Automated chemiluminescent dual-analyte immunoassay based on resolved immunosensing channels

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ABSTRACT

A novel system of series-wound immunosensing channels (SWIC) was proposed for automated chemiluminescent (CL) dual-analyte immunoassay by immobilizing respectively different capture antibodies on the inner walls of series-wound glass channels. This system could use a single enzyme as label to perform multiplex immunoassay in one fluid way. Using α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) as model analytes, the mixture including AFP, horseradish peroxidase (HRP)-labeled anti-AFP antibody, CEA and HRP-labeled anti-CEA antibody was introduced into the SWIC for carrying out the on-line incubation. Upon injection of CL substrate the CL signals from the two immunosensing channels were conveniently resolved and near-simultaneously collected with the aid of optical shutter. AFP and CEA could be rapidly assayed in the ranges of 1.0–100 and 1.0–80 ng/ml with detection limits of 0.41 and 0.39 ng/ml, respectively. The assay results of clinical serum samples were in an acceptable agreement with the reference values. This designed flow-through immunosensing system based on SWIC provided an automated, reusable, simple, sensitive and low-cost approach for multianalyte immunoassay.

1. Introduction

Tumor markers are chemical substances produced by tumor cells, or generated by the reactions of human body to certain tumors [1]. Abnormally elevated levels of tumor markers in human serum are significantly related with certain tumors [2]. Thus the immunoassay of tumor markers in serum has been extensively used for early disease screen, evaluating the extent of disease, monitoring the response of disease to therapy, and predicting recurrence [3,4]. Due to the insufficiency to detect a single tumor marker for diagnosis purpose, the multiplex immunoassay of tumor markers panel has attracted considerable interest for clinical purpose [5–7]. Compared with the traditional parallel single-analyte immunoassay, multiplex immunoassay can provide higher sample throughput, shorter assay time, less sample consumption, lower cost of per assay and more efficient diagnosis for the disease [8–10].

The multiplex immunoassay methods developed previously are mainly based on multilabel and spatial resolution modes. The multilabel mode typically employs several labels to tag antibodies or antigens corresponding to the analytes. These labels include enzymes [11–13], fluorescent dyes [14,15], metal ions [16–18] and nanoparticles [19–21]. However, the combination of multi-ple labels in a single run often suffers from incompatible optimal assay conditions [3,8] and the signal overlapping of different labels [19]. Furthermore, the number of labels available for simultaneous determination is restricted [10]. The spatial resolution mode uses a universal label for optical [22–29], electrochemical [30,31] and piezoelectric [32] detection of antigen or antibody array. These methods require expensive array detectors and often suffer from potential cross-talk [5,31]. Therefore, it is still a great challenge to develop novel single-label and non-array based multiplex immunoassays.

Chemiluminescent (CL) immunoassay combines good specificity of immunoreaction with high sensitivity of CL detection and has become a powerful analytical means [33–36]. This technique has been extensively used for development of multiplex immunoassay [3,6,13,37]. Our previous work proposed an automated support-resolution strategy by immobilizing the capture antibodies on the inner wall of a glass tube and the surface of magnetic microspheres (PMs) [37]. The immunoassay process needed the help of magnet to capture the immunocomplex immobilized PMs, thus was obviously inconvenient. Here, a novel system based on series-wound immunosensing channels (SWIC) constructed by immobilizing the capture antibodies on the inner walls of different glass tubes was developed for automated CL multiplex immunoassay. After the sandwich immunocomplexes were formed, the enzymatic CL signals corresponding to different analytes could be near-simultaneously collected from the SWIC with the aid of opti-

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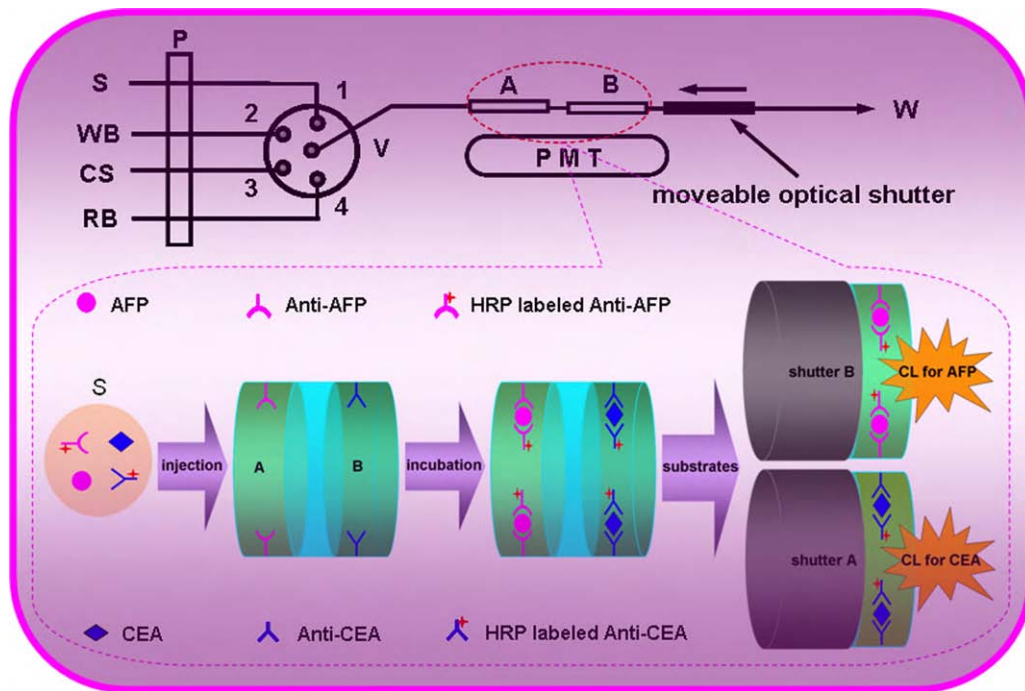
Fig. 1. Scheme of automated CL multiplex immunoassay based on series-wound immunosensing channels: (A, B) glass tube, (S) incubation mixture, (WB) wash buffer, (CS) HRP substrates, (RB) regeneration buffer, (V) multiposition valve, (P) peristaltic pump, (PMT) detector, and (W) waste.

cal shutter. This proposed method overcame the drawbacks existed in the previous developed strategies [6,37] and showed a series of advantages such as simple manipulation, high sensitivity, acceptable linear calibration range and reproducibility, low-cost detector, and ease to achieve automation and high analyte throughput.

2. Experimental

2.1. Reagents and materials

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 AFP and CEA, including standard solutions of AFP and CEA, HRP-labeled anti-AFP and anti-CEA antibodies, HRP substrate solutions (luminol-p-iodophenol-H_2O_2), were obtained from Autobio Diagnostics Co., Ltd. (China). Electrochemiluminescent immunoassay (ECLIA) reagent kits used for the reference detection of AFP and CEA were supplied by Roche Diagnostics GmbH (Germany). Bovine serum albumin (BSA) and 3-glycidoxypropyltrimethoxysilane (GPTMS, 98%) were purchased from Sigma (St. Louis, MO). Blocking buffer was PBS containing 1% bovine serum albumin. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into PBS as wash buffer (PBST). Regeneration buffer was 0.1 M glycine–HCl at pH 2.2. Ultrapure water obtained from a Millipore water purification system ($\geq$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.

2.2. Equipments

The automated flow-through CL dual-analyte immunoassay system was constructed as illustrated in Fig. 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 solutions were delivered with a multichannel bidirectional peristaltic pump of an IFFM-D

Luminescent Analyzer (Remax, China). A multiposition valve with five inlets and one outlet was used to switch different solutions into flow system sequentially. Two silicon rubber tube-connected glass tubes A and B of about 30 μl (i.d. 1.4-mm, length 2.0-cm) for anti-AFP and anti-CEA immobilization, respectively, were positioned in front of end-on photomultiplier (PMT, CR 105), which comes from Beijing Hamamatsu Photon Techniques Inc. (China). A black plastic tube (i.d. 3.0-mm, length 3.0-cm) was used as the moveable tubular optical shutter to resolve the CL signals produced in the series-wound channels A and B, which were measured with the PTM operated at −800 V. Instrument control and data record were performed using IFFM software package run under Windows 2000.

The reference ECLIA was performed with a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH). Atomic force microscopy (AFM) images were obtained with an Agilent 5500 atomic force microscopy (U.S.A.).

2.3. Preparation of anti-AFP and anti-CEA immobilized SWIC

The glass tubes A and B were firstly dipped in piranha solution (H_2SO_4/30% H_2O_2, 7:3 in volume) for 12 h. After washing thoroughly with ultrapure water, their inner walls were dried under a stream of nitrogen and silanized by immersing them in toluene solution of 1% GPTMS overnight at room temperature (RT, 25 °C) [38]. After the coupling reaction, the modified glass tubes A and B were 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. Upon this process, many exposed active epoxy groups were formed on the inner wall of glass tubes. By injecting 30 μl of 200 μg/ml anti-AFP and anti-CEA solution in the glass tubes A and B, respectively, these active epoxy groups further reacted with anti-AFP and anti-CEA antibodies at RT for 2 h and 4 °C overnight. After washing three times with PBST, the left epoxy groups were blocked with blocking buffer for 6 h at 4 °C. The anti-AFP and anti-CEA immobilized glass tubes were dipped in PBS containing 0.1% NaN_3 for storage at 4 °C.

Table 1
Details of automated immunoassay protocol based on series-wound immunosensing channels for the determination of CEA and AFP.

Step no.	Valve position	Step	Starting time (min:s)
1	1	Introduce mixture of 30 μl of sample (15 μl for each), 15 μl of AFP tracer antibody and 15 μl of CEA tracer antibody into channels A and B	00:00
2		Stop flow and incubate at room temperature	00:30
3	2	Wash the channels with wash buffer at a flow rate of 0.5 ml/min	20:30
4	3	Introduce 30 μl of HRP substrate into channel A and 30 μl of HRP substrate into channel B	22:30
5		Stop flow and trigger the HRP catalyzed CL reaction	23:00
6		Collect CL signal from channel A for AFP detection while shadow channel B with optical shutter	24:00
7		Collect CL signal from channel B for CEA detection while shadow channel A with optical shutter	24:10
8	4	Introduce regeneration buffer to regenerate channels A and B at a flow rate of 0.5 ml/min	24:20
9	2	Introduce wash buffer to recondition channels A and B at a flow rate of 0.5 ml/min	26:20
10	1	Ready for the next assay cycle	26:50

2.4. Immunoassay protocol

A detailed description of the assay process for AFP and CEA was given in Table 1. The whole immunoassay process was performed at room temperature and controlled automatically by personal computer equipped with IFFM software package instead of manually manipulation. A mixture of AFP, CEA, HRP-labeled anti-AFP antibody, and HRP-labeled anti-CEA antibody (15 μl for each) was firstly introduced into glass tubes A and B, and incubated under stop flow for 20 min. PBST was then delivered at a flow rate of 0.5 ml/min into the system to wash the series-wound immunosensing channels. Next, CL substrate solutions were introduced into the two channels. When the HRP catalyzed CL reaction was triggered under stop flow, with the aid of a moveable optical shutter, the CL signals from channels A and B were near-simultaneously collected in a short period of 20 s while shadow another channel, respectively. The whole procedure from sample injection to signal detection could be finished within 25 min. Afterwards, regeneration buffer and PBS passed through the channels for two cycles to regenerate channels A and B. At a time of about 26.8 min, the multiplex analytical system was ready for next assay cycle.

2.5. 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.

3. Results and discussion

### 3.1. Characterization of antibodies immobilized SWIC

This work used epoxy group modified glass tubes to immobilize anti-AFP and anti-CEA, respectively, and thus proposed a SWIC-based CL multiplex immunoassay. After the bare glass tubes were treated with piranha solution, a complete, smooth and homogeneous inner surface could be obtained (Fig. 2a). Different from the treated glass tubes, the inner surface of GPTMS-silylanized glass tubes formed homogeneous expoxysilane layer film (Fig. 2b). After the immobilization of anti-AFP (Fig. 2c) and anti-CEA (Fig. 2d) antibodies, the inner surface showed obviously different surface morphologies, indicating AFP and CEA antibodies were successfully bound to inner surface of the glass tubes.

3.2. Kinetic characteristics of CL reaction

This work employed HRP as an enzyme label to examine the designed CL multiplex immunoassay system. The kinetic behavior of the CL reaction catalyzed by HRP labeled to the sandwich immunocomplexes of AFP in glass tube A and CEA on glass tube B was studied with a static method. The CL reaction occurred immediately after the introduction of the CL substrate into detection channels. The intensity of CL emission from glass tubes A and B
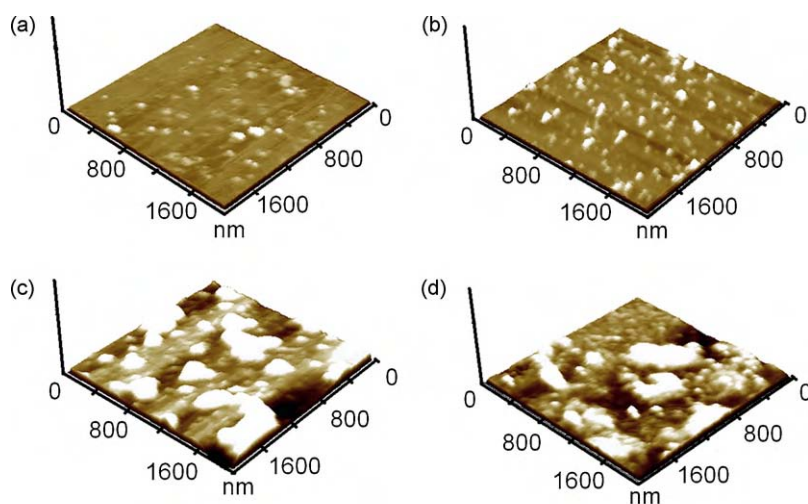
Fig. 2. AFM images of bare (a), GPTMS-silylanized (b), anti-AFP immobilized (c) and anti-CEA immobilized (d) glass tubes.
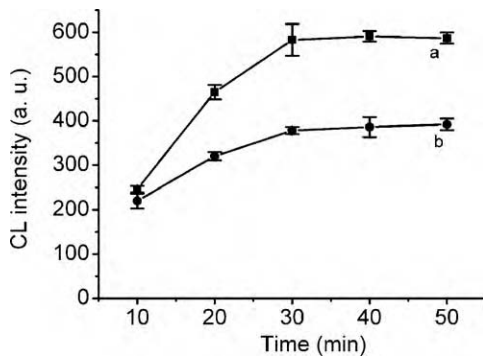
Fig. 3. Effect of incubation time on CL intensity in the presence of 10 ng/ml AFP (a) and CEA (b) ($n = 5$ for each point).

increased quickly and both trended to its maximum value within 30 s (not shown here). Obviously, the large inner surface area of glass tube led to rapid mass transfer of enzyme substrate and CL reaction. In order to acquire high detection sensitivity, 30 s was chosen as the optimal CL reaction time to perform the CL signal collection for near-simultaneous detection of AFP and CEA.

3.3. Effect of 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 [13,37]. Compared to the microwell used in conventional ELISA, the antibody immobilized inner surface of glass tube with i.d. of 1.4 mm increased the reaction area and was beneficial to accelerating the immunoreaction for formation of sandwich immunocomplex. As shown in Fig. 3, with the increasing incubation time the CL signals from glass tubes A and B, which were examined at 10 ng/ml AFP and CEA, increased and trended to the maximum values at 30 min,
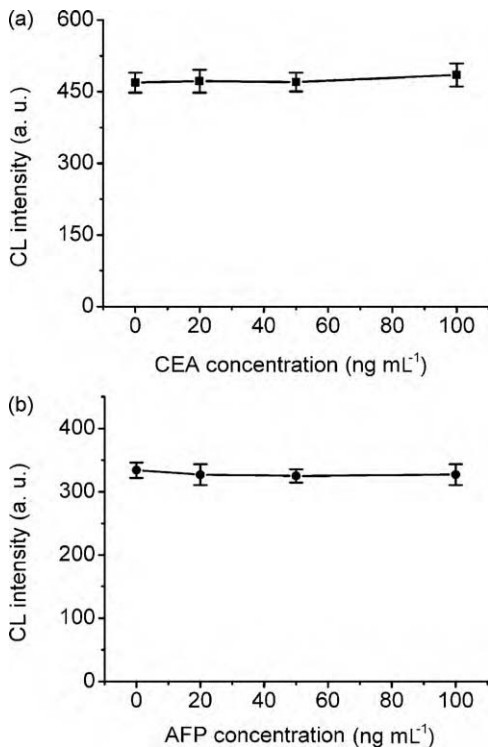
Fig. 4. Cross-reactivity of another analyte to AFP (a) and CEA (b) antibodies in the presence of 10 ng/ml AFP and CEA, respectively ($n = 5$ for each point).

respectively, indicating the maximum formation of the sandwich immunocomplexes. The times for the maximum formation of these immunocomplexes at RT using this system were obviously shorter than that of 1–3 h at 37 °C for the conventional microwell plate ELISA method. Although the CL signals at the incubation time of 20 min were 78 and 81% of the maximum values for AFP and CEA, respectively, the high sensitivity of CL assay made detection limits low enough for clinical detection. 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 multiplex immunoassay.

3.4. Evaluation of cross-reactivity

The cross-reactivity was examined by comparing the change of CL signal at a definite concentration of analyte with the increasing concentration of another analyte as interferent. When the concentration of interferent changed in the range of 20–100 ng/ml, the changes in CL signal for 10 ng/ml AFP and CEA were less than 2.7 and 3.5%, respectively (Fig. 4), indicating that the cross-reactivity between AFP and CEA antibodies and the other noncognate antigens was negligible. This result suggested that the two tumor markers could be assayed in a single run using the designed multiplex immunoassay system.

3.5. Regeneration of antibodies immobilized SWIC

With a simple and short period treatment step, the antibodies covalently bound on inner wall of glass tubes could be regenerated with regeneration reagent for reuse. However, it 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 was calculated according to the reported method [39]. 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 regeneration efficiency of 98.0 and 97.8% for AFP and CEA, respectively. As a mild regeneration reagent, glycine–HCl buffer showed good activity retention for the immobilized antibodies. After used for 10 times, the activity of the regenerated anti-AFP and anti-CAE immobilized inner wall of glass tubes did not show obvious decrease. Thus glycine–HCl buffer was chosen as the regeneration buffer for the regeneration of the antibodies immobilized in glass tubes.
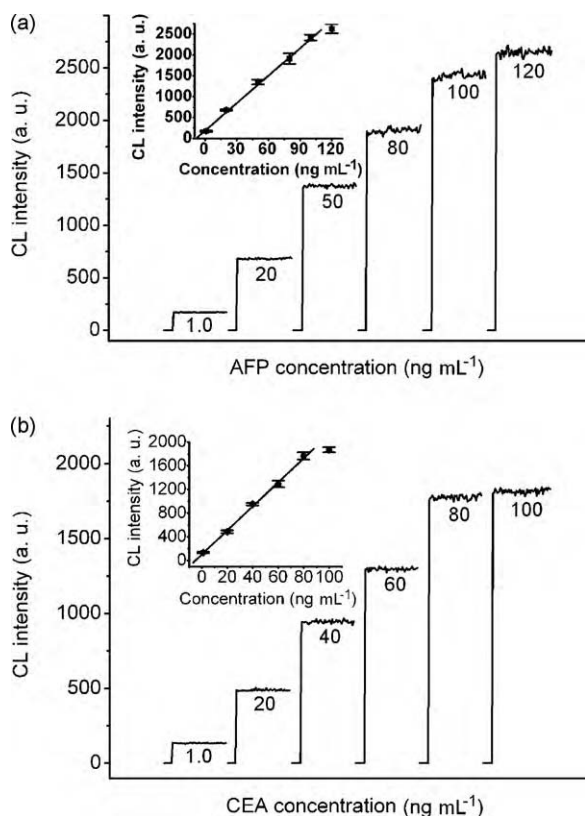
3.6. Dose–response curves

Under the optimum conditions, the CL intensity from SWIC for both AFP and CEA increased with the increasing concentration of analytes (Fig. 5). The linear ranges for AFP and CEA were 1.0–100 ng/ml and 1.0–80 ng/ml with limits of detection of 0.41 and 0.39 ng/ml at a signal-to-noise ratio of 3, respectively. The linear regression equations were $I = 186.9 + 21.9$ [AFP](ng/ml) ($R^2 = 0.9978$) and $I = 105.7 + 20.3$ [CEA](ng/ml) ($R^2 = 0.9958$), respectively, where I is CL intensity. The obtained limits of detection for CEA and AFP were lower than those of the previously reported multiplex immunoassay methods [2,3,37,40,41]. 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 to practical application. When more glass tubes were used for immobilization of more deferent capture antibodies to construct a series-wound immunosensing channel array, the proposed system can detect above two analytes in a single run. Here, one tubular optical shutter with a suited hole, which covers the whole series-wound channel, can conveniently resolve

Table 2
Assay results of clinical serum samples using the proposed and reference methods (ng/ml).

	Analyte					
	AFP			CEA		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Proposed method	3.3	39.8	88.6	5.8	49.0	69.5
Reference method	3.6	41.7	83.4	5.4	51.3	63.4
Relative error (%)	−8.3	−4.6	6.2	7.4	−4.5	9.6

Fig. 5. Calibration curves for AFP (a) and CEA (b). Inset: dose–response curves ($n = 5$ for each point).

the signals from deferent channels through shadowing the other channels respectively. However, the maximum number of analyte which can be detected in a single run is restricted because of the limited interior space of CL detector.

3.7. Reproducibility and stability of the multiplex immunosensing system

The reproducibility of the automated dual-analyte immunoassay system was evaluated using samples containing 10 ng/ml AFP and CEA. The intra-assay coefficient of variation (CV) was the difference among five measurements of the same sample incubated with the antibody immobilized glass tubes A and B prepared in the same batch. The inter-assay CV was the difference among the measurements of the samples incubated with the antibody immobilized glass tubes A and B prepared in five batches. The intra- and inter-assay CVs obtained were 3.9 and 4.7% for AFP and 6.2 and 7.3% for CEA, respectively, showing acceptable detection and fabrication reproducibility.

When the antibody immobilized glass tubes were not in use, they could be stored in PBS containing 0.1% NaN_3 at 4 °C for 20 days without obvious signal change. The acceptable retention of

immunoreactivity was very important for the development of the proposed method in low-cost application.

3.8. Detection of tumor markers AFP and CEA in clinical serum samples

Under the optimum conditions, the analytical reliability and application potential of SWIC-based dual-analyte immunoassay system was evaluated by assaying clinical serum samples using the proposed method as well as the reference ECLIA method. The latter was carried out in Jiangsu Institute of Cancer Prevention and Cure. 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 assay. The results gave the relative errors less than 8.3% for AFP and less than 9.6% for CEA, showing an acceptable agreement between the two methods (Table 2).

4. Conclusions

A novel strategy of series-wound immunosensing channels coupled with flow-injection chemiluminescent system is proposed for automated multiplex immunoassay. This method uses series-wound glass tubes as supports for immobilization of antibodies. The developed method can near-simultaneously resolve CL signals from the two immunosensing channels for single-label multiplex immunoassay. The proposed method is simple, fast, sensitive and low-cost and can accomplish the whole assay including the incubation, detection and regeneration steps within 27 min. Compared with the traditional sequential single-analyte immunoassay, the proposed system can provide higher sample throughput, shorter assay time, less sample consumption, lower cost of per assay and more efficient diagnosis for the disease. The multiplex immunosensing system shows acceptable detection and fabrication reproducibility and accuracy, and easy automation for high sample throughput, indicating a promising practicality in clinical diagnosis. Thus this strategy could easily achieve high analyte and sample throughput.

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