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Simplified transient isotachophoresis/capillary gel electrophoresis method for highly sensitive analysis of polymerase chain reaction samples on a microchip with laser-induced fluorescence detection

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ABSTRACT

We present a sensitive, simple and robust on-chip transient isotachophoresis/capillary gel electrophoresis (tITP/CGE) method for the analysis of polymerase chain reaction (PCR) samples. Using chloride ions in the PCR buffer and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) in the background electrolyte, respectively, as the leading and terminating electrolytes, the tITP preconcentration was coupled with CGE separation with double-T shaped channel network. The tITP/CGE separation was carried out with a single running buffer. The separation process involved only two steps that were performed continuously with the sequential switching of four voltage outputs. The tITP/CGE method showed an analysis time and a separation efficiency comparable to those of standard CGE, while the signal intensity was enhanced by factors of over 20. The limit of detection of the chip-based tITP/CGE method was estimated to be 1.1 ng/mL of DNA in 1× PCR buffer using confocal fluorescence detection following 473 nm laser excitation.

1. Introduction

Microchip electrophoresis has been accepted as a powerful tool for DNA analysis [1–7], with advantages of short analysis times, small amounts of samples and reagents consumption, and the capability for construction of integrated and high-throughput systems.

Polymerase chain reaction (PCR) is one of the most widely used techniques in nucleic acid research. The sizing of PCR fragments is important for genetic and diagnostic research in pursuit of sensitive and accurate qualitative analysis [3,8]. Microfluidic devices are attractive for the analysis of PCR products because of their potential capability of integrating PCR amplification and capillary gel electrophoresis (CGE) analysis in a single device [9–14], which lead to shorter analysis time and lower reagents consumption. With the development of PCR-CE integrated microchip technology, simply designed microfluidic devices that can analyze PCR samples with high sensitivity are highly desired. As shown by the previous works, highly sensitive microchip electrophoresis method effectively reduced PCR time and were suitable for detection of trace samples [15,16].

The sizes of micro-channels are typically under 100 μm, providing the rapid separation and high resolving power. However, with the most widely used cross-shaped microchip, the sample amounts injected impact the detection sensitivity. In addition, as the dependence of sample injection on the conductivity of the sample buffer and the electrophoresis buffer [17,18], the highly saline PCR buffer also reduces the amounts of DNA that is electrokinetically loaded into the buffer channel, and thus the detection sensitivity of PCR sample is usually limited with a microchip with cross geometry. Therefore, the limited detection sensitivity of microchip hinders its application to trace PCR sample analysis.

Isotachophoresis (ITP) is an effective technique for on-chip double-stranded DNA (dsDNA) stacking [16,19–21], with which the detection sensitivity is improved with the increased amounts of sample loading. However, the typical ITP/CGE operation is cumbersome as it needs discontinuous buffers and includes multi steps such as sample injection, TE buffer migration, stacking and separation. As a result, the conventional ITP/CGE is difficult to be integrated into the miniaturized PCR-CE system.

Transient isotachophoresis (tITP) is a preconcentration technique that compatible with high salt samples, as ions in the sample matrix may serve as leading or terminating electrolytes [22–26]. In tITP, a large volume of sample and leading/terminating ions are injected to background electrolyte (BGE). By applying current, ana-

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lytes in the sample zone are stacked into narrow bands one after another according to their mobility, and at a certain time separation proceeds by CGE in the same channel. tITP can be a simpler scheme for coupling ITP and CGE, by which the high-salt PCR sample can be directly analyzed with high sensitivity. As examples, Xu [21] and Wang et al. [27] reported microchip electrophoresis tITP DNA analysis with UV and laser-induced fluorescence (LIF) detection.

In this paper, we describe a simple, sensitive and robust tITP/CGE method for the analysis of PCR sample. Using chloride ions in the PCR buffer and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) in the BGE respectively as the leading and terminating electrolytes, the PCR sample was stacked and separated on a double-T structured microchip. The tITP/CGE analysis is as simple as that of standard CGE. The whole operation is carried out with single running buffer and includes only two steps. We demonstrate effectively improved peak intensity with the simplified tITP/CGE method.

2. Experimental

2.1. Materials and reagents

Imidazole, HEPES, 1× TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3), acrylamide, ammonium persulfate (APS), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Sigma (St. Louis, MO, USA). 3-(Trimethoxysilyl)propyl methacrylate was from Acros Organics. (Hydroxypropyl)methyl cellulose (HPMC, 50 cP, 2% in H_2O) was from Aldrich (Milwaukee, WI, USA). The *Φ*X 174/*Hae*III digest was from Fermentas (Burlington, Canada). The 10× PCR buffer (1× PCR buffer containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM, $MgCl_2$) and standard PCR products were from Tiangen (Beijing, China). The intercalating DNA dye GeneFinder was purchased from Biovision (Xiamen, Fujian, China).

2.2. Instrumentation

The microchip experiments were carried on a microfluidic chip analyzer with a confocal LIF detector and four individually controlled high voltage modules.

The optical system was installed on a three-dimensional translational stage driven by three stepper motors. The stepper motors are responsible for positioning the detector head, allowing detection at selected channel location and focusing laser beam. The light source was a 20 mW 473 nm air-cooled diode solid-state laser (Bangshou, Beijing, China). The laser beam passes through a 470 ± 10 nm band pass filter (Omega Optical, Brattleboro, VT, USA) and is reflected off a dichroic mirror (Omega Optical, 505 DRLP 02) set at 45° to the incident beam and focused into the center of the microchannel by a 20× microscope objective (0.4 numerical aperture). The emitted fluorescence is collected and collimated by the same microscope objective and focused by a 200 mm lens onto a spatial pinhole filter (600 μm). An imaging charge coupled device (CCD, Sony, HZ-670P) is installed for monitoring the focusing of laser beam in the channel. The fluorescence is spectrally filtered by a 520 nm band pass filter (Omega Optical) and detected by a photomultiplier tube (PMT, Hamamatsu, H9306, Hamamatsu, Japan).

A computer controlled high-voltage power supply provides sequential voltage supplies. Data acquisition and processing are carried out using an analog-to-digital (A/D) converter. The detection system and the high-voltage system are synchronized by the operation software, which also displays current, voltage and electropherogram, identifies peaks and calculates migration times, half-height peak widths, peak areas and heights.

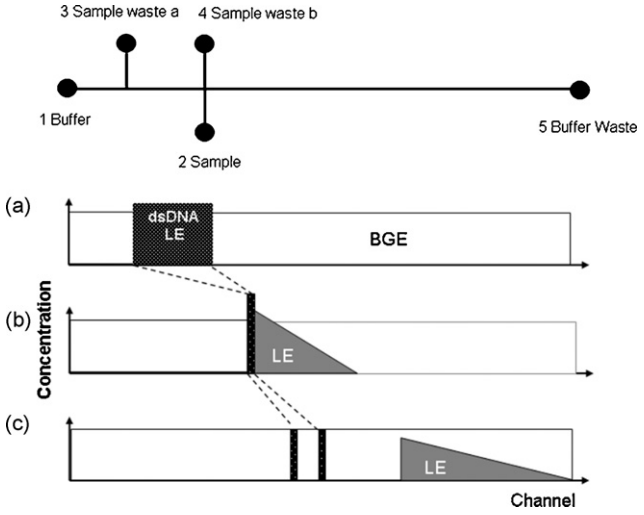

Fig. 1. Layout of the microfluidic chip and schematic demonstration of the chip-based tITP/CGE method for analysis of PCR samples. (a) LE and dsDNA are loaded to fill the offset by electrokinetic injection; (b) tITP preconcentration starts with the application of electrical current; (c) the dsDNA destack and was separated in CGE mode. LE = leading electrolyte; BGE = background electrolyte.

2.3. Microchip electrophoresis

The layouts of the glass microfluidic chip developed for standard CGE and tITP/CGE separation are shown in Fig. 1. The microchip was fabricated with a standard lithography, wet-etching and thermo-bonding method [16,28]. The microchannel was coated with linear polyarcylamides using Hjertén's method [29]. The separation channel was 30 μm deep and 50 μm wide at half-height. The total length of the separation channel was 5.8 cm and the effective separation distance was 4.5 cm. Sample injection segment of the double-T structure was 7.5 mm, corresponding to an approximate sample volume of 11 nL.

The background buffer for microchip electrophoresis was 20 mM HEPES/40 mM imidazole, pH 7.5. The running buffer contained various concentrations of HPMC-50 as sieving matrix and 1× GeneFinder was used as intercalating dye. All buffers were prepared with double distilled water (ddH_2O). The digested DNA samples were diluted to 1 μg/mL in 1× PCR buffer. Before each run, the running buffer was loaded to the reservoirs defined as buffer, buffer waste and sample waste (Fig. 1). By addressing negative pressure with a vacuum pump at the sample reservoir, all channels were filled with buffer. Finally, the PCR sample was loaded to the sample reservoir. Both tITP/CGE and standard CGE analysis included two steps, sample injection and separation. Sample injection was performed by applying a 300 V (300 V/cm) potential across the sample and sample-waste-b reservoir in the standard CGE mode and 600 V (340 V/cm) potential across the sample and sample-waste-a reservoirs in the tITP/CGE mode, with the sample at ground. For separation, the sample and sample waste were floating and high voltage (430 V/cm in standard CGE and desired values in tITP/CGE mode) was applied to the buffer waste with ground at the buffer. Sequential steps for microchip electrophoresis in standard CGE and tITP/CGE modes are given in Table 1.

3. Results and discussion

3.1. Principle of the tITP/CGE method

The tITP/CGE method consists of a single background electrolyte, used for both ITP preconcentration and CGE separation. As shown