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Aptamer-based microchip electrophoresis assays for amplification detection of carcinoembryonia antigen

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Abstract

Background—Carcinoembryonic antigen (CEA) as one of the most widely used tumor marker is used in the clinical diagnosis of colorectal, pancreatic, gastric, and cervical carcinomas. We developed an aptamer-based microchip electrophoresis assay technique for assaying CEA in human serum for cancer diagnosis.

Methods—The magnetic beads (MBs) are employed as carriers of double strand DNA that is formed by an aptamer of target and a complementary DNA of aptamer. After the aptamer in MB-dsDNA conjugate binds with target, the complementary DNA was released from MB-dsDNA conjugate. The released complementary DNA hybridizes with a fluorescein amidite (FAM) labeled DNA, and forms DNA duplex, which triggers the selective cleavage of FAM labeled DNA by nicking endonuclease Nb.BbvCI, and generating FAM labeled DNA segment. The released complementary DNA hybridizes with another FAM labeled DNA, resulting in a continuous cleavage of FAM labeled DNA, and the generation of large numbers of FAM labeled DNA segments. In MCE laser induced fluorescence detection (LIF), FAM labeled DNA segment is separated and detected.

Results—The linear range for CEA was 130 pg/ml~8.0 ng/ml with a correlation coefficient of 0.9916 and a detection limit of 68 pg/ml. The CEA concentration in the serum samples from healthy subjects was found be in the range 1.3 ng/ml to 3.2 ng/ml. The CEA concentration in the samples from cancer patients was found to be >15 ng/ml.

Conclusions—This method may become a useful tool for rapid analysis of CEA and other tumor markers in biomedical analysis and clinical diagnosis.

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Keywords

aptamer; carcinoembryonic antigen; microchip electrophoresis; cancer

1. Introduction

Micro-total analysis system (μ TAS) has served as a focal point to bring together the different research fields [1–3]. Microchip electrophoresis (MCE) has been the most successful analytical technique for practical applications of the μ TAS, and has exhibited prominent advantages in terms of low sample consumption, rapid analysis times, easy operation, efficient resolution of compounds, and increased throughput. In the past decade, many academic laboratories have demonstrated the potential of MCE as a powerful analytical tool leading to the next generation in chemical separation/detection technologies [4]. This technology has lead to more research focus on analysis particularly in hospital settings for clinical diagnostics [5] including the detection of disease biomarkers [6], DNA [7], RNA [8] and proteins [9]. However, since the microfluidic channel for the detection was extremely small, the sensitivity of MCE methods reported for clinical analysis was in the range of 10^{-6} – 10^{-9} M (limit of detection, LOD), which was hardly sufficient for quantifying trace components in biological samples. Therefore, effectively detecting the separated trace analytes by MCE remains still a challenge.

In order to obtain high sensitivity for the detection of trace proteins, aptamer based amplification assays have been paid more and more attention [10], such as rolling circle amplification [11,12], isothermal circular strand-displacement polymerization [13], and DNzyme based isothermal amplification [14,15]. Although many aptamer based amplification methods have been reported for sensing of various targets, few methods are currently available for amplified MCE-laser induced fluorescence (LIF) assay.

Carcinoembryonia antigen (CEA) as one of the most widely used tumor marker is used in the clinical diagnosis of colorectal, pancreatic, gastric and cervical carcinomas [16–19]. Meanwhile, the CEA concentration in serum is also related to the stage of the tumor, the outcome of therapy and the prognosis [20], so it can be used as a marker to directly evaluate curative effects, recrudescence, and metastasis [21]. Various immunoassays, ranging from the radioimmunoassay [22], enzyme-linked immunosorbent assay [23], time-resolved fluoroimmunoassay [24], chemiluminescence immunoassay [25] and MCE-based immunoassay [26] to impedimetric immunoassay [27] were developed for CEA detection. However, the conventional immunoassay requires a relatively long assay time, and involves troublesome liquid-handling procedures and many expensive antibody reagents.

Aptamers are single stranded oligonucleotides selected in vitro by the systematic evolution of the ligand by the exponential enrichment (SELEX) process from random-sequence nucleic acids libraries [28]. Compared to other recognition elements, such as antibodies, aptamers have many advantages, such as simple synthesis, good stability, high affinity, excellent selectivity and wide applicability, making them suitable candidates for biological application [29].

2. Materials and Methods

2.1 Materials and reagents

All oligonucleotides and fluorescein amidite (FAM) labeled DNA probe used in this work were synthesized and HPLC-purified by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The sequences of oligonucleotides were 5'-NH₂-ATA CCA GCT TAT TCA ATT-3' and 3'-T CGA ATA AGT TAA CGA CTC CTC GTT A-5'. The sequence of FAM labeled DNA probe was 5'-FAM-ATT GCT GAG GAG C-3'. CEA, carboxyl-modified MBs (75 µm, 50.0 mg/ml), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS), thiazole orange (TO), bovine serum albumin (BSA) and tetrabutylammonium phosphate (TBAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxypropyl methylcellulose (HPMC) was from Dow Chemical Co. The nicking enzyme Nb.BbvCI and 10×NEB buffer 2 (500 mmol/l NaCl, 100 mmol/l Tris-HCl, 100 mmol/l MgCl₂, and 10 mmol/l dithiothreitol, pH 7.9) were from the New England Biolabs, Inc. 2-(N-morpholino)-ethanesulfonic acid monohydrate (MES) buffer (pH 6.0) was from Fluka BioChemika (Buchs, Switzerland). All the other chemicals used in this work were of analytical grade. Water was purified by employing a Milli-Q plus 185 equip from Millipore, and used throughout the work.

The oligonucleotides were used as provided and diluted in 20 mmol/l Tris-HCl buffer solution (pH 7.4) to give a stock solutions of 20 µmol/l. 20 µmol/l 5'-NH₂-ATA CCA GCT TAT TCA ATT-3' and 20 µmol/l 3'-T CGA ATA AGT TAA CGA CTC CTC GTT A-5' solution were mixed and heated to 95 °C for 3 min, and slowly cooled down to room temperature to obtain dual stranded DNA (dsDNA) before use. The electrophoretic buffer was 20 mmol/l phosphate buffer solution (pH 7.4) containing 0.8% HPMC and 15 mmol/l TBAP. All solutions for MCE 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). 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 [30]. The layout and dimensions of the chip was shown in Fig. 1. All channels etched in glass substrates were 25 µm deep and 45 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 Preparation of dsDNA immobilized MBs

The dsDNA was immobilized on the MBs according to the procedure described by Bi et al. [31] with a slight modification. Briefly, a suspension of MBs (200 µl, 50.0 mg/ml) in a 5 ml eppendorf tube was separated magnetically. The MBs were washed 3 times with MES buffer (3×300 µL), and then suspended to a final volume of 200 µl in same buffer solution. A 0.2 M

NHS solution (200 μ l) and a 0.8 M EDC solution (200 μ l) were added to the eppendorf tube, and the mixture was incubated at room temperature for 2 h to activate the carboxylate groups on the MBs. The MBs were then washed 3 times with 10 mmol/l phosphate buffer solution (3 $\times$ 300 μ l), and resuspended to a final volume of 2.0 ml. An aqueous solution of the dsDNA (200 μ l, 6.25×10^{-7} mol/l) was then added to 500 μ l activated MBs solution, and the resulting suspensions were allowed to stand for 24 h at 25 $^{\circ}$ C for the immobilization of the dsDNA on the surface of the activated MBs. Finally, the resulting MBs-dsDNA conjugates were separated magnetically, and washed three times with 10 mmol/l phosphate buffer solution (3 $\times$ 300 μ l), and then resuspended in 400 μ l of 10 mmol/l phosphate buffer solution containing 1.0% BSA. The solution was incubated at 25 $^{\circ}$ C for 2 h to eliminate the risk of unspecific binding, and separated magnetically. Then, MBs-dsDNA conjugate was washed three times with 10 mmol/l phosphate buffer solution (3 $\times$ 300 μ l), and resuspended to a final volume of 1.0 ml with 10 mmol/l phosphate buffer solution. This suspension was stored at 4 $^{\circ}$ C for further use.

2.4 Preparation of human serum samples

Human blood samples were kindly provided by the No. 5 People's Hospital. Human blood samples were centrifuged at 2000 rpm for 15 min to obtain serum. These samples were stored at -20° C until analysis, and diluted 10 fold with 10 mmol/l phosphate buffer solution before analysis.

2.5 Amplification reaction

A 10 μ L volume of standard CEA solution or sample solution was mixed with 90 μ l of MBs-dsDNA conjugate solution in a 0.5-ml centrifuge tube. This mixture solution was then incubated for 1 h at 37 $^{\circ}$ C. The resulting solution was separated magnetically, and 95 μ l of supernatant was transferred into a 0.5-ml centrifuge tube. Subsequently, 5 μ l of 50 μ mol/l FAM labeled DNA probe solution was added, and incubated for 1 h at 37 $^{\circ}$ C. Finally, 11 μ l of 10 $\times$ NEB buffer 2 and 3 μ l of 10 u/ μ l Nb.BbvCI solutions were added, and incubated for 2 h at 37 $^{\circ}$ C. The resulting solution was analyzed by MCE-LIF.

2.6 MCE-LIF procedure

Before repetitive runs started, the microfluidic channel was rinsed sequentially with 0.1 mol/l 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 500 V, reservoir B at 250 V, reservoir BW at 350 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 2500 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 1500 V to prevent the sample entering the separation channel.

3 Results and discussion

3.1 Principle of magnetic bead assisted target-induced strand circle signal amplification

In this work, MBs are employed as carriers of dsDNA formed by an aptamer of the target and its complementary DNA strand. FAM labeled DNA is used as the signaling probe. The principle for signal amplification is illustrated in scheme 1. Firstly, the aptamer hybridizes with its complementary DNA strand to form dsDNA, the carboxyl-modified MBs are then conjugated with dsDNA. MB-dsDNA conjugates are incubated with the target to release the complementary DNA of aptamer. The released complementary DNA hybridizes with FAM labeled DNA probe, and forms a DNA duplex, which triggers the selective cleavage of the FAM labeled DNA probe by nicking endonuclease Nb.BbvCI, leading to a continuous release of the complementary DNA. The released complementary DNA then hybridizes with another FAM labeled DNA probe, and the cycle starts anew, resulting in a continuous cleavage of the FAM labeled DNA, and the generation of large numbers of FAM labeled DNA segments, which result in amplification of fluorescence signal. Superfluous FAM labeled DNA probe and FAM labeled DNA segment are then separated by MCE, and detected by LIF. The peak height (fluorescence intensity) of FAM labeled DNA segment is used as quantification of the target.

3.2 Characterization of MB-dsDNA conjugates

TO, an asymmetric cyanine dye is essentially nonfluorescent in aqueous solution. It combines with the minor groove, intercalates between base pairs of dsDNA, resulting in a high fluorescence emission, which can be used to detect different DNA and RNA structures. Therefore, the fluorescence spectrum of TO intercalating dsDNA was used to characterize the formation of MBs-dsDNA conjugates. As shown in Fig. 2, a fluorescence peak at 530 nm was appeared in curve c (containing TO and MBs-dsDNA), which was corresponding to the fluorescence peak of TO after intercalating between base pairs of dsDNA. While no fluorescence peak was observed in curve a (only containing TO) and curve b (containing TO and MBs). These results indicate the dsDNA has been immobilized on the MBs, and demonstrated the formation of the MBs-dsDNA conjugates.

3.3 Effect of Nb.BbvCI concentration on the amplification reaction

The nicking enzyme used in this work is Nb.BbvCI, which recognizes a simple asymmetric sequence, 5'-GCTGAGG-3', and cleaves DNA at a fixed position relatively to the recognition sequence. Therefore, the concentration of Nb.BbvCI plays an important role in the amplified process. To investigate the effect of the concentration of Nb.BbvCI, complementary DNA and FAM labeled DNA probe were mixed with different concentrations of Nb.BbvCI ranging from 0 U to 50 U. The fluorescence intensities of FAM labeled DNA segment increased rapidly with increasing concentration of Nb.BbvCI to 30 U, and then remained constant until 50 U.

3.4 Effects of the incubation time on the amplification reaction

The test for the effects of incubation time on the amplification reaction was performed by monitoring the fluorescence intensity of FAM labeled DNA segment as a function of time. It

is found that the fluorescence intensity of FAM labeled DNA segment increased rapidly with increasing the incubation time up to 120 min, and then remained constant. Therefore, 120 min was selected for the cleaving reaction in this work.

3.5 Optimization of MCE conditions

The conditions for MCE separation including the concentrations of the electrophoretic buffer, HPMC and TBAP, as well as separation potential were optimized, and the separations were characterized by resolution (R_s) values ($R_s = 2(T_2 - T_1)/(W_2 + W_1)$), where T_1 and T_2 are the migration time of peak-1 and peak-2, W_1 and W_2 are the bottom width of peak-1 and peak-2).

The influence of buffer concentration on the R_s was investigated with a phosphate buffer of pH 7.4 containing 0.8 % HPMC and 15 mmol/l TBAP. The separation of FAM labeled DNA probes and FAM labeled DNA segments was enhanced with the increase of buffer concentration in the range of 5 mmol/l to 20 mmol/l, the R_s value is more than 1.5 at 20 mmol/l. Further increasing the concentration of phosphate resulted in the decrease of R_s value. Therefore, a concentration of 20 mmol/l phosphate was chosen as the optimization.

HPMC is one of the most frequently used sieving matrices for the separation of DNA fragments by CE. The effect of HPMC concentration in a range of 0.2–1.0% (w/v) was investigated. The results indicate that R_s value for the separation of FAM labeled DNA probe and FAM labeled DNA segment increased with the increase of HPMC concentrations. However, the migration times of both FAM labeled DNA probe and FAM labeled DNA segment were increased significantly at high HPMC concentrations. A concentration of 0.8% HPMC was selected for an acceptable R_s and a favorable separation time.

TBAP as an ion pair reagent was employed as an additive in the electrophoretic buffer solution to improve the separation of DNA fragments because TBAP having a positive charge can interact with DNA taking negative charges through electrostatic interaction. The effect of TBAP concentration in a range of 3–18 mmol/l was investigated. The results indicate that a maximal R_s value for the separation of FAM labeled DNA and FAM labeled DNA segment was obtained when the concentration of TBAP was 15 mmol/l.

Separation voltage affected the migration rate and the R_s value for the separation of FAM labeled DNA and FAM labeled DNA segment. It was examined in the range of 1500–3000 V. The results showed that migration times decreased and the R_s increased with the increase of separation voltage from 1500 V to 2500 V. Further increasing the separation voltage resulted in the decrease of R_s value. Therefore, 2500 V was chosen as optimal separation voltage.

According to the experimental results described above, optimal conditions for the separation of FAM labeled DNA probe and FAM labeled DNA segment were confirmed as following: 2500 V separation voltage and a running buffer containing 20 mmol/l phosphate, 0.8 % HPMC and 15 mmol/l TBAP at pH 7.4. Under the optimized conditions, the typical electrophorogram for the separation of FAM labeled DNA probes and FAM labeled DNA

segment was shown in Fig. 3. As can be seen, FAM labeled DNA probes and FAM labeled DNA segment were well separated within 1.5 min.

3.6 Analytical figures of merit

The method was evaluated in terms of response linearity, limit of detection (LOD), and reproducibility. Under the selected MCE assay conditions, nine standard CEA solutions at various concentrations were analyzed, and the electropherograms for standard CEA solutions at various concentrations were shown in Fig. 4. Linear regression analysis of data yielded the following equation: $H=241.96C+3.25$, where H is the fluorescence intensity (μV), and C is the concentration of CEA (ng/ml). The calibration curve showed excellent linearity in the range of 0.13–8.0 ng/ml with a correlation coefficient of 0.9916. Based on a signal/noise ratio (S/N) of 3, LOD for CEA was estimated to be 0.068 ng/ml (3.4×10^{-13} mol/l). This LOD value was far below the threshold of CEA (2.5 ng/ml) in normal human serum [32]. It was also below the LOD values obtained from inductively coupled plasma mass spectrometry (0.14 ng/ml) [33], capillary-based three-dimensional immunosensor (0.2 ng/ml) [34], size exclusion chromatography with inductively coupled plasma mass spectrometric detection (2.6 ng/ml) [35], rolling circle amplification for protein detection (6.8×10^{-12} M) [11], and isothermal circular strand-displacement polymerization for protein detection (12 ng/ml) [13]. Assay reproducibility was estimated by analyzing a standard solution of CEA at 5.0 ng/ml (n=7). The results showed that the relative standard deviations (RSDs) of the migration time and peak height of the FAM labeled DNA segment were 3.5% and 2.6%, respectively.

3.7 Specificity of aptamer-based MCE method for CEA detection

To evaluate the specificity of proposed aptamer-based MCE method for CEA detection, some other nontarget tumor markers such as alpha-fetoprotein (α -AFP), carcinoma antigen 125 (CA-125), carbohydrate antigen 15-3 (CA15-3) and neuron-specific enolase (NSE) at the same concentration to CEA were further investigated. It was found that the target CEA can present two peaks, while all nontarget tumor markers can only present one peak in the electropherogram. These results clearly demonstrate the high specificity of the proposed aptamer-based MCE method to CEA.

3.8 Quantification of CEA in human serum

Human serum samples taken from three healthy volunteers and three colon cancer patients were analyzed to demonstrate the feasibility of proposed MCE–LIF method for the determination of CEA in clinical samples. The electropherograms obtained upon the analysis of the studied samples were shown in Fig. 5. The results were summarized in Table 1. The CEA concentration in the serum samples from healthy subjects was found to be in the range 1.3 ng/ml to 3.2 ng/ml. The CEA concentration in the samples from colon cancer patients was found to be >15 ng/ml. CVs for the determination of CEA in human serum samples were in the ranges of 1.8–4.2%. The feasibility of the current method for clinical applications was also investigated by analyzing clinical serum samples, in comparison with the ELISA method. Table 1 shows also the results obtained by the proposed method and ELISA method. The results exhibit an acceptable agreement between the 2 methods. Recovery of CEA from these samples was studied. CEA was spiked to serum sample and

analyzed (Fig. 4C). Recoveries were found to be in the range of 95–103% (Table 2). The results suggested a potential applicability of the proposed assay in express primary diagnosis of cancer diseases.

4 Conclusions

A novel strategy for improving detection sensitivity in MCE assays based on magnetic bead assisted target-induced strand circle has been developed. As a proof of concept, we demonstrate that the proposed MCE-LIF assay can detect CEA in human serum samples taken from healthy subjects and cancer patients with very high sensitivity and specificity. The developed MCE-LIF platform may provide a cost-effective means for reliable point-of-care diagnostics of cancers. This amplified MCE-LIF assay has several excellent features. Firstly, by using the MBs assisted target-induced strand circle approach, the sensitivity of method is at least two orders of magnitude higher than that of traditional immunoassay. Secondly, by taking advantage of affinity and selectivity of aptamer, the proposed MCE-LIF assay showed significantly high specificity toward target and could distinguish the target molecules from their analogues. In addition, the proposed signal amplification strategy would open new avenues on the developing highly sensitive MCE-LIF methods. We anticipate that this strategy can be applied readily to detecting other biomarkers by simply changing relevant aptamer sequences in MB-dsDNA conjugates, and thus finds a wide application in disease diagnosis.

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Abbreviations

MCE	microchip electrophoresis
LIF	laser induced fluorescence
FAM	fluorescein amidite
MBs	magnetic beads
TO	thiazole orange
HPMC	Hydroxypropyl methylcellulose
TBAP	tetrabutylammonium phosphate

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Highlights

A novel strategy for improving detection sensitivity in MCE assays was developed.

This strategy was based on the aptamer recognition and target-induced strand circle.

This assay is recommended for amplification detection of carcinoembryonia antigen.

This method may become a useful tool for rapid analysis of tumor markers.

Fig. 1. Dimensions and layout of the glass/PDMS microchip used in this work: S, sample reservoir; B, buffer reservoir; SW, sample waste reservoir; BW, buffer waste reservoir.

Fig. 2.
Fluorescence spectra of TO (a), TO+MBs (b) and TO+MBs-dsDNA (c).

Fig. 3.

Electropherogram obtained from a CEA standard solution at 6.0 ng/ml. MCE conditions were as following: 2500 V separation voltage and a running buffer containing 20 mmol/l phosphate, 0.8 % HPMC and 15 mmol/l TBAP at pH 7.4. Peak identification: (1) FAM labeled DNA segment; (2) FAM labeled DNA.

Fig. 4. Electropherograms for CEA standard solutions at various concentrations. The concentrations of CEA were 0 (a), 0.13 (b), 0.30 (c), 0.40 (d), 0.60 (e), 0.80 (f), 2.0 (g), 4.0 (h), 6.0 (i) and 8.0 (j) ng/ml. Peak identification: (1) FAM labeled DNA segment; (2) FAM labeled DNA. MCE conditions were as in Fig. 3.

Fig. 5. Electropherograms obtained from the serum samples of a healthy volunteer (A), a cancer patient (B) and a cancer patient spiked with CEA at 2.0 ng/ml (C). Peak identification: (1) FAM labeled DNA segment; (2) FAM labeled DNA. MCE conditions were as in Fig. 3.

Scheme 1.

Schematic illustration of detection principle with the proposed aptamer- based microchip electrophoresis assays for amplification detection of carcinoembryonia antigen.

Table 1

Analytical results of CEA in human serum.

Sample	Found by present method (ng/ml)	RSD (%, n=5)	Found by ELISA* (ng/ml)
Normal person 1	1.35	3.8	1.33
Normal person 2	2.12	3.6	2.14
Normal person 3	3.20	4.2	3.41
Colon cancer patient 1	40.11	1.8	40.00
Colon cancer patient 2	18.40	2.5	18.24
Colon cancer patient 3	68.62	3.5	67.69

Table 2

Recovery of CEA in human serum samples by the proposed MCE-LIF assay

Sample	Original Concentration (ng/ml)	Added concentration (ng/ml)	Found total concentration (ng/ml)	Recovery (%)
1	1.35	2.0	3.40	103
		20.0	21.3	99.8
2	40.1	2.0	42.1	100
		20.0	59.3	96.0
3	18.4	2.0	20.3	95.0
		20.0	38.5	101