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Automated Support-Resolution Strategy for a One-Way Chemiluminescent Multiplex Immunoassay

Zhanjun Yang,[†] Hong Liu,[†] Chen Zong,[†] Feng Yan,*[,‡] and Huangxian Ju*[,†]

Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing 210093, and Jiangsu Institute of Cancer Research, Nanjing 210009, P.R. China

An automated support-resolution strategy was designed to couple with a flow-through immunosensing system for performing a one-way chemiluminescent (CL) multiplex immunoassay. Different from multilabel and multichannel-based detection techniques, this immunoassay method employed a single horseradish peroxidase (HRP) label in one way. With the use of carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) as model analytes, the capture antibodies for CEA and AFP were immobilized on the inner wall of a glass tube and the surface of paramagnetic microspheres (PMs), respectively. The on-line incubation could be performed in the glass tube after introducing the mixture of CEA, HRP-labeled anti-CEA antibody, AFP, anti-AFP immobilized PMs, and HRP-labeled anti-AFP antibody. With the use of a wash step, the formed sandwich immunocomplexes were separated automatically and the immunocomplex immobilized PMs were captured in another unmodified glass tube with a magnet. The CL signals from the two glass tubes were near-simultaneously collected with the aid of an optical shutter to perform quantitative detection. CEA and AFP could be rapidly assayed in the ranges of 1.0−60 and 1.0−80 ng/mL within 27 min. The assay results of clinical serum samples with the proposed method were in an acceptable agreement with the reference values. This system provides a promising multiplex immunoassay approach for clinical applications.

Recently, the multiplex immunoassay has attracted considerable interest in clinical, environmental, and biodefense applications due to the growing demand for quantitative detection of multiple markers and the unique advantages of this technique.[1] Great effort has mainly been focused on developing spatial resolution and multilabel modes for multiplex immunoassay. The spatial resolution mode uses a universal label for fluorescent,[2−5] chemiluminescent (CL),[6−8] spectrophotometric,[9,10] electrochemical,[11,12] and piezoelectric[13] detection of antigen or antibody array. Commonly, these methods require expensive array detectors for spatial resolution of different immunoreaction areas, such as a charge-coupled device (CCD) camera or multichannel electrochemical workstation. The CCD camera used in optical detection needs to be cooled for lowering the thermal background, and the electrochemical sensor array often suffers from cross-talk potentially occurring due to the diffusion of electroactive product generated at one electrode to a neighboring electrode.[11,14] Although some nonarray detector-based immunoassay methods have been reported for spatially-resolved multiplex assays by moving the affinity microcolumn composed of discrete segments of beads bearing distinct receptors[15] or multiple-band disposable optical capillary immunosensors[16] with motorized translational stages, they make the detection devices more complicated. The multilabel mode typically employs more than one label, such as several enzymes,[17,18] fluorescent dyes,[19] metal ion labels,[20] or nanoparticles[21,22] for the

* Corresponding author. Phone/fax: +86-25-83593593. E-mail: hxju@nju.edu.cn (H.J.); yanfeng2007@sohu.com (F.Y.).
[†] Nanjing University.
[‡] Jiangsu Institute of Cancer Research.

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multiplex immunoassay. However, the number of labels available for simultaneous determination is restricted.[23] It is still a great challenge to develop novel single-label-based multianalyte immunoassays, though it is also important to further extend the applications of current multilabel-based detection techniques.

Automated immunoassay possesses the advantages in reducing cost, labor, and turnaround time[24] and improving performance.[25] The automation of the immunoassay can be achieved by combining a flow injection system with a microarray,[7] surface-renewable immunosensors,[26,27] paramagnetic microparticles (PMs),[28,29] and polymer beads.[30] PMs coupled with immunoreagent can freely suspend in solution and conveniently be captured with a magnet, thus the immunoreaction can be performed in a relatively short incubation time and the formed immunocomplex can conveniently be separated from the reaction mixture. With the flow injection technique, this work developed an automated support-resolution strategy for single-label-based multianalyte CL immunoassays.

CL detection has extensively been used in the sensitive immunoassay due to its wide calibration range, need of simple instrumentation, and suitability for miniaturization.[7,31−34] Recently a temperature-triggered chemiluminescent immunoassay for sequential determination of two proteins has been reported.[35] This method introduced poly(*N*-isopropylacrylamide) (PNIP) and magnetic beads as antibody immobilizing carriers to separate two proteins, which needed several temperature-controlled and centrifugation procedures to separate PNIP from magnetic beads and thus made the whole assay system more complicated, labor-intensive, and time-consuming. Here, a novel automated support-resolution strategy coupled with a simple and low-cost flow-through immunosensing system using a single photomultiplier (PMT) was designed for performing one-way chemiluminescent multiplex immunoassay. In this proposed method, a single horseradish peroxidase (HRP) label and capture antibodies immobilized glass tube (GT) and PMs as supports were employed in one fluidic way. With the use of carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) as model analytes, the incubation mixture, containing CEA, HRP-labeled anti-CEA antibody, AFP, anti-AFP immobilized PMs, and HRP-labeled anti-AFP antibody, was first introduced into the modified GT for online incubation. By virtue of a simple wash and capture step, the two formed sandwich immunocomplexes could then be separated in the GT and another unmodified GT applied with a magnet. The CL signals from the two GTs, corresponding to two analytes, were thus near-simultaneously collected with the aid of an optical shutter. This proposed system provided an automated, reusable, simple, sensitive, and low-cost approach for a multiplex immunoassay. The whole immunoassay could be finished in 27 min with high sensitivity, acceptable linear calibration range, and reproducibility.

EXPERIMENTAL SECTION

Materials and Reagents. Rabbit polyclonal anti-CEA (bs-0060R) and mouse monoclonal anti-AFP (D08081901) antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. and Xiamen Boson Biotechnology Co., Ltd., respectively. Chemiluminescent ELISA kits of CEA and AFP, including standard solutions of CEA and AFP, HRP-labeled anti-CEA and anti-AFP antibodies, HRP substrate solutions (luminol-*p*-iodophenol-H_2O_2), were supplied by Autobio Diagnostics Co., Ltd. (China). Electrochemiluminescent immunoassay (ECLIA) reagent kits used for reference detection of CEA and AFP were provided by Roche Diagnostics GmbH (Germany). Carboxyl group modified PMs (magnetic iron oxide) in an aqueous suspension with the mean diameter of 1.5 μm and concentration of 20 mg/mL were obtained from Bangs Laboratories Inc. *N*-Hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Aldrich and Merck, respectively. 3-Glycidoxypropyltrimethoxysilane (GPTMS, 98%) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all runs. The clinical serum samples were from Jiangsu Institute of Cancer Research. All other reagents were of the best grade available and used as received.

Buffers. Activation buffer for carboxyl group was 0.01 M 2-morpholinoethanesulfonic acid, adjusted to pH 5.5 with NaOH. Coupling buffer for antibody immobilization was 0.01 M pH 7.4 phosphate buffer solution (PBS). Blocking buffer was PBS containing 1% bovine serum albumin. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into PBS as a wash buffer (PBST). The regeneration buffer was 0.1 M glycine-HCl at pH 2.2.

Apparatus. The automated support-resolved flow-through CL multiplex immunoassay system was constructed as illustrated in Figure 1. The Teflon tubes (0.8 mm i.d.) and silicon rubber tubes (1.0 mm i.d.) were used to connect all components in this system. All fluids were delivered with a multichannel bidirectional peristaltic pump of an IFFM-D luminescent analyzer (Remax, China). The introduction of different solutions into the one-way channel were performed using a multiposition valve with five inlets and one outlet. Two silicon rubber tube-connected GTs of about 50 μL (i.d. 1.5 mm, length 2.8 cm) and 30 μL (i.d. 1.5 mm, length 1.7 cm) for anti-CEA immobilization and PMs capture, respectively, were positioned in front of the photomultiplier. One moveable tubular optical shutter was used to resolve the CL signals

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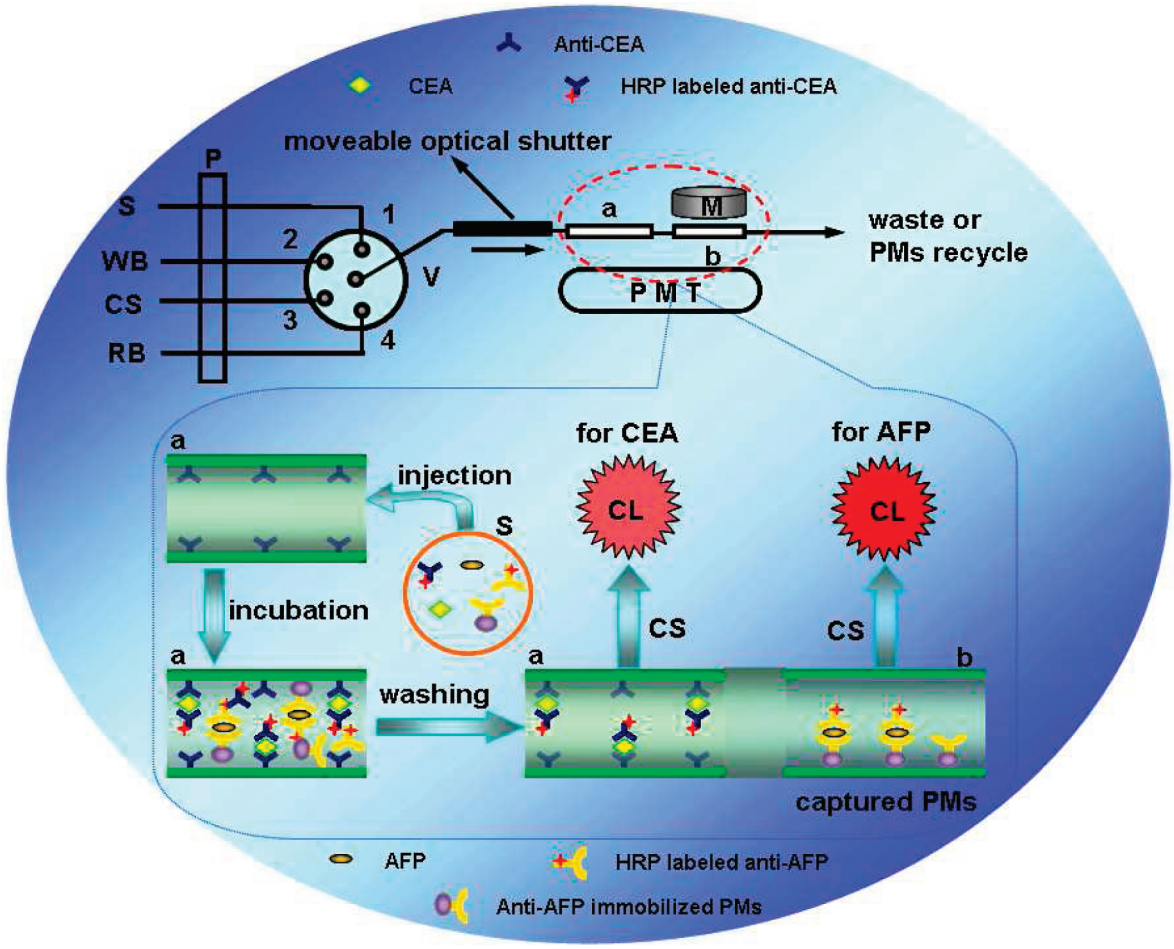

Figure 1. Scheme of the automated support-resolved flow-through CL multiplex immunoassay system: (a, b) GT; (S) incubation mixture; (WB) wash buffer; (CS) CL substrate for HRP; (RB) regeneration buffer; (V) multiposition valve; (P) peristaltic pump, (M) magnet; (PMT) photomultiplier.

produced in channels a and b, which was measured with the PTM operated at −800 V. Instrument control and the data recording were performed using the IFFM software package run under Windows 2000.

The reference ECLIA was performed with a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH). Scanning electron micrographs (SEM) were obtained with a Hitachi S-3000N scanning electron microscope (Japan) at an acceleration voltage of 10 kV. IR spectra were recorded on a Nicolet 400 Fourier transform infrared (FT-IR) spectrometer (Madison, WI).

Preparation of Anti-CEA Immobilized GT and Anti-AFP Immobilized PMs. The GT was first dipped in piranha solution (H_2SO_4/30% H_2O_2, 7:3 in volume) for 12 h. After washing thoroughly with ultrawater, its inner wall was dried under a stream of nitrogen and silanized by injecting 1% GPTMS toluene solution in the tube overnight at room temperature (RT, 25 °C).[36] After the coupling reaction, the modified GT was rinsed several times with pure toluene and ethanol to remove the physically absorbed silane from the inner surface and dried under a stream of nitrogen. As shown in Figure 2a, many exposed active epoxy groups were formed on the inner wall of GT and further reacted with anti-CEA antibody by injecting 50 μL of 20 μg/mL anti-CEA solution in the GT at RT for 2 h and 4 °C overnight. The left epoxy groups were blocked with blocking buffer for 6 h at 4 °C after washing three times with PBST. The anti-CEA immobilized GT was dipped in PBS containing 0.1% NaN_3 for storage at 4 °C.

A volume of 0.5 mL of PMs suspension was first washed three times 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 30 mg of EDC and 15 mg of NHS were added to activate the carboxyl groups for 1 h under constant stirring at RT (Figure 2b). The activated PMs were washed three times with coupling buffer and resuspended in 0.5 mL of coupling buffer, and 0.5 mL of 200 μg/mL anti-AFP solution was added and reacted with the activated PMs under gentle stirring at RT for 2 h followed with standing overnight at 4 °C. The resulting antibody immobilized PMs were thoroughly washed with washing buffer, blocked with blocking buffer for 6 h at 4 °C, and dispersed in pH 7.4 PBS containing 0.1% NaN_3 for storage at 4 °C.

Immunoassay Protocol. The assay process for CEA and AFP was illustrated in Figure 1. A mixture of CEA, AFP, HRP-labeled anti-CEA antibody, anti-AFP immobilized PMs, and HRP-labeled anti-AFP antibody (10 μL for each) was first delivered into GT a and incubated under a stop flow at RT for 20 min. PBST was then delivered at a flow rate of 0.5 mL/min into the system to wash the channel, and the formed immunocomplex immobilized PMs

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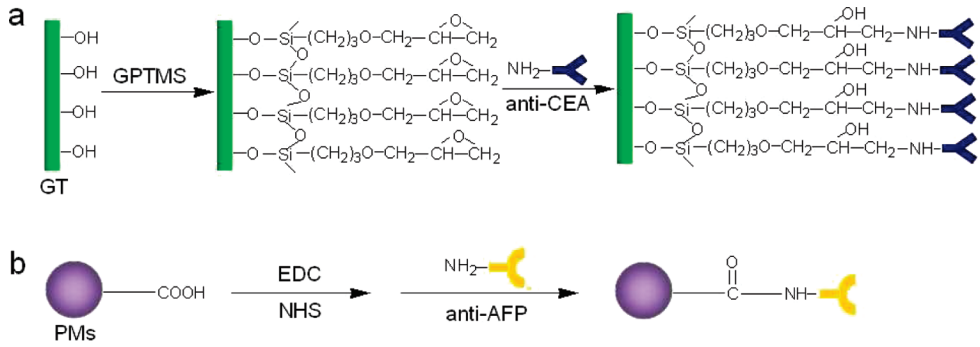

Figure 2. Scheme for immobilization of antibodies on the inner wall of GT and the surface of PMs.

were collected in GT b with a magnet. Next, CL substrate solutions were introduced into the two GTs. When the HRP catalyzed CL reaction was triggered under a stop flow, the magnetic field was withdrawn. With the aid of an optical shutter, the CL signals from GTs a and b were near-simultaneously collected. The whole procedure from sample injection to signal detection could be finished within 25 min. Afterward, regeneration buffer and PBS passed through the channel, respectively, for two cycles to regenerate GT a and expel PMs. At a time of about 26.8 min, the multiplex analytical system was ready for the next assay cycle. After the detection, the expelled PMs were collected and regenerated in regeneration buffer for 10 min followed by washing using the blocking buffer three times.

Specimen Collection and Safety Consideration. According to the rules of the local ethical committee, blood specimens were collected using a standard venipuncture technique and the sera were centrifugally separated from the cells, without hemolysis. The serum samples could be directly assayed with the proposed and reference methods. If necessary, the samples 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. For safety consideration, all handling and processing were performed carefully, and all tools in contact with patient specimens and immunoreagents were disinfected after use.

RESULTS AND DISCUSSION

Characterization of Antibodies Immobilized GT and PMs. This work used epoxy group modified GT and carboxyl group activated PMs as the anti-CEA and anti-AFP immobilizing supports, respectively. From SEM images of the GT treated with piranha solution, a complete, smooth, and homogeneous inner surface could be obtained (Figure 3a). After the immobilization of anti-CEA antibody, the inner surface showed obviously different surface morphology from that treated with piranha solution (Figure 3b), indicating CEA antibody was successfully linked to the inner surface of GT.

FT-IR spectrum of carboxyl group activated PMs showed two absorption peaks at 1624 and 1187 cm^{-1} (Figure 3c), which were attributed to the C=O and C−O stretching vibrations of the carbonyl group, respectively.[37] The wide band around 3348 cm^{-1} was attributed to the stretching vibration of the O−H group on PMs. The FT-IR spectrum of anti-AFP modified PMs showed

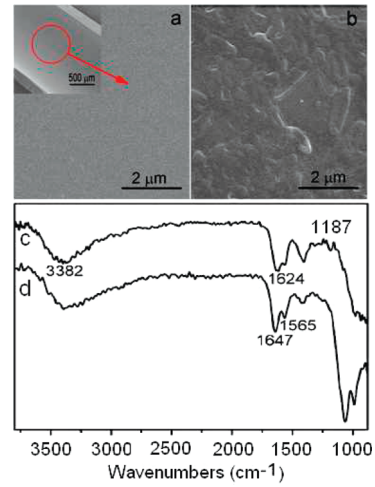

Figure 3. SEM images of bare (a) and anti-CEA immobilized (b) GTs and FT-IR spectra of bare (c) and anti-AFP immobilized (d) PMs.

the absorption of amide I band (1700−1600 cm^{-1}) and amide II band (1600−1500 cm^{-1}) with peaks at 1642 and 1538 cm^{-1} (Figure 3d), indicating that the antibody molecules were covalently bound to PMs.

Kinetics of the CL Reaction. HRP and alkaline phosphatase (ALP) are two major labels used in enzyme-catalyzed CL immunoassay. The CL signal from the HRP-catalyzed reaction reaches its maximum value much faster than that from the ALP-catalyzed reaction.[18] Thus this work used HRP as an enzyme label to examine the designed support-resolution strategy. The kinetic behavior of the CL reaction catalyzed by HRP labeled to the sandwich immunocomplexes of CEA in GT a and AFP on PMs in GT b were studied with a static method. The CL reaction both on the inner wall of GT and PMs occurred immediately after injection of the CL substrate. The intensity of CL emission from GT a increased quickly and reached its maximum value within 30 s, while the emission from GT b reached its maximum value in undetectable time and then decreased slowly. Obviously, the suspension of HRP-immobilized PMs in the CL substrate solution led to a more rapid mass transport and CL reaction. In order to acquire high detection sensitivity, the CL signal collection was performed from GT b at the beginning and then GT a at 30 s for near-simultaneous detection of AFP and CEA.

Incubation Time. The incubation time is a bottleneck to the improvement of immunoassay efficiency, which is usually controlled by mass transport of immunoreagents and kinetics of immunoreaction.[18] Compared to the microwell used in conven-

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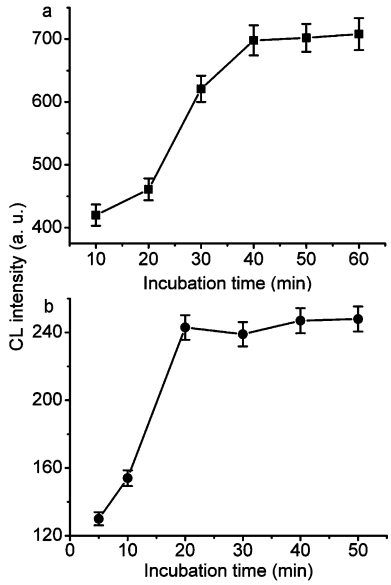

Figure 4. Effects of incubation time on CL intensity in the presence of 20 ng/mL CEA (a) and AFP (b) ($n = 5$ for each point).

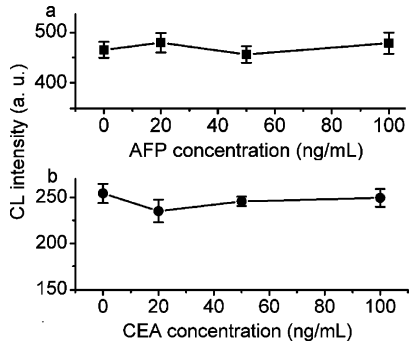

Figure 5. Cross-reactivity of another analyte to CEA (a) and AFP (b) antibodies in the presence of 20 ng/mL CEA (a) and AFP (b), respectively ($n = 5$ for each point).

tional ELISA, the antibody immobilized inner surface of GT with an i.d. of 1.5 mm increased the reaction area for formation of the sandwich immunocomplex, and the suspension of antibody immobilized PMs in the incubation mixture was beneficial to accelerating the immunoreaction. As shown in Figure 4, with the increasing incubation time, the CL signals from GTs a and b, which were examined at 20 ng/mL CEA and AFP, increased and trended to the maximum values at 40 and 20 min, respectively, indicating the maximum formation of the sandwich immunocomplexes. At the incubation of 20 min, the CL intensity for CEA was 66% of the maximum value. Considering the optimal analytical performance and further development of this method to high sample throughput, 20 min of incubation time was used in the immunoassay.

Evaluation of Cross Reactivity. The cross reactivity was evaluated by comparing the change of CL signal at a definite concentration of analyte with the increasing concentration of another analyte as an interfering agent. When the concentration of interfering agent changed in the range of 20−100 ng/mL, the change in CL signal for 20 ng/mL CEA and AFP was less than 3.5% and 3.0%, respectively (Figure 5), indicating that the cross reactivity between CEA and AFP antibodies and the other noncognate antigens was negligible. This result suggested that

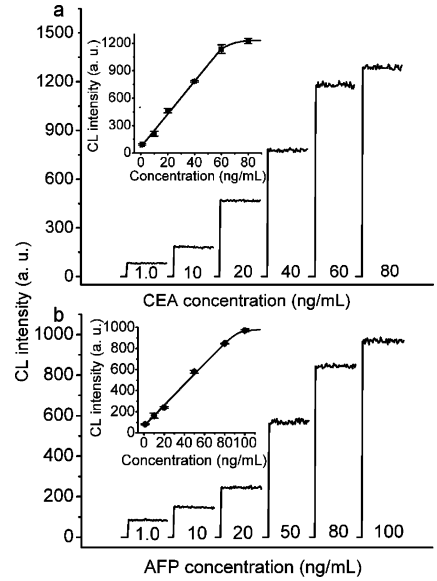

Figure 6. Calibration curves for CEA (a) and AFP (b). Inset: Dose−response curves ($n = 5$ for each point).

the two tumor markers could be assayed in a single run using the designed multiplex immunoassay system.

Regeneration of Antibody Immobilized GTs and PMs. The regeneration of covalently bound antibodies for reuse must avoid loss of their activity and their leakage out of the support surface. Different regeneration reagents, including buffer with low pH value (0.1 M glycine/HCl, pH 2.2), organic solvent (CH_3OH-H_2O, 1:1), and diluted alkali (50 mM NaOH), were used to perform the regeneration. The regeneration efficiency (RE) was calculated with the following the equation.[38]

$$RE = [1 - A/A_0] \times 100\% \quad (1)$$

where A represents the CL signal obtained after the regeneration cycle and A_0 is the signal before applying the regeneration step. The most efficient dissociating reagent was found to be 0.1 M glycine-HCl (pH 2.2), which allowed a fast and complete dissociation of the immunocomplexes with a RE of 97.6% and 98.2% for CEA and AFP, respectively. As a mild regeneration reagent, glycine-HCl buffer showed good activity retention for the immobilized antibodies. The activity of the regenerated anti-CAE immobilized inner wall of GT and anti-AFP immobilized PMs did not show obvious decrease after being used 10 times. Thus glycine-HCl buffer was chosen as the regeneration buffer for the regeneration of the antibodies immobilized in GTs and on PMs.

Analytical Performance. Under the optimum conditions, the CL intensity for both CEA and AFP increased with increasing concentration of analytes (Figure 6). The dose−response curves for CEA and AFP showed the linear ranges from 1.0 to 60 and 1.0 to 80 ng/mL with limits of detection of 0.6 and 0.89 ng/mL at a signal-to-noise ratio of 3, respectively. The linear regression equations were $I = 67.6 + 17.9$[CEA] ($R^2 = 0.9962$, SD = 30.3) and $I = 64.1 + 9.9$[AFP] ($R^2 = 0.9974$, SD = 18.5), respectively. The obtained limits of detection for CEA and AFP were lower

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than those reported previously in multiplex immunoassay methods.[39−41] Since the cutoff values of the two tumor markers in diagnostic are 5 and 25 ng/mL, respectively, the sensitivity of the proposed method was enough for practical application. When this proposed strategy was combined with other techniques, such as substrate,[18] channel,[39] and sampling resolutions,[28] more analytes could be detected in a single run.

With the use of samples containing 20 ng/mL CEA and AFP, the reproducibility of the automated support-resolved multiplex immunoassay system was evaluated by the intra- and interassay coefficients of variation (CV). The intra-assay CV was the difference among five measurements of the same sample incubated with the antibodies immobilized GTs and PMs prepared in the same batch. The interassay CV was the difference among the measurements of the samples incubated with the antibodies immobilized GTs and PMs prepared in five batches. The intra- and interassay CVs obtained were 1.8 and 3.1% for CEA and 3.9 and 5.4% for AFP, respectively, showing acceptable detection and fabrication reproducibility.

When the antibodies immobilized GT and PMs were not in use, they could be stored in PBS containing 0.1% NaN_3 at 4 °C for at least 20 days without obvious signal change. The acceptable retention of immunoreactivity was very important for the development of the proposed method in low-cost applications.

Method Comparison. To evaluate the analytical reliability and application potential of the support-resolved multiplex immunoassay system, the assay results of practical serum samples using the proposed method were compared with those detected with the reference ECLIA method. When the levels of tumor markers were over the calibration ranges, serum samples were appropriately diluted with 0.01 M pH 7.4 PBS prior to the assay. The results were listed in Table 1, which showed an acceptable agreement with relative errors less than 9.4%.

Table 1. Assay Results of Clinical Serum Samples Using the Proposed and Reference Methods (in ng/mL)

analyte	CEA			AFP		
sample	1	2	3	1	2	3
proposed method	4.2	26.8	49.0	8.6	43.3	55.2
reference method	3.9	26.2	51.4	9.5	41.7	58.1
relative error (%)	7.6	2.3	−4.7	−9.4	3.8	−5.0

CONCLUSION

A novel automated support-resolution strategy and flow-through chemiluminescent multiplex immunoassay system have been developed. This strategy uses GT and PMs as supports for antibodies immobilization to automatically separate different analytes by a simple wash and magnetic capture step. The developed method employs a single HRP label for a simple and convenient one-way near-simultaneous CL detection. The whole immunoassay, including incubation, capture, wash, detection, and regeneration, can be finished in 27 min, which is obviously faster than those reported previously.[12,16,39] This method does not show any matrix effect and is sensitive and low-cost. It shows acceptable detection and fabrication reproducibility and accuracy, indicating a promising practicality in automated rapid screening and clinical diagnosis.

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