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

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ABSTRACT

Background: Carcinoembryonic antigen (CEA) as one of the most widely used tumor markers 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 the target and a complementary DNA of the aptamer. After the aptamer in the MB–dsDNA conjugate binds with the target, the complementary DNA was released from the MB–dsDNA conjugate. The released complementary DNA hybridizes with a fluorescein amidite (FAM) labeled DNA, and forms a DNA duplex, which triggers the selective cleavage of FAM labeled DNA by nicking endonuclease Nb.BbvCI, and generating a 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), the 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 to 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.

1. Introduction

A micro-total analysis system (μTAS) 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 μTAS, 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 of chemical separation/detection technologies [4]. This technology has led 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 DNAzyme 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) assays.

Carcinoembryonic antigen (CEA) as one of the most widely used tumor markers 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

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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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 acid 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 the 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′-NH_2-ATA CCA GCT TAT TCA ATT-3′ and 3′-T CGA ATA AGT TAA CGA CTC CTC GTT A-5′. The sequence of the 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-hydroxysuccinimide (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 $MgCl_2$, and 10 mmol/l dithiothreitol, pH 7.9) were from 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 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 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

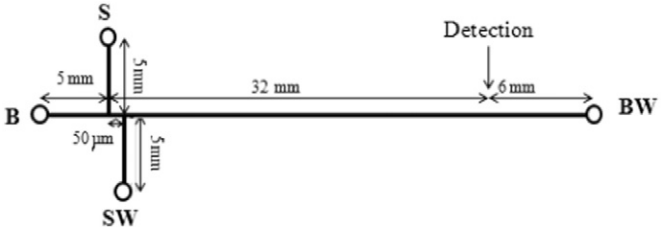

Fig. 1. Dimensions and layout of the glass/PDMS microchip used in this work: S, sample reservoir; B, buffer reservoir; SW, sample waste reservoir; and BW, buffer waste reservoir.

"T" microchip was fabricated according to the procedures previously reported [30]. The layout and dimensions of the chip were 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 reservoirs 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 the same buffer solution. A 0.2 M NHS solution (200 μl) and a 0.8 M EDC solution (200 μl) 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 × 300 μl), and resuspended to a final volume of 2.0 ml. An aqueous solution of the dsDNA (200 μl, 6.25×10^{-7} mol/l) was then added to 500 μl activated MB solution, and the resulting suspensions were allowed to stand for 24 h at 25 °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 × 300 μl), and then resuspended in 400 μl of 10 mmol/l phosphate buffer solution containing 1.0% BSA. The solution was incubated at 25 °C for 2 h to eliminate the risk of unspecific binding, and separated magnetically. Then, the MBs–dsDNA conjugate was washed three times with 10 mmol/l phosphate buffer solution (3 × 300 μl), and resuspended to a final volume of 1.0 ml with 10 mmol/l phosphate buffer solution. This suspension was stored at 4 °C for further use.

2.4. Preparation of human serum samples

Human blood samples were kindly provided by 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 μl volume of standard CEA solution or sample solution was mixed with 90 μl of MBs–dsDNA conjugate solution in a 0.5-ml centrifuge tube. This mixture solution was then incubated for 1 h at 37 °C. The resulting solution was separated magnetically, and 95 μl of supernatant was transferred into a 0.5-ml centrifuge tube. Subsequently, 5 μl of 50 μmol/l FAM labeled DNA probe solution was added, and incubated for 1 h at 37 °C. Finally, 11 μl of 10 × NEB buffer 2 and 3 μl of 10 u/μl Nb.BbvCI solutions were added, and incubated for 2 h at 37 °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 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