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Short communication

Microchip fluorescence-enhanced immunoaasay for simultaneous quantification of multiple tumor markers

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ABSTRACT

A microchip fluorescence-enhanced immunoassay method was developed for simultaneous detection of carcinoma antigen 125 (CA125) and carbohydrate antigen 15-3 (CA15-3). In this method, CA125 and CA15-3 react with excess amount of fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (Ab^*) of CA125 and CA15-3 to form CA125-Ab^*_{125} and CA15-3-$Ab^*_{15\text{-}3}$ complexes. Microchip electrophoresis (MCE) separation of free Ab^*_{125}, $Ab^*_{15\text{-}3}$, and CA125-Ab^*_{125}, CA15-3-$Ab^*_{15\text{-}3}$ complexes were then performed. The separated species were sensitively detected by laser-induced fluorescence detection (LIF). CA125 and CA15-3 were quantified simultaneously by measuring the fluorescence intensity of CA125-Ab^*_{125} and CA15-3-$Ab^*_{15\text{-}3}$ complexes, respectively. Under the optimum conditions, the limits of detection were 0.23 U/mL for CA125 and 0.09 U/mL for CA15-3. The present MCE-LIF method was applied to the determination of CA125 and CA15-3 in serum from healthy subjects and cancer patients. The levels of CA125 and CA15-3 in these sera samples were found to be in the ranges of 15.6–36.1 U/mL and 13.8–28.4 U/mL for healthy subjects, and 192.5–368.3 U/mL and 63.3–198.4 U/mL for cancer patients.

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1. Introduction

The detection of tumor markers is essential for early screening of cancer disease, evaluating the extent of cancer disease and monitoring the response of disease to therapy. Carcinoma antigen 125 (CA125) and carbohydrate antigen 15-3 (CA15-3) are quantified as important tumor markers in clinical diagnosis of ovarian cancer and breast cancer. A variety of methods have been reported for the determination of CA125 and CA15-3, such as immunoradiometric assay [1,2], enzyme immunoassay [3–7], microparticle enzyme immunoassay [8], immunofluorometric assay [9,10], electrochemical immunosensor [11,12], electrochemiluminescence immunoassay [13] and capillary electrophoretic enzyme immunoassay [14,15]. However, these conventional immunoassay methods require relatively long assay time, expensive antibody reagents, and involve troublesome liquid-handling procedures. Also, a single marker is often not sufficient for diagnosis purpose due to limited specificity and sensitivity. The assay of cancer marker panel can improve the diagnosis value of disease. Thus, multiplexed immunoassay has attracted considerable interest to meet the demand for early screening of disease [16,17].

Microfluidic technology has been demonstrated to be a promising approach for various applications [18–22]. Microchip electrophoresis (MCE) based on microfluidic devices has been one of the best candidates for fast microanalysis due to its small volume and portability. MCE exhibits potential advantages including shorter analysis time, lower sample and reagent consumption, good integration and disposability. In recent years, MCE-based immunoassays have gained popular consideration [23–29]. Although a number of MCE-based immunoassay has been used for biochemical applications, to the best of our knowledge, there have been few attempts employing this technique for simultaneous quantification of multiple tumor markers.

In this research, an MCE system with laser induced fluorescence detection (LIF) was utilized for simultaneous immunoassay of CA125 and CA15-3. The immunoassay was based on the noncompetitive immunoreaction of CA125 and CA15-3 with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (Ab^*) of CA125 and CA15-3. After off-line incubation of CA125, CA15-3 with Ab^*, MCE was used for the separation of immunological reactants. The present method is suitable for the determination of CA125 and CA15-3 in biological fluids, and the

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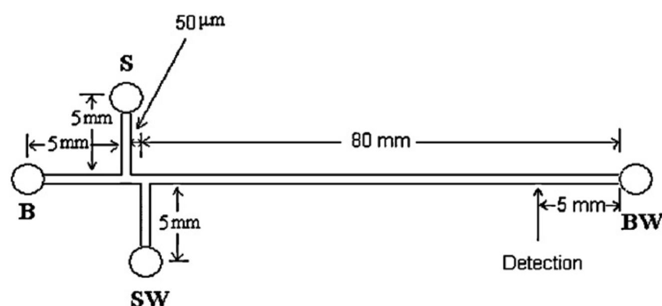


Fig. 1. Dimensions and layout of glass/PDMS microchip. S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir.

feasibility for clinical diagnosis of cancer patients was demonstrated.

2. Experimental

2.1. Chemicals and solutions

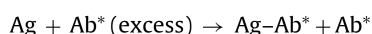
CA125 and CA15-3 were purchased from Saibo Biochem. Co. (Zhengzhou, China). FITC-labeled monoclonal antibodies (Ab^*) of CA125 and CA15-3 were purchased from Kezhou Marine Biotechnology Center (Beijing, China). SDS obtained from Shanghai Chemical Reagents Corporation (Shanghai, China). All other chemicals used in this work were of analytical grade. The CA125, CA15-3, Ab^*_{125} and $Ab^*_{15\text{-}3}$ solutions were prepared by dissolving the reagents in 20 mM phosphate buffer solution (PBS, pH 7.4). The electrophoretic buffer was 30 mM tris (hydroxymethyl) aminomethane (Tris) solution (pH 9.0) containing 60 mM SDS. Deionized water was used as the solvent throughout the work. All solutions were filtered through 0.22 μm membrane filters before use.

2.2. Apparatus

The microchip electrophoresis-confocal laser induced fluorescence detection system with 473 nm semiconductor laser was built by the Shandong Normal University. A multi-terminal high voltage power supply, variable in the range of 0–8000 V, was used for sample loading and MCE separation. The output signal was recorded and processed with a computer using a chromatography data system (Zhejiang University Star Information Technology, Hangzhou, China). A home-made glass/PDMS hybrid microfluidic chip was used for the separation of samples. The double "T" microchip was fabricated according to the procedures previously reported by Zhou et al. [30]. The layout and dimensions of the chip is shown in Fig. 1. All channels etched in glass substrates were 25 μm deep and 60 μm wide. The diameter of all reservoirs was 3.5 mm in diameter and 1.5 mm deep. The channel between reservoir S and SW was used for sampling, and the channel between B and BW was used for the separation.

2.3. Immunological reactions

Ten microlitres solution containing different concentrations of CA125 and CA15-3 or sample solution were mixed with 10 μL solution containing 0.2 μg/mL Ab^*_{125} and $Ab^*_{15\text{-}3}$ in a 300 μL reaction vial. 30 μL PBS was added. The mixture was vortexed and incubated with a dry heating block at 37 °C for 40 min. The whole procedure is illustrated in the following equations:



$$Ag + Ab^*(\text{excess}) \rightarrow Ag\text{–}Ab^* + Ab^*$$

2.4. Preparation of human serum samples

Human blood samples were kindly provided by the No. 5 People's Hospital (Guilin, China). Human blood samples were centrifuged at 2000 rpm for 15 min to obtain serum. These samples were stored at −20 °C until analysis. The serum samples were diluted 4 times for normal person and 10 times for cancer patients with PBS before being injected into the MCE-LIF system.

2.5. MCE procedure

Before repetitive runs started, the microfluidic channel was rinsed sequentially with 0.1 M NaOH, water, and electrophoretic buffer solution for 10 min each. Prior to electrophoresis, all reservoirs were filled with the electrophoretic buffer. Vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoretic buffer. Then, the electrophoretic buffer solution in reservoir S was replaced by sample solution. For loading the sample solution, a set of electrical potentials were applied to four reservoirs: reservoir S at 900 V, reservoir B at 250 V, reservoir BW at 400 V and reservoir SW at grounded. The sample solution was transported from reservoir S to SW in pinched mode. After 15 s, potentials were switched to reservoir B at 2800 V, while reservoir BW was grounded for separation and detection. During separation, the sample between the S reservoir and SW reservoir was drawn back by applying a pinched voltage of 1800 V to prevent the sample entering the separation channel.

All experiments that have been carried out for optimization/application were under experimental.

3. Results and discussion

3.1. Effects of incubation time

The incubation time had a great influence on the immunocomplex formation. In this experiment, CA125 and CA15-3 were determined by noncompetitive immunoassay. The excess Ab^*_{125} and $Ab^*_{15\text{-}3}$ were added to the sample solution containing CA125 and CA15-3 to obtain a mixture including CA125-Ab^*_{125} complex, CA15-3-$Ab^*_{15\text{-}3}$ complex, Ab^*_{125} and $Ab^*_{15\text{-}3}$. The incubation time of Ab^*_{125} and $Ab^*_{15\text{-}3}$ to CA125 and CA15-3 binding reaction was studied based on analysis of single CA125-Ab^*_{125} and CA15-3-$Ab^*_{15\text{-}3}$ complexes. Seven identical samples were incubated for 20–50 min, and then the solution was injected into the MCE system to determine the optimal incubation time when the maximum binding yield was obtained. It was found that increasing the incubation time from 20 min to 35 min increased the fluorescence intensity of CA125-Ab^*_{125} and CA15-3-$Ab^*_{15\text{-}3}$ complexes. When the incubation time was over 35 min, the fluorescence intensity of CA125-Ab^*_{125} and CA15-3-$Ab^*_{15\text{-}3}$ complexes was constant (data not shown). So an incubating time of 40 min was selected as this study.

3.2. Optimization of separation conditions

The parameters affecting the resolution such as concentration of electrolyte, pH of running buffer, SDS concentration and applied voltage were optimized. The pH of the running buffer has an important effect on the channel-wall surfaces characteristics of microchip and the effective electric charge of the analytes. In this work, the Tris buffer was used as background electrolyte for the separation of CA125-Ab^*_{125} complex, CA15-3-$Ab^*_{15\text{-}3}$ complex, Ab^*_{125} and $Ab^*_{15\text{-}3}$, and the concentration and pH of Tris buffer were optimized. The results indicate that the resolutions (R_s) values of CA125-Ab^*_{125}/Ab^*_{125}, CA15-3-$Ab^*_{15\text{-}3}$/$Ab^*_{15\text{-}3}$ and CA125-Ab^*_{125}/$Ab^*_{15\text{-}3}$ increases with the increase of running buffer pH from 7.8 to 9.0, while they decrease with further increasing of

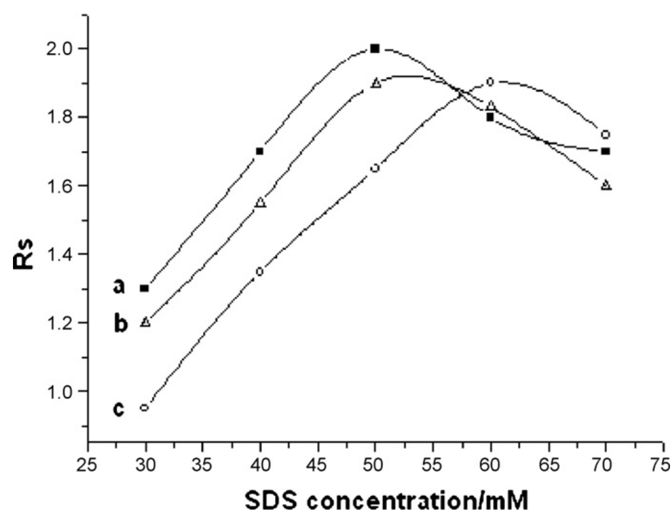


Fig. 2. Effects of SDS concentration on the separation. Sample solution contains 10 U/mL Ab^*_{125}, $Ab^*_{15\text{-}3}$ and 0.25 μg/mL CA125, CA15-3. Electrophoretic buffer was 30 mM Tris solution (pH 9.0) containing different concentrations of SDS. Separation voltage was 2800 V. R_s values are (a) for CA125-Ab^*_{125}/Ab^*_{125}, (b) for CA15-3-$Ab^*_{15\text{-}3}$/$Ab^*_{15\text{-}3}$, and (c) for CA125-Ab^*_{125}/$Ab^*_{15\text{-}3}$.

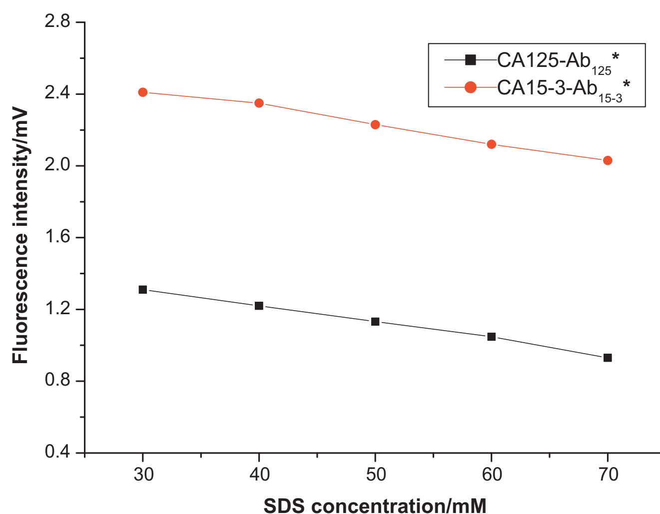


Fig. 3. Effects of SDS concentration on formation of immune complexes. A mixture solution containing 10 U/mL Ab^*_{125}, $Ab^*_{15\text{-}3}$ and 0.25 μg/mL CA125, CA15-3. Electrophoretic buffer was 30 mM Tris solution (pH 9.0) containing different concentrations of SDS. Separation voltage was 2800 V.

running buffer pH to 9.4 (data not shown). Therefore, the running buffer with pH value of 9.0 was used for further experiments.

The effect of the different buffer concentrations on R_s was also investigated. The R_s values for CA125-Ab^*_{125}/Ab^*_{125} and CA125-Ab^*_{125}/$Ab^*_{15\text{-}3}$ increases with the increase of buffer concentration from 20 mM to 30 mM, while for CA15-3-$Ab^*_{15\text{-}3}$/$Ab^*_{15\text{-}3}$ is from 20 mM to 35 mM. A further increase of the buffer concentration resulted in significantly decreased R_s (data not shown). Therefore, a concentration of 30 mM Tris buffer was used for further experiments.

The separation of protein on MCE often suffers from analyte adsorption onto channel-wall surfaces. One way to overcome this problem is to use surface modifications. Surfactants are the most widely used dynamic coating reagent in MCE-based immunoassay [31,32]. In this work, a popular surfactant, SDS, was selected as a dynamic coating reagent added into the running buffer [33], and the influence of the SDS concentration on R_s ranging from 30 mM to 70 mM was investigated. The results are shown in Fig. 2, the R_s values for CA125-Ab^*_{125}/Ab^*_{125} (Fig. 2, trace a) and CA15-3-$Ab^*_{15\text{-}3}$/$Ab^*_{15\text{-}3}$ (Fig. 2, trace b) increases with the increase of SDS concentration from 30 mM to 50 mM, while for CA125-Ab^*_{125}/$Ab^*_{15\text{-}3}$ (Fig. 2, trace c) is from 30 mM to 60 mM. When SDS concentration was 60 mM, all R_s values are higher than 1.8, which has reached baseline separation. With further increases in the SDS concentrations, the R_s decreased gradually. On the other hand, the introduction of SDS may induce structural changes in proteins which could severely affect the molecular recognition process between Ag–Ab. Therefore, the influence of SDS concentration on the immune complexes recognition was also investigated. It was found that the fluorescence intensity of immune complexes decreases gradually with the increase of SDS concentration from 30 mM to 70 mM (Fig. 3), but this influence is slight when SDS concentration was lower than 60 mM. Base on above experiment results, a 60 mM SDS concentration was employed in this study.

Separation voltage also affected R_s. It was examined in the range of 2300–3000 V. The results showed that the migration times and R_s decreased with the increase in separation voltage (data not shown). Considering both the analysis time and R_s, a separation voltage of 2800 V is considered optimal.

According to the experimental results described above, the optimal conditions for the separation of CA125-Ab^*_{125} complex, CA15-3-$Ab^*_{15\text{-}3}$ complex, Ab^*_{125} and $Ab^*_{15\text{-}3}$ were confirmed as following: 2800 V separation voltage and a running buffer containing 30 mM Tirs and 60 mM SDS at pH 9.0. Under this optimized condition, the typical electropherograms of the noncompetitive immunoassay of CA125 and CA15-3 was shown in Fig. 4. It can be seen from the electropherograms that the CA125-Ab^*_{125} complex, CA15-3-$Ab^*_{15\text{-}3}$ complex, Ab^*_{125} and $Ab^*_{15\text{-}3}$ were all well separated within 80 s. Trace A was obtained from a solution containing both Ab^*_{125} and $Ab^*_{15\text{-}3}$ only. Trace B was obtained from a solution containing Ab^*_{125}, $Ab^*_{15\text{-}3}$, CA125-Ab^*_{125} complex and CA15-3-$Ab^*_{15\text{-}3}$ complex. Two new peaks that were from the CA125-Ab^*_{125} complex and CA15-3-$Ab^*_{15\text{-}3}$ complex were observed in the trace B. It is worth noting that CA125-Ab^*_{125} complex, CA15-3-$Ab^*_{15\text{-}3}$ complex, Ab^*_{125} and $Ab^*_{15\text{-}3}$ were well separated each other, making the assay very selective and useful for simultaneous determination of CA125 and CA15-3 in human serum.

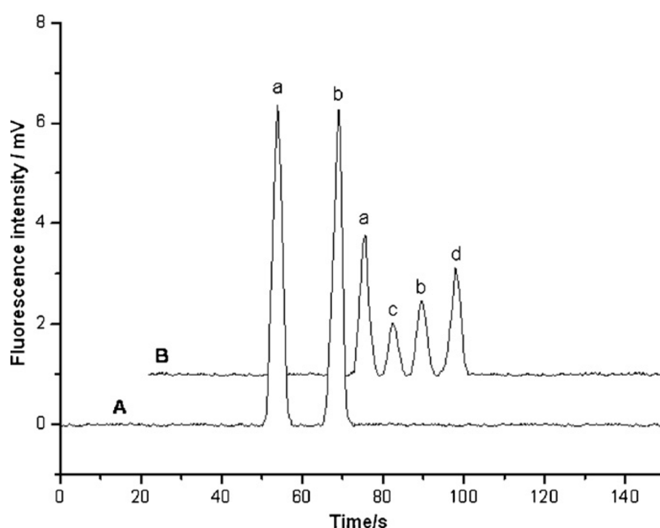


Fig. 4. Electropherograms from separating the noncompetitive immunoreaction solutions. (A) A mixture solution containing 10 U/mL Ab^*_{125} and $Ab^*_{15\text{-}3}$; (B) a mixture solution containing 10 U/mL Ab^*_{125}, $Ab^*_{15\text{-}3}$ and 0.25 μg/mL CA125, CA15-3. Electrophoretic buffer was 30 mM Tris solution (pH 9.0) containing 60 mM SDS. Separation voltage was 2800 V. Peak identification: (a) Ab^*_{125}; (b) $Ab^*_{15\text{-}3}$; (c) CA125-Ab^*_{125} complex; and (d) CA15-3-$Ab^*_{15\text{-}3}$ complex.

3.3. Analytical figures of merit

The present noncompetitive immunoassay method based on MCE-LIF was evaluated in terms of the response linearity, limit of detection (LOD) and reproducibility. Under the optimized analysis conditions, a series of CA125 and CA15-3 standard solutions were tested to determine the linearity between CA125, CA15-3 concentrations and fluorescence intensity (peak high) of CA125-Ab^*_{125} complex, CA15-3-$Ab^*_{15\text{-}3}$ complex. Linear regression analysis of the results yielded the following equations:

CA125 $H = 89.991C - 2.882 \quad r^2 = 0.9934$
CA15-3 $H = 208.13C - 0.264 \quad r^2 = 0.9961$

where H is the fluorescence intensity (μV), and C is the concentration of CA125 and CA15-3 in the derivative solution (U/mL). The calibration curves exhibited an excellent linear behavior over the concentration range of from 0.5 U/mL to 40 U/mL for CA125 and 0.2 U/mL to 20 U/mL for CA15-3. Based on S(signal)/N(noise) = 3, the detection limits for CA125 and CA15-3 were estimated to be 0.23 U/mL and 0.09 U/mL, respectively. Assay reproducibility was studied by analyzing a standard solution containing 2 U/mL CA125 and 2 U/mL CA15-3 ten times and recording the migration times and peak heights. The relative standard deviations (RSD) for the peak heights and migration times were all lower than 4.6%.

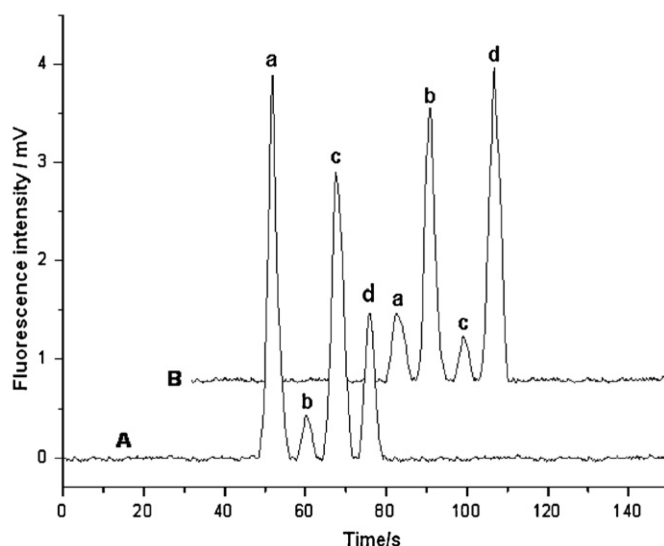


Fig. 5. Electropherograms from the separating a serum sample of normal person (A) and a serum sample of patient with ovarian cancer (B). MCE conditions were as in Fig. 2. Peak identification: (a) Ab^*_{125}; (b) CA125-Ab^*_{125} complex; (c) $Ab^*_{15\text{-}3}$; and (d) CA15-3-$Ab^*_{15\text{-}3}$ complex.

3.4. Analysis of human serum

Human sera samples taken from five healthy volunteers (normal person) and nine patients suffering from various cancer diseases were analyzed. Before analysis, the samples were diluted 4 times for normal person and 10 times for cancer patients with PBS. Electropherograms obtained from analyzing the serum samples of a normal person and a cancer patient are shown in Fig. 5. As can be seen by comparing the two traces, the peaks of CA125-Ab^*_{125} and CA15-3-$Ab^*_{15\text{-}3}$ complexes in cancer patient serum were higher than that in normal person serum. Table 1 summarizes the analytical results of human sera samples. In the sera of normal persons, CA125 and CA15-3 had the serum levels ranged from 15.6 U/mL to 36.1 U/mL and 13.8 U/mL to 28.4 U/mL. While in the sera of cancer patients, CA125 and CA15-3 had the serum levels ranged from 92.5 to 368.3 U/mL and 63.3 to 198.4 U/mL, respectively. These results demonstrated that the serum CA125 and CA15-3 levels were higher remarkably in cancer patients compared to normal persons, which was in agreement with previous reports [34,35].

Table 1
Analytical results of human sera samples.

Sample	CA125 (U/mL)	RSD (%, n = 5)	CA15-3 (U/mL)	RSD (%, n = 5)
1	163.5	4.3	198.4	2.6
2	127.2	3.8	160.6	3.9
3	368.3	3.1	145.0	4.7
4	286.1	2.4	113.8	3.4
5	156.9	5.1	109.6	2.9
6	178.2	3.5	132.5	2.8
7	121.0	1.	82.4	5.0
8	101.5	2.2	63.3	3.3
9	92.5	2.7	74.9	4.4
10	34.8	3.9	13.8	2.6
11	25.6	2.1	17.3	3.6
12	36.1	3.6	20.4	2.0
13	20.2	3.7	15.1	2.3
14	15.6	4.0	28.4	3.8

1 and 2: breast cancer; 3 and 4: ovarian cancer; 5 and 6: liver cancer; 7 and 8:lung cancer; 9: thyroid cancer; 10–14: healthy person.

3.5. Method validation and comparison

To verify the experimental reliability, the human sera samples taken from five healthy volunteers were analyzed by an approved enzyme-linked immunoassay (ELISA) method. Analytical results were comparable with of presented method (Table 2). The CA125 and CA15-3 concentrations determined in five human sera samples gave good agreement between MCE-LIF immunoassay and the validated ELISA procedure. The assay precision was evaluated by repeatedly analyzing each human serum sample five times within a working day. RSDs for CA125 and CA15-3 detection were between 1.9% and 5.1%. Recoveries of CA125 and CA15-3 from serum sample were also studied. CA125 and CA15-3 were spiked to three portions of serum sample of a normal person at 1.0, 5.0 and 10 U/mL, and the samples were then analyzed again. Recoveries were found to be in the range of 91.4–105.3% and 93.6–107.2%, respectively.

Table 2
The results for the determination of CA125 and CA15-3 in human sera samples from healthy person by MCE-LIF immunoassay and enzyme-linked immunoassay (ELISA) methods.

Sample number	Present method		ELISA		Relative error	
	CA125 (U/mL)	CA15-3 (U/mL)	CA125 (U/mL)	A15-3 (U/mL)	CA125 (%)	CA15-3 (%)
1	34.8	13.8	32.9	14.5	5.8	−4.8
2	25.6	17.3	26.8	18.8	−4.5	−8.0
3	36.1	20.4	38.0	19.5	−5.0	4.6
4	20.2	15.1	19.2	15.8	5.2	−4.4
5	15.6	28.4	16.1	28.5	−3.1	−0.4

4. Conclusions

Sera CA 125 and CA15-3 have been widely considered to be two especially valuable serum markers for clinical diagnosis of ovarian cancer and breast cancer. However, a single marker is often not sufficient for diagnosis purpose due to limited specificity and sensitivity. The assay of cancer marker panel can improve the diagnosis value of disease. The present protocol combines the immunoassay and MCE technique to achieve a multiplexed immunoassay of CA125 and CA15-3 in human serum. The assay was successfully used for simultaneous determination of CA125 and CA15-3 contents in sera from healthy controls and patients with ovarian cancer, breast cancer, liver cancer, lung cancer and thyroid cancer. The analysis results showed that the levels of CA125 and CA15-3 in the sera of cancer patients were significantly elevated over that of the healthy controls. Compared with conventional methods, the proposed MCE-LIF immunoassay offers significant advantages over the methods that have previously been used to detect the levels of CA125 and CA15-3 in the human serum. The method requires shorter analysis time (less 1.5 h), lower reagent consumption (less 0.5 μL) and simple operation (ca. two steps). This may be contrasted with enzyme immunoassay based methods that was time-consuming (ca. 4 h), high reagent consumption (ca. 5 μL) and complicated operation (over 10 steps). Especially, a multiplexed immunoassay could be achieved. Combined with commercial immunoassay kits, MCE-LIF immunoassay will become a useful tool in clinical diagnosis.

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