Talanta 189 (2018) 437–441

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A novel multiplex signal amplification strategy based on microchip electrophoresis platform for the improved separation and detection of microRNAs

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ARTICLE INFO

Keywords:
Microchip electrophoresis
Laser induced fluorescence detection
Multiplex amplification
MicroRNAs

ABSTRACT

A multiplex fluorescence signal amplification method based on microchip electrophoresis (MCE) platform was developed for the improvements in the separation and detection of microRNAs. The method used two kinds of fluorescein amidite labeled DNA signal probes to hybridize with its target microRNAs, utilizing T7 exonuclease assisting target circling realized the fluorescence signal amplification. Then, two kinds of fluorescein amidite labeled DNA segments with different size were separated and detected on the MCE-laser induced fluorescence detection platform. The microRNAs-126 and microRNAs-141 were used as model analytes in the proof-of-concept experiments. Two calibration curves between the fluorescence intensity and microRNAs concentration all showed good linearity in the range of 0.025–20 nM. The correlation coefficients obtained were 0.9975 and 0.9925, respectively. The limits of detection for two kinds of microRNAs were estimated to all be 15 pM. By spiking T24 cell lysate samples with varying amounts of miRNA-126 and miRNA-141, the recovery of analytes ranged from 96.0% to 115%, and the relative standard deviations are lower than 5.5%. The present method showed high sensitivity and selectivity, which has a promising application in biomedical research.

1. Introduction

MicroRNAs (miRNAs) are a kind of short, noncoding RNAs that contain about 18–22 nucleotides [1]. The miRNA genes are believed to exist in all multicellular organisms, and play very important roles in a wide range of physiologic and pathologic processes [2]. The anomaly of the miRNA expression level is associated with several diseases especially cancer [3]. As promising next-generation biomarkers for diagnosis and prognosis, the miRNAs assays have attracted more and more attention, and impressive progress has been made with the development of novel strategies for the sensitive detection of miRNA [4–10]. Although these strategies have become increasingly popular in the detection of miRNAs, these methods can only detect one kind of miRNA in every assay. Because many diseases are associated with multiple miRNAs, the detection of one kind of miRNA often limits its diagnostic value. Therefore, sensitive detection of multiplex miRNAs has obtained great significance.

Capillary electrophoresis (CE) is a wider accepted analytical technique, and has become a powerful micro analytical platform in biology and medicine due to its high separation efficiency, low reagent and sample consumption, and fast analysis speed [11,12]. In the past five years, several studies have been reported using CE technique for the detection of multiplex miRNAs. Krylov et al. developed a CE method for direct detection of multiplex miRNAs based on hybridization between DNA probe and miRNAs [13]. Song et al. designed three fluorescein amidite (FAM) and Cy5 labeled DNA probes to specifically bind to different target miRNA, achieved multiplex detection of miRNAs based on the CE separation [14]. By using the ligase chain reaction (LCR) combine with CE separation, Li et al. have achieved highly sensitive analysis of three miRNAs [15]. Lee et al. developed a multiplex miRNA profiling method using modified isothermal exponential amplification reaction combined with high-resolution CE-based single-strand conformation polymorphism [16]. Microchip electrophoresis (MCE) technology was evolved from CE, and has become an attractive separation and analysis technique [17–19]. Compared to the CE, MCE exhibits numerous superiority, such as integrating sample loading, pre-enrichment, separation and detection onto a single chip, and with much lower sample consumption, much higher separation efficiency, and easy operation etc [20–22]. Therefore, simultaneous, highly sensitive detection of multiple miRNAs in biotechnological applications and medical diagnostics is one of important goals for MCE.

The separation and simultaneous signal amplification of multiple

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https://doi.org/10.1016/j.talanta.2018.07.037
Received 11 April 2018; Received in revised form 8 July 2018; Accepted 11 July 2018
Available online 20 July 2018

miRNAs in MCE has not been reported. In this work, we developed a multiplex signal amplification strategy based on MCE platform for the improvement in separation and detection of miRNAs. The miRNA-126 and miRNA-141 were used as model analytes in the proposed proof-of-concept experiments. The present method for multiplexed miRNAs detection showed high sensitivity and selectivity, which has a promising application in disease diagnosis and biomedical research.

2. Experimental

2.1. Reagents and solutions

The miRNAs and FAM-labeled DNA signal probes (FAM-DNA) used in this study were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of these miRNAs and FAM-DNA probes were listed at Table S1. T7 exonuclease (T7-Exo) and 10 × NEB (New England Biolabs) buffer 4 were purchased from New England Biolabs (Ipswich, MA, USA), the T7 Exo was stored at −20 °C, and the 10 × NEB buffer 4 was stored at 4 °C. RPMI (Roswell Park Memorial Institute) 1640 medium was from BRL Life Sciences Technologies (GIBCO, Netherlands). SDS and borate were obtained from Shanghai Chemical Reagent (Shanghai, China). Other reagents were of analytical grade and used without further purification. Milli-Q ultrapure water (18.2 MΩ) was used in all experiments. Which was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA). The miRNAs and FAM-DNA probes were dissolved in 10 mM Tris-HCl (pH = 7.4) buffer solution. The electrophoresis buffer solution was 30 mM borate solution (pH = 9.2) containing 30 mM SDS. All electrophoresis buffer solutions and microchip channel rinsing solutions were filtered through a 0.22 μm membrane filter.

2.2. Apparatus and microfluidic chip

The MCE-LIF detection system using a 473 nm semiconductor laser as exciting light source was built by our laboratory. A multi-terminal high voltage power supplier with voltage output range from 0 to 5000 V used for sample loading and electrophoresis separation was also obtained from our laboratory. The fluorescence signal was collected and recorded by using a chromatography data system (Zhejiang University Star Information Technology, Hangzhou, China). A glass microchip was fabricated by Dalian Tuowei Technology Co., Ltd. (Dalian, China). The layout and dimensions of the microchip was shown in Fig. S1. All the channels were 25 μm deep and 45 μm wide. The distance between the cross point and SW (sample waste reservoir) was 45 mm. All the reservoirs were 3 mm in diameter and 2 mm in depth. The effective length of separation channel was 40 mm. The channel between S (sample reservoir) and SW was used for sample loading, and the channel from B (electrophoresis buffer reservoir) to BW (electrophoresis buffer waste reservoir) was used for separation.

2.3. Cells culture and treatment

Human bladder carcinoma T24 cells (purchased from China Center for Type Culture Collection) were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C, and RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, invitrogen), 100 mg/mL streptomycin and 100 units/mL penicillin for 48 h. The cells were detached with a 5% trypsin/EDTA solution and centrifuged, then dispersed in the phosphate buffered saline (PBS) solution, repeat the operation procedure three times. The cells were broken by the ultrasonic wave for 10 min then centrifuged to obtain the supernatant. The supernatant was filtered by 10 kD hydrosart to remove the protein; finally the samples were store at 4 °C to be used.

2.4. Signal amplification reaction

The FAM-DNA probe was heated at 95 °C for 5 min, and slowly cooled down to room temperature before use. Then, 2.5 μL FAM-DNA probes (2.5 μM) solution, 2.5 μL 10 × NEB buffer 4 solution, 6.0 μL T7 Exo and various concentrations of miRNAs were mixed, the mixed solution was diluted with DEPC (diethypyrocarbonate)-treated water to 25 μL, and the final concentration of FAM-DNA probes was 0.25 μM. The above prepared solution was incubated for 80 min at 37 °C. After that, MCE-LIF measurement was performed to execute the separation and detection operation of miRNAs.

2.5. Agarose gel electrophoresis

The gel electrophoresis assay was carried out on 4% agarose gels and run in 1 × TBE buffer (90 mM Tris, pH 8.3, 90 mM boric acid, 0.1 mM EDTA) solution. 20 μL different reaction products were mixed with 2 μL loading buffer, and added to each lane, respectively. Electrophoresis was performed with a 100 V constant voltage for 90 min at room temperature. After stained with ethidium bromide, the gel was scanned and recorded by using the Omega 16ic Gel imaging system.

2.6. Microchip electrophoresis

The procedure for MCE-LIF operation was similar to that described previously [23]. Briefly, before the electrophoresis separation, the microchip channel was rinsed in sequence with 0.1 M NaOH for 10 min, water, and electrophoretic buffer solution for 5 min each by applying a vacuum pump. Then all the four reservoirs were filled with the electrophoresis buffer solution and vacuum was applied to the reservoir BW to fill the micro-channel with the electrophoresis buffer solution. Then, the solution in reservoir S was replaced with sample solution. The microchip was placed on the X-Y movable operating desk, and a laser beam was focused at the detection point. Then four platinum electrodes were inserted into the four reservoirs. The electrophoresis separation and detection operation were conducted in a dark box, and the layout and dimensions of the microchip was shown in Fig. S1. A group of electrical potentials (500 V for reservoir S, 250 V for reservoir B, 350 V for reservoir BW, and reservoir SW at grounded) were set at the mode of loading sample. The sample solution was transported from reservoir S to reservoir SW in pinched mode. After 20 s, potentials at reservoir B, S and SW were set to be 2500, 1900 and 1900 V, and reservoir BW at grounded for sample separation and fluorescence detection.

The peak 1 and peak 2 in the electropherogram were used to recognize the miRNA-141 and miRNA-126, and peak height of two peaks was used for the quantification of two microRNAs, respectively. The reproducibility of the assay was evaluated by analyzing a mixture solution of 1 nM miRNA-141 and miRNA-126 for five times. The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the signal-to-noise (S/N) ratio. The standard deviation of the S/N ratio was calculated and multiplied by the factors of 3 and 10 for the LOD and LOQ.

3. Results and discussion

3.1. Design principle of method

The principle of proposed structural transformation consorting with multiplexed fluorescence signal amplification strategy on the microchip platform is outlined in Scheme 1. The multiplexed fluorescence signal amplification system consists of two kinds of FAM-labeled DNA signal probes (FAM-DNA), two kinds of target miRNAs and T7 Exo. Two kinds of FAM-DNA signal probes can hybridize with its target miRNA respectively, and forming miRNA/DNA duplexs. The miRNA/DNA duplexes were then digested by T7 Exo from 5′→3′ of the FAM-DNA probes in miRNA/DNA duplexes, which releases target miRNAs and