, and inductively coupled plasma mass spectrometric (ICPMS). Among these detectors the optical measurements commonly use different wavelengths[13,15,16] or decay times[17] to distinguish the signal of one label from the others. Such multicolor immunoassays are often limited by signal overlapping of different labels due to the broad absorption band[13] or complicated by the requirement of an elaborate excitation and detection scheme and by the broad emission bands.[15,18] Electrochemical immunoassay has been extensively developed for multianalyte detection using different electrochemical active labels such as metal ions,[21] complexes,[19] and inorganic nanocrystal tracers.[18] The distinct manipulation potentials of these labels reflect the identity of every component. With the similar principle, the multianalyte immunoassay based on different metal ions with ICPMS detection has also been proposed.[20] It has been noted that the different labels often need markedly different optimal assay conditions, and traditionally, simple combination of multiple labels often leads to loss of assay performance.[1]

Besides the two dominant modes, a neural network based on one label using cross-reactive antibodies has been proposed for multianalyte immunoassay.[22,23] However, this method shows great relative error up to 670%[22] and poor limit of detection.[23] Another method coupled with the separation techniques such as capillary electrophoresis[24,25] and liquid chromatography[26] can be used for multianalyte immunoassay. Obviously this strategy makes the detection system more complicated and often encounters the adsorption of immunoreagents on the inner wall of the capillary and chromatographic column. Here one new strategy, substrate zone-resolved technique, is constructed. Coupled with a chemiluminescent (CL) immunoassay, this proposed method shows a series of advantages such as simple manipulation, high sensitivity, good accuracy, ideal selectivity, low-cost detector, and ease to achieve automation and high sample throughput.

CL immunoassay, combining good specificity of immunoreaction with high sensitivity of the CL assay, has proved to be a powerful analytical tool.[6,9,27−30] The array CL immunoassay with a sensitive CCD camera for screening antibiotics in milk[6] and CL multichannel immunosensors in combination with three fused-silica capillaries for biodetection[9] have been developed. However, the later needs three devices combined to a detector array for parallel detection of three different analytes. Different from the spectrophotometric and fluorometric approach, CL intensity is the only considered factor in a CL assay, while wavelength is not considered; thus, it is very difficult to distinguish CL signals of different labels. To solve this problem, this work designed a substrate zone-resolved technique to develop a novel flow-through multianalyte immunosensing system. Using two tumor markers, carcinoma antigen 125 (CA 125) and carcinoembryonic antigen (CEA), as model analytes, this system was demonstrated to be promising.

CA 125 is a surface antigen associated with epithelial ovarian cancer. CEA is a cell surface glycoprotein related to lung, liver, pancreas, breast, cervix, and prostate cancer.[31,32] It also occurs in the serum of epithelial ovarian carcinoma patients.[33] The simultaneous quantitation of the two tumor markers is of great significance in clinical tumor diagnosis and evaluating curative effect. This work constructed a CA 125 and CEA antibody-modified membrane as a reusable immunoreactor to capture CA 125 and CEA for further binding of horseradish peroxidase (HRP)-labeled CA 125 and alkaline phosphatase (ALP)-labeled CEA antibodies. Following the sequential introduction of the substrates for HRP and ALP, the CA 125 and CEA levels were detected in a single run. The proposed method possesses attractive characteristics and will be suitable for fast detection of multianalyte and mass production.

EXPERIMENTAL SECTION

Apparatus and Device. The flow device was fabricated with a modified method reported previously.[34] Its schematic diagram is shown in Figure 1a. A Teflon cover (4.0 cm × 2.5 cm × 0.8 cm) with inlet and outlet, a silicon rubber spacer (1.0-mm thickness), and a transparent plexiglass slice 0.5 cm thick were used to produce a flow cell of 50 μL (2.5 cm × 0.5 cm × 0.04 cm). An antibody immobilized membrane used as immunoreactor was immobilized on the center area of the inner side of the Teflon cover. The flow-through analysis system was constructed as illustrated in Figure 1b. Teflon tubes (0.8-mm i.d.) were used to connect all the components in the flow system. All solutions were delivered with a multichannel peristaltic pump of the IFFM-D luminescence analyzer (Remax). A multiposition valve with five inlets and one outlet was used to switch different solutions into the flow system sequentially. The flow device was positioned in front of the photomultiplier of the luminescence analyzer. The CL intensity was measured with the photomultiplier biased at −800 V, which was sensitive to photons with a wavelength range of 200−800 nm. Instrument control and data record were performed via IFFM software package.

The reference electrochemiluminescence immunoassay was performed with a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH). Scanning electron micrograph (SEM) of antibody immobilized membrane was obtained with a LEO-1530 scanning electron microscope (LEO Electron Microscopy Inc.) at an acceleration voltage of 5 kV. A VG ESCALAB MK2 spectrometer working in an ultrahigh vacuum (5×10^{-8} mbar)

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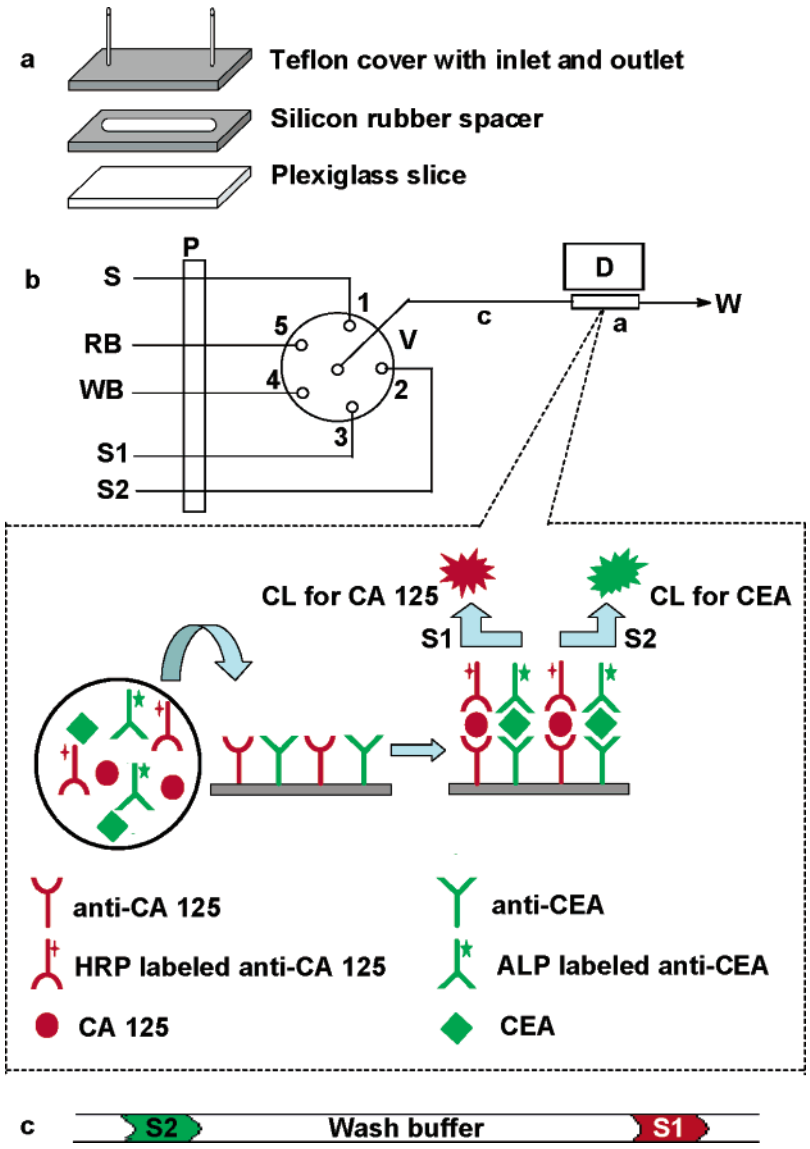

Figure 1. Scheme of the flow-through multianalyte immunosensing system based on substrate zone-resolved technique: (a) flow device; (b) flow-through analysis system and reactions occurred in the flow cell; (c) substrate zones in the flow path. (S) Sample, (RB) regeneration buffer, (WB) wash buffer, (S1) HRP substrate, (S2) ALP substrate, (V) multiposition valve, (P) peristaltic pump, (D) detector, and (W) waste.

with an Mg Kα X-ray source was employed for the X-ray photoelectron spectroscopy (XPS) analysis of antibody immobilized membrane.

Reagents. Mouse monoclonal anti-CA 125 and anti-CEA used as capture antibodies were purchased from Lab Vision & Neomarkers Corp. and Xiamen Boson Biotechnology Co. Ltd., respectively. CA 125 and HRP-labeled mouse monoclonal anti-CA 125 were provided by CanAg Diagnostics AB. CEA and ALP-labeled mouse monoclonal anti-CEA were obtained from Xiamen Boson Biotechnology Co. Ltd. The HRP and ALP labeled to antibodies were reported by their manufacturers to retain about 72 (180 units/mg) and 76% (1900 units/mg) of their original activities, respectively. HRP substrate based on the luminol−*p*-iodophenol−H_2O_2 CL system was supplied by Autobio Diagnostics Co. Ltd. ALP substrate based on the disodium 3-(4-methoxyspiro-(1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl) phenyl phosphate CL system was obtained from Xiamen Boson Biotechnology Co. Ltd. Electrochemiluminescence immunoassay reagent kits for CA 125 and CEA were purchased from Roche Diagnostics GmbH. UltraBind aldehyde-actived membrane was obtained from Pall Gelman Science Corp. The serum samples were provided by Jiangsu Institute of Cancer Prevention and Cure. All other reagents were of the best grade available and used as received.

Buffers. Doubly distilled water was used to prepare all buffers. Coupling buffer for antibody immobilization was 0.01 M pH 7.4 phosphate buffer solution (PBS). Blocking buffer for blocking the residual reactive sites on the antibody immobilized membrane was PBS containing 0.5% bovine serum albumin and 0.5% casein. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into 0.01 M pH 7.4 PBS as wash buffer (PBST). Regeneration buffer was 0.1 M glycine-HCl at pH 2.0.

Immunosensor Preparation. A 20-μL sample of mixture solution of anti-CA 125 and anti-CEA (50 μg/mL each in coupling buffer) was dropped on a piece of UltraBind aldehyde-actived membrane (2.5 cm × 0.5 cm), reacted at room temperature (25 °C) for 30 min, and followed in refrigerator at 4 °C overnight. The membrane was then blocked with blocking buffer for 12 h and treated with 1 mg/mL $NaCNBH_3$ solution for 40 min at 4 °C.

Table 1. Details of Flow-Through Analysis Protocol for Multianalyte Immunoassay

step no.	valve position	step	starting time (min)
1	1	introduce mixture of 25 μL of sample, 12.5 μL of CA 125 tracer and 12.5 μL of CEA trace into the flow cell	00:00
2	1	stop flow and incubate at room temperature	00:30
3	4	wash the flow cell with PBST at a flow rate of 1.0 mL/min	20:30
4	3	introduce HRP substrate into the flow cell and stop flow to collect data	22:00
5	4	wash the flow cell with PBST at a flow rate of 1.0 mL/min	26:30
6	2	introduce ALP substrate into the flow cell and stop flow to collect data	27:00
7	5	introduce regeneration buffer to regenerate the immunoreactor at a flow rate of 0.5 mL/min	31:30
8	4	introduce PBST to recondition the immunoreactor at a flow rate of 0.5 mL/min	33:00
9	5	regenerate the immunoreactor for the second cycle	33:30
10	4	recondition the immunoreactor for the second cycle	34:30
11	1	ready for the next assay cycle	35:00

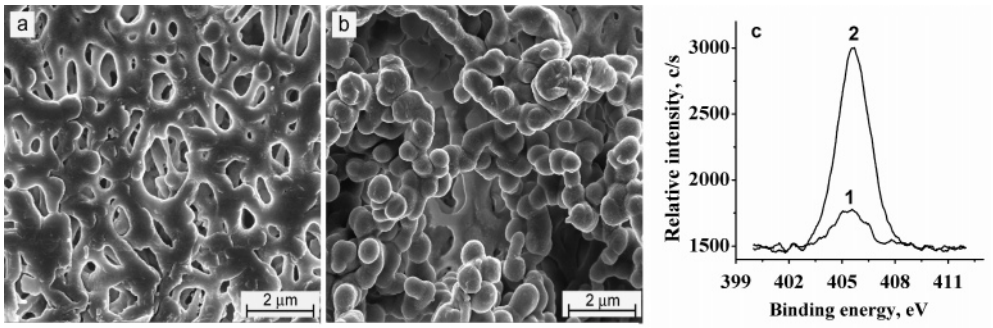

Figure 2. SEM and N(1s) XPS spectra of UltraBind aldehyde-actived (a, curve 1 in c) and antibody immobilized (b, curve 2 in c) membranes.

The modified membrane was washed with PBS, dried in air, and fixed on the center area of the inner side of the Teflon cover with double-side glue tape. The total thickness of the double-side glue tape and the membrane was less than 0.6 mm.

Immunoassay Protocol. A description of the events in the assay process is given in Table 1. The mixture of sample, enzyme-labeled CA 125, and CEA antibodies was first delivered into the flow cell and incubated at room temperature for 20 min. PBST was then delivered into the system to wash the immunoreactor. Following step 3 finished at a time of 22 min, HRP and ALP substrates were introduced into the cell with a separation zone of PBST to distinguish two substrate zones (Figure 1c). The CL signals corresponding to the two components were detected, respectively. The zone separation of the two substrates eliminated the incompatibility of the assay conditions for the two CL systems, for example, optimal pHs of 8.5 and 9.5 for substrates of HRP and ALP, respectively. The whole procedure from sample injection to signal collection for two components could be finished in 31.5 min. Afterward, regeneration buffer and PBST passed through the cell respectively for two cycles to regenerate the immunosensor. At 35 min, the regenerated immunosensor was ready for the next assay cycle.

Switching the flow cell to different flow paths to introduce each reagent was performed with the aid of a multiposition valve. All manipulations were performed automatically by personal computer equipped with IFFM software package.

Safety Considerations. Handle all patient specimen and immunoreagents as potentially infectious. All manipulation must be performed carefully, and all tools in contact with patient specimen and immunoreagents must be disinfected after use.

RESULTS AND DISCUSSION

Characterization of Immunoreactor. The UltraBind membrane used for antibody immobilization was an aldehyde-actived polyethersulfone material. Many uniform distribution pores could be observed on its SEM (Figure 2a). This uniform open structure provided a significant increase of the binding capacity. Thus, the surface and these pores had already shown good binding capacity to protein.[35] The binding site could react with the amino group of the antibody to form a reversible Schiff base, which was then reduced by a reductive alkylation process using $NaCNBH_3$ to obtain a stable antibody immobilized membrane. This membrane showed obviously different surface morphology from the aldehyde-actived membrane. The aggregates of the bound antibodies were distributed regularly and showed an incompact islandlike porous structure (Figure 2b), which facilitated the approach of immunoreagents to the immobilized antibodies and access of the substrates to their corresponding enzymes and resulted in fast immunoreaction and sensitive CL response.

After the antibody immobilized membrane was ultrasonically cleaned and thoroughly rinsed, it was characterized with XPS. As seen in Figure 2c, a remarkable N(1s) peak was observed (curve 2), which was 5.8 times larger than that of the naked membrane, and resulted from the trace amount of nitrogen (curve 1), indicating the presence of a large amount of nitrogen. This

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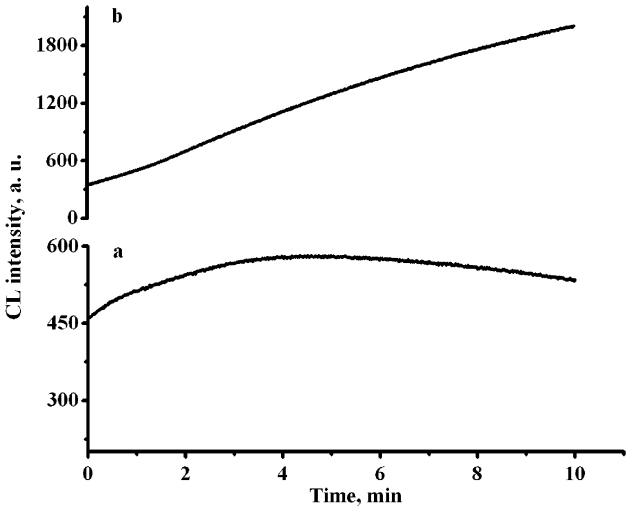

Figure 3. Kinetic curves of CL reactions catalyzed by HRP (a) and ALP (b) labeled to CA 125 and CEA sandwich complexes at 50 units/mL CA 125 and 50 ng/mL CEA concentrations.

confirmed that antibody molecules were covalently bound to the membrane.

Kinetic Characteristics of CL Reaction. The kinetic behaviors of the two CL reactions catalyzed by the two enzymes labeled to the sandwich complexes of CA 125 and CEA were studied with a static method. Figure 3 shows the typical kinetic curves. Both CL reactions occurred immediately after injection of the substrates. The intensity of CL from HRP-labeled CA 125 complex increased with time and reached a maximum value within 4 min (Figure 3a). The CL intensity slowly decreased after 4 min due to the inhibition of HRP activity by H_2O_2 after a long time exposure.[36,37] Therefore, CL measurement was performed at 4 min. The CL reaction of ALP substrate (Figure 3b) showed some difference from that catalyzed by HRP labeled to the CA 125 complex; its intensity increased with time in the whole measurement period, indicating that improved sensitivity could be obtained with longer measurement time. Considering the analytical time, sensitivity, and convenience of the multianalyte immunoassay, 4 min was selected again for CEA immunoassay.

Incubation Time. The incubation time is a bottleneck to the improvement of assay speed in an immunoassay, which is usually controlled by mass transport of immunoreagents and kinetics of immunoreaction. The incompact porous structure of the immunoreactor and the 0.04-cm thickness of the flow cell comparable with the diffusion boundary layer were beneficial to the mass transport of the immunoreagents. The immunoreagents in bulk could easily diffuse to the immunoreactor surface and access the bound antibodies; thus, the rate of immunoreaction increased. The incubation process for the membrane immunoassay needed shorter time compared with 1−3 h at 37 °C for the traditional multiwell plate approach. The effect of incubation time on the immunoreaction was examined by mixing 25 μL of sample containing 50 units/mL CA 125 and 50 ng/mL CEA with their enzyme tracers and carrying the mixture into the flow cell to react

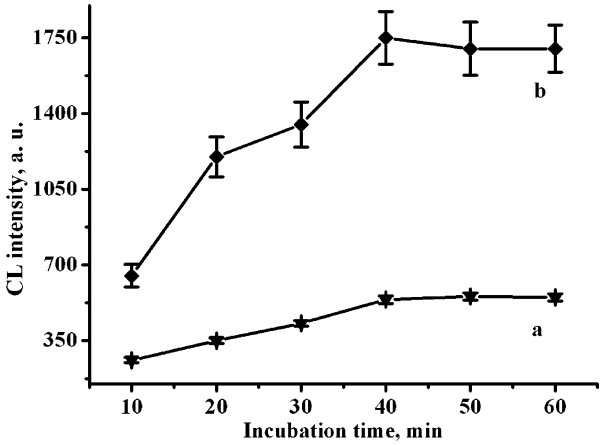

Figure 4. Effects of incubation time on CL intensity at 50 units/mL CA 125 (a) and 50 ng/mL CEA (b) concentrations, where $n = 5$ for each point.

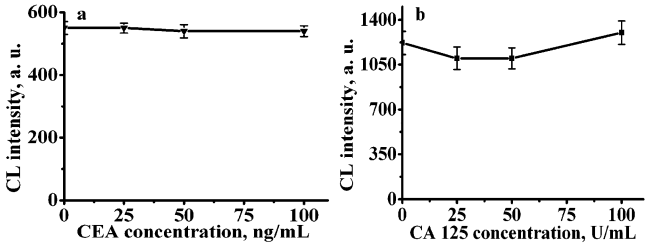

Figure 5. Cross reactivity of CEA to anti-CA 125 in the presence of 50 units/mL CA 125 (a) and CA 125 to anti-CEA in the presence of 50 ng/mL CEA (b), where $n = 5$ for each point.

with the fixed immunoreactor at room temperature for different time intervals. With an increasing incubation time, both CL signals of the two components increased and trended to their maximum values after an incubation time of 40 min (Figure 4), indicating the maximum formation of these sandwich complexes. Although the CL signals at 20 min were 65 and 69% of the maximum values for CA 125 and CEA, respectively, the high sensitivity of CL detection provided detection limits low enough for clinical diagnosis. Considering the optimal analytical performance and the further development of this method to high sample throughput, 20 min of incubation time was used in the further study.

Evaluation of Cross Reactivity. To examine the cross reactivity between CEA and anti-CA 125 or CA 125 and anti-CEA, the mixture of 50 units/mL CA 125 and HRP-labeled anti-CA 125 or 50 ng/mL CEA and ALP-labeled anti-CEA was spiked with variable amounts of CEA or CA 125 and carried into the immunosensor to incubate for 20 min, respectively. The CL intensity obtained for every concentration of CEA or CA 125 is shown in Figure 5. The presence of 50 ng/mL CEA in 50 units/mL CA 125 did not change the CL signal from HRP substrate. When the concentration of CEA was 100 ng/mL, the CL signal decreased by only 1.4%, indicative of negligible cross-reaction between CEA and anti-CA 125. Similarly, the presence of variable amounts of CA 125 in 50 ng/mL CEA led to a change of less than 8.0% of the CL signal from ALP substrate. The change was close to the coefficient of variation (CV) of 7.5% for CEA intra-assay (described in the following Reproducibility and Stability of the Immunosensing System section). Thus, the cross-reaction between CA 125 and anti-CEA was also negligible.

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Table 2. Regeneration Efficiency of Different Dissociation Reagents (n = 5, %)

	glycine-HCl (0.1 M, pH 2.0)	acetonitrile (30%)	NaOH (50 mM)	NaCl (1 M)
CA 125	97.4 ± 1.6	92.1 ± 2.0	50.4 ± 3.1	41.6 ± 1.3
CEA	96.4 ± 1.8	92.8 ± 2.1	34.1 ± 2.5	44.0 ± 1.5

Furthermore, the HRP-labeled CA 125 complex was not found to affect the CL signal from the ALP substrate, and vice versa. These results suggested that CA 125 and CEA could be assayed in a single run using the described system without interference to each other.

Regeneration of Immunoreactor. The immunosensor could be regenerated for reuse by a simple and short-period regeneration step with the regeneration buffer, which is very important for the further development of this strategy in low-cost application and automation multianalyte immunoassay. Of course, the regeneration step must avoid affecting the activity of immobilized proteins or damaging the bonds between the proteins and support surface when the dissociation of the bound immunocomplex occurs.[38] Using 50 units/mL CA 125, 50 ng/mL CEA, and enzyme tracers to measure the CL signals from HRP and ALP substrates, this work tested different dissociation reagents for the regeneration of immunosensor, including salt solution of high concentration, organic solvent, buffer with low pH value, and diluted alkali solution. The regeneration efficiencies, calculated according to the method reported by Yakovleva et al.,[39] are shown in Table 2. With two regeneration cycles in 3 min, 0.1 M glycine-HCl (pH 2.0) showed the best regeneration efficiency at more than 96%, which was chosen as the regeneration buffer for the regeneration of the immunosensor. With the regeneration procedure, this immunoreactor could be used for 20 cycles with an acceptable reproducibility (described in following Reproducibility and Stability of the Immunosensing System section).

Dose−Response Curves. Under the selected conditions, the substrate zone-resolved CL intensity for both CA 125 and CEA increased with increasing concentration of analytes (Figure 6). The dose−response curves showed the linear ranges from 5.0 to 100 units/mL with a correlation coefficient of 0.9954 for CA 125 and 1.0 to 120 ng/mL with a correlation coefficient of 0.9995 for CEA. The linear range for CA 125 was much wider than that up to 14 units/mL reported for single-analyte electrochemical immunosensor.[40] The other for CEA was also much wider than that up to 25 ng/mL reported for single-analyte CL immunoassay.[41] When the concentrations of analytes were over the linear ranges, an appropriate dilution of sample could extend the detectable concentration ranges.

The limits of detection for CA 125 and CEA were 1.5 units/mL and 0.6 ng/mL at a signal/noise ratio of 3, respectively. The former was close to that reported previously,[40] but the latter was lower than that of 1.2 ng/mL with a linear range of 25−150 ng/mL reported for CEA in a simultaneous two-analyte electrochemical immunoassay system,[10] showing better sensitivity. The cutoff values of the two tumor markers in clinical diagnostic are 35 units/mL and 5 ng/mL, respectively.[31] Therefore, the sensitivity of the described system was enough to practical application.

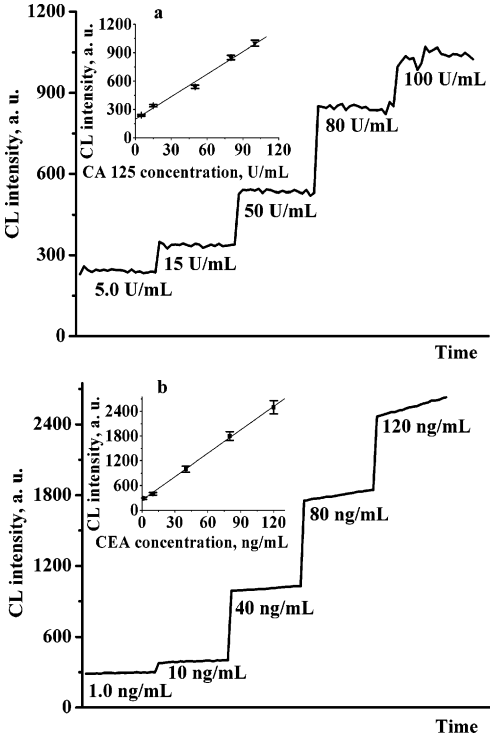

Figure 6. Dose−response curves for substrate zone-resolved immunoassay of CA 125 (a) and CEA (b), where n = 5 for each point. The values indicated below the curves correspond to the total concentrations.

Table 3. Assay Results of Clinical Sera Using the Proposed and Reference Methods (n = 5)

	CA 125 concentration (units/mL)		CEA concentration (ng/mL)	
sample	proposed method	reference method	proposed method	reference method
1	22.5 ± 0.6	24.9 ± 0.4	3.0 ± 0.2	3.3 ± 0.1
2	38.6 ± 1.5	40.3 ± 2.0	3.6 ± 0.3	3.8 ± 0.2
3	100.2 ± 4.4	122.2 ± 3.7	13.1 ± 0.7	12.8 ± 0.5
4	21.9 ± 0.7	20.1 ± 0.6	10.8 ± 0.7	11.6 ± 0.5
5	80.8 ± 2.3	86.9 ± 2.6	14.7 ± 1.2	15.3 ± 0.4

Reproducibility and Stability of the Immunosensing System. The reproducibility of the substrate zone-resolved multianalyte immunosensing system was assessed by intra- and interassay CVs. The intra-assay CV was the difference between five determinations of one sample on the same immunoreactor with regeneration procedure between each measurement. The interassay CV was the difference between the measurements of the same sample on five immunoreactors prepared in batch. The 50 units/mL CA 125 and 50 ng/mL CEA were used in the reproducibility evaluation. The intra- and interassay CVs obtained were 3.2 and 8.2% for CA 125 and 7.5 and 11.0% for CEA, respectively. These results showed acceptable detection and fabrication reproducibility of this strategy.

When the immunosensor was not in use, it was filled with PBS containing 0.1% sodium azide and stored at 4 °C. No obvious change was observed after storing for at least 10 days.

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Detection of CA 125 and CEA in Clinical Serum Samples. Five clinical sera were assayed using the proposed substrate zone-resolved multianalyte immunoassay method as well as the reference electrochemiluminescence method; the latter was carried out with parallel single-analyte test by Jiangsu Institute of Cancer Prevention and Cure. The results were shown in Table 3, the agreement between the two methods was acceptable.

CONCLUSION

A strategy of the substrate zone-resolved multianalyte immunoassay has been designed. The proposed system is simple, convenient, fast, sensitive, low cost, reusable, and automated for high sample throughput. The substrate zone-resolved multianalyte analytical technique solves two key problems: one is to obtain distinguishable CL signals without consideration of wavelength and the other is to provide each CL reaction catalyzed by the label in its optimal assay condition without loss of assay performance.

To achieve high sample throughput and automated test, short analytical time and simple testing procedure are necessary. The proposed method can control all steps by automatic programmed manipulations, and the total assay process including incubation, detection, and regeneration can be completed in 35 min, which is much shorter than those of 1 h to more than 10 h with other methods.[3,13,15,18] The short test time makes it possible to perform high sample throughput. Our further work is to prepare the immunoreactors with more analytes and combine more than one immunosensor in parallel by a multioutlet valve for higher throughput detection.

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