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Analysis of multiplex PCR fragments with PMMA microchip

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Abstract

Microchip electrophoresis is a promising technique for analysis of bio-molecules. It has the advantages of fast analysis, high sensitivity, high resolution and low-cost of samples. Plastic chip has the potential of mass production for clinical use for its advantages in biocompatibility and low cost. In this work, the method for fabrication of poly(methyl methacrylate) (PMMA) chip was described, and conditions for DNA separation were investigated with the chip. The PMMA microchip was used for detection of multiplex PCR products of 18 and 36 cases with SARS and hepatitis B virus infection under optimized separation conditions. Microchip electrophoresis showed higher sensitivity, higher resolution and less time consumption when compared with gel electrophoresis. The microchip electrophoresis with PMMA chip provided a rapid, sensitive and reliable method for analysis of multiplex PCR products.

Keywords: Microchip electrophoresis; Microchip fabrication; Multiplex PCR

1. Introduction

Microchip electrophoresis is a promising technique for separation; it has been applied to analyze various biological samples. The microchips have been fabricated in silicon, glass, quartz and plastic substrates. Plastic chip has been frequently used recently because of its advantages in biocompatibility and lower cost as well as increased flexibility with respect to fabrication methods and substrate dimensions [1]. Plastic chips can be made of a variety of polymeric materials, such as poly(methyl methacrylate) (PMMA) [2], polycarbonate [3], SU-8 photoresist [4], Zeonor 1020 [5] and the elastic poly(dimethylsiloxane) (PDMS) [6,7]. Polymeric substrates have then been patterned by laser ablation [1], X-ray lithography [8], injection-molding [9] and imprinting with master templates [10].

Mass production of plastic chip may effectively reduce the cost. There are many methods for mass production of polymeric devices, such as the devices of silicone structures fabricated by a casting protocol [11], the production of microseparation channels in plastic substrates via laser ablation [12], the fabrication of micro-channel chips in acrylic substrate with an injection-mold method [9] and rapid prototyping for injection-molded integrated micro-fluidic devices and diffractive element arrays [13]. In summary, injection-molding, compression-molding or stamping processes provide the possibility of mass production of essentially evenly fabricated plastic micro-devices from a single master in a cost-effective manner.

The microchip electrophoresis with plastic chip, which has the advantage of high sensitivity, fast separation, little con-

Abbreviations: HPMC, hydroxyprolymethyl cellulose; PDMS, poly(dimethylsiloxane); PMMA, poly(methyl methacrylate); SARS, severe acute respiratory syndrome

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sumption of samples and the possibility of mass-production for disposable use, has the potential to be used in clinical diagnosis.

Multiplex PCR analysis, which is widely used in genetic diagnosis and genotyping, offers advantages including greater speed and simplicity, reduced labor requirement and lesser risk of cross-contamination. Detection methods feature less time, high resolution and high sensitivity and are highly wanted for analysis of multiple PCR products. However, the currently used slab gel electrophoresis is low sensitive and time consuming. In this study, we described the method for fabrication of PMMA chips, and the microchip electrophoresis under optimal separation conditions was applied to determination of multiplex PCR products of samples with SARS and hepatitis B virus infection.

2. Experimental

2.1. Fabrication of microchip

A multistep fabrication process was developed for the production of the poly(methyl methacrylate) (PMMA, Meiqi Co., Taiwan, China) chips. Three micro-channels were fabricated on an electrophoresis chip (Fig. 1). Initially, the silicon template was fabricated with a previously described method [2]. Briefly, the schematic of the channels was first drawn using the CAD software. The image was reproduced on the chrome glass to serve as mask for photolithography. An oxide silicon layer was produced on the surface of a 4 in. silicon wafer with crystal orientation ⟨1 0 0⟩. Subsequently, a layer of photoresist was spin-coated over the oxide silicon layer, followed by being exposed to UV light through the mask aligned to the wafer flat. The photoresist was then developed to reveal the transferred image. Therefore, all the area defining the channels was exposed in this step. The exposed silicon oxide layer was etched with hydrofluoric acid before serially rinsed with distilled water and acetone to remove the photoresist.

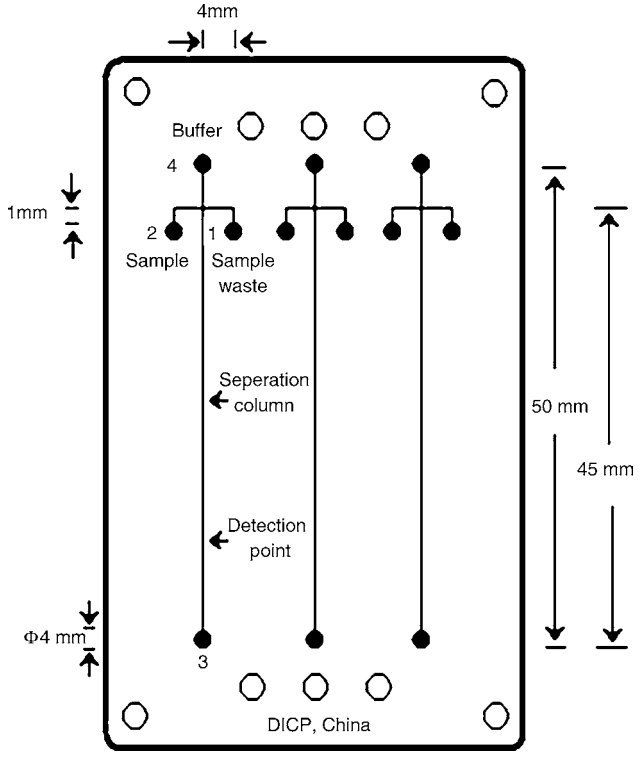

Fig. 1. Dimension and layout of the PMMA chip. The figure in black spots indicate the reservoir numbers: 1, sample waste; 2, sample; 3, buffer waste; 4, buffer. The small figures denote the channel lengths and device dimensions (mm).

The remaining oxide layer was used as a mask for anisotropic etching of the silicon wafer, which was later etched in KOH solution to produce the three-dimensional sunken images of channels. The depth and width of the sunken channels were controlled by etching time. The silicon template was served as the master for subsequent steps.

Subsequently, a layer of metal was plated on the surface of silicon template before it was immerged in electroforming solution. After the silicon template was applied to 220 V for 48 h, a "positive" nickel mold with 1.5 cm thickness was produced. Metal columns with 1.5 mm height, 4 mm diameter were mounted at the terminals of channels to produce reservoirs. The nickel mold was then machined to match the injection insert, which was inlayed in a metal mold. After the metal mold was installed in the injection machine, the plastic PMMA substrate was molded to make chips.

The channels of PMMA microchip were sealed with a 50 μm cover PMMA film (Goodfellow Corporation, England). The sealing reagent consists of alcohol, ketene and water was first sprinkled on the surface of chip, and then the film was immediately attached. A 0.15 kg/cm^2 (14.7 kPa) pressure was applied to the chip surface for 30 min at 25 °C. Excessive sealing reagent was eliminated by the vacuum pump. The film seal forms the fourth wall of the separation channels and bottoms of reservoirs.

The dimension of the PMMA chip was showed Fig. 1. The upper width, the bottom width and depth of the trapezoid profile of the channel was 38, 110 and 30 μm, respectively. The injection volume was calculated to be 177 pL. The volume of each reservoir was 13 μL. Reservoirs 1 and 2 are assigned for loading sample and sample waste, while reservoirs 3 and 4 for running buffer.

2.2. Microchip electrophoresis analyzer

The dimensions of the homemade chip analyzer were 50 cm × 50 cm × 45 cm. The related information was described in details previously [14]. In brief, the output radiation (532 nm) from an air-cooled laser diode (LD)-pumped solid-state laser (20 mW) (Mektec Seiwa Corporation, Beijing, China) passes through a 532 nm filter (Omega Optical, Brattleboro, VT). The beam is reflected by a dichroic beam splitter (Omega Optical) and focused into the channel through a 20× microscope objective (0.4 NA). The emission signal is

collected by the same objective and transmitted back through the dichroic beam-splitter. The emission beam passes through a bandpass filter (Omega Optical), which may be alternated easily to fit a wide selection of dyes, and is focused by a focusing lens through a 400 μm pinhole. The photo-multiplier tube (Hamamatsu Photonics R212, Japan) is mounted in an integrated detection module including high voltage power supply, voltage divider and amplifier. The charge coupled device (CCD) camera is fixed at the same board as the photo-multiplier tube to focus and observe the channel. The whole optical system is installed on the *X–Y–Z* translational stage (3-D micromanipulator, which adjusting precision is 1 μm), and the focus can be controlled via picture displayed on the screen.

2.3. Samples

Nasopharyngeal swabs from 18 SARS patients and serum samples from 36 patients with hepatitis B were studied. All the patients were confirmed seropositive for virus infection, and the SARS patients fulfilled the WHO case definition [15]. Samples were kept in sterile containers and stored at −20 °C until analysis.

Nasopharyngeal swabs were suspended in viral transport medium and centrifuged for 1 min with 10,000 × *g*. The supernatant was collected for RNA extraction. Total SARS–coronavirus (SARS–CoV) RNA was isolated from 210 μL samples using an RNAeasy Mini Kit (Qiagen, Hilden Germany). The extracted RNA was eluted in 40 μL of RNase-free water before amplification. This operation was performed in the Biosafty P3 Level Laboratory in Institute of Microbiology and Epidemiology, Chinese Academy of Military Medical Sciences. The hepatitis virus B (HBV) DNA was extracted from 100 μL serum samples with the phenol–chloroform method. The resulting pellets were dissolved in 100 μL RNase free water and stored at −20 °C.

2.4. RT-PCR and PCR

All thermal reactions were performed with the GeneAmp PCR System 2700 (Applied Biosystems, Singapore). The SARS–CoV RNA was first reversely transcribed to cDNA. Reaction was performed with the following conditions: 10 min at 30 °C, 30 min at 50 °C, 5 min at 99 °C and 5 min at 4 °C.

SARS–CoV and HBV infected samples were amplified by triplex and duplex PCR, respectively. Primers were designed to conserved regions. The theoretical annealing temperature was set between 55 and 60 °C. The sizes of resulted PCR fragments were 153, 216 and 318 bp for SARS–CoV, 320 and 462 bp for HBV (Table 1).

The HBV DNA and SARS cDNA were added to multiplex PCR reaction mixtures. Positive and negative PCR controls, containing the control templates and sterile water, were included in each run. The thermocycler was programmed to first incubate the sample for 5 min at 94 °C, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. All PCR reactions were carried out twice, if the results of the two runs were different, a third reaction was carried out.

2.5. Microchip electrophoresis

Hydroxyprolymethyl cellulose (HPMC)-50 (Sigma, St. Louis, MO) was dissolved in 1× TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.5). SYTOX Orange nucleic acid stain dye (Molecular Probes, Eugene, OR) solution was prepared in the separation matrix to label DNA online. The buffer was prepared in doubly distilled water and filtered (0.22 μm filters) before introduction into the chip. Before each run, separation channel of the PMMA chip was washed by doubly distilled water and infused with sieving buffer from the buffer reservoir (reservoir 3, see Fig. 1) with a vacuum pump. The sample was first added to the sample reservoir and then injected by applying 400 V at the sample waste and grounding the sample reservoir for 30 s. During separation, the voltage at buffer waste reservoir (reservoir 3, see Fig. 1) was adjusted to a desired value and potentials at the buffer, sample and sample waste reservoirs (reservoirs 4, 2 and 1, see Fig. 1) were switched to 0, 0.3 and 0.3 kV. The effective separation length was 40 mm.

In parallel experiments, 10 μL of PCR products were also loaded onto 1% agarose gel, followed by 30 min separation under 90 V and visualize the product by staining with ethidium bromide.

3. Results and discussion

3.1. Determination of detection limit

The detection limit of the microchip electrophoresis system was tested with serially diluted φX-174/HaeШ digests (50 ng/μL, Takara Biotech, Dalian) in which the 310 bp frag-

Table 1
Primers for multiplex PCR

	Sense	Antisense	Amplicon size (bp)
HBV primers	5′-TGGCCAAAATTCGCAGTCC-3′	5′-GGCCCCCAATACCACATCAT-3′	462
	5′-CACCGCCTCTGCTCTGTATCG-3′	5′-TAGGGGCATTTGGTGGTCTGTAAG-3′	320
SARS primers	5′-GTCAAGCTGTTACAGCCAAT-3′	5′-AAGCGTAAAACTCATCCACG-3′	153
	5′-TAGGATTGCCTACGCAGACT-3′	5′-CCACATTGCGACGTGGTATT-3′	216
	5′-CTGGTCTTCATCCTACAC-3′	5′-TCGGTACAGCTACTAAGT-3′	318

ment was 2.9 ng/μL. The microchip electrophoresis is able to detect the 310 bp fragment as low as 0.005 ng/μL with signal-to-noise ratio >3, while the slab gel electrophoresis can only detect a 0.2 ng/μL dilution (data not shown).

3.2. Optimization of separation conditions

The φX-174/HaeIII digests containing 11 DNA fragments at 1.0 ng/μL concentration was used for optimization of separation conditions. The resolution (R_s) and theoretical plate number, which characterize the efficiency of the separation system were used. The theoretical plate number was calculated according to the equation, $N = 8 \ln 2(t_m/w)^2$, where t_m is migration time and w is the widths at half height. The resolution $R_s = (2 \ln 2)^{1/2} \Delta t_m/(w_1 + w_2)$, where w_1 and w_2 are the widths at half height of peaks corresponding to two fragments, and Δt_m is the retention difference of the two peaks.

3.2.1. Effect of sieving matrix concentration

The microchip separations were performed with various concentrations of HPMC-50 ranging from 0.5 to 3.0% presented in running buffer (Fig. 2). Higher concentration of HPMC facilitates the resolution increasing of DNA fragments but increases separation time. The 11 fragments of φX-174/HaeIII digests could be separated with sieving matrix concentration higher than 1.0%, and baseline separation of 271 and 281 bp fragment could be attained with sieving matrix concentration higher than 2.5%. The sieving matrix with 2.5% showed enough separation efficiency and higher concentration obviously prolonged analysis time. So, 2.5% HPMC was used in subsequent experiments.

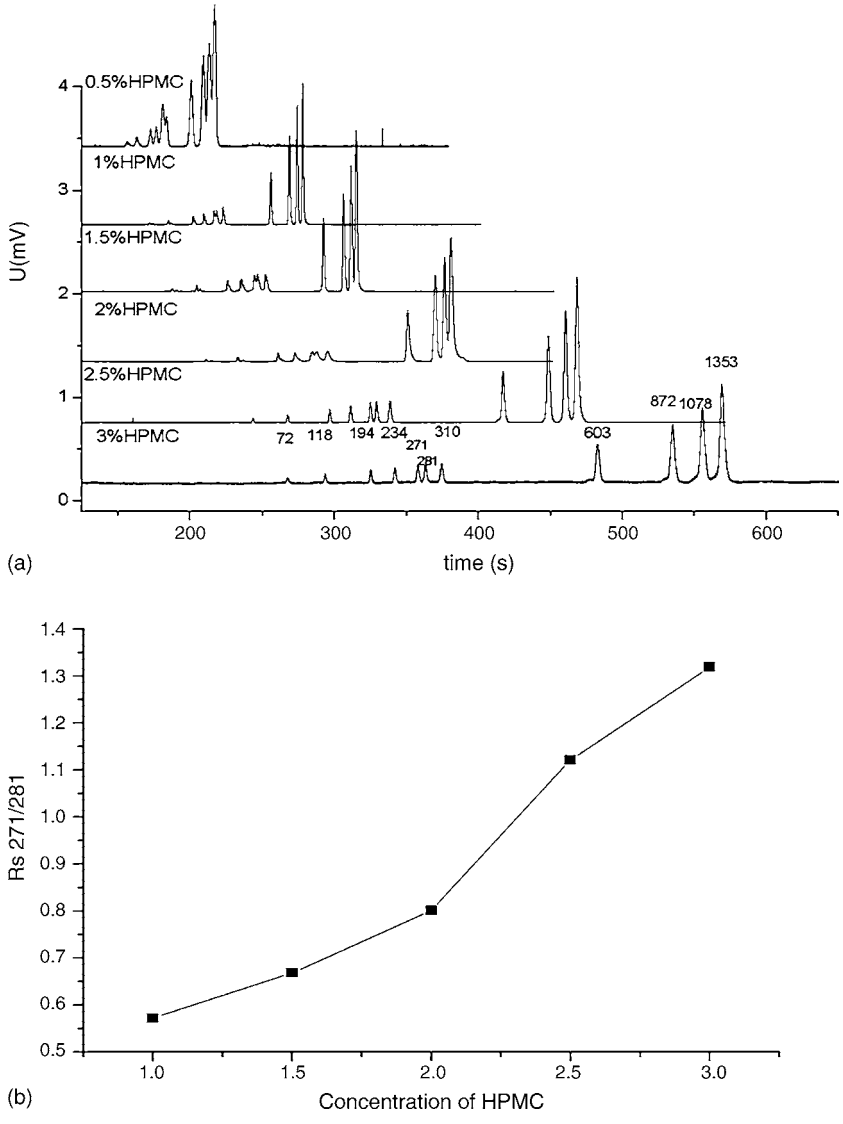

Fig. 2. (a) Electropherograms of φX-174/HaeIII digests on the microchip using various HPMC concentrations ranging from 0.5 to 3.0%. Numbers on the electropherograms correspond the size of DNA fragments in bp. Applied field strength was 120 V/cm. (b) HPMC concentration dependence of resolution (R_s, 271/281) of the 310 bp fragments.

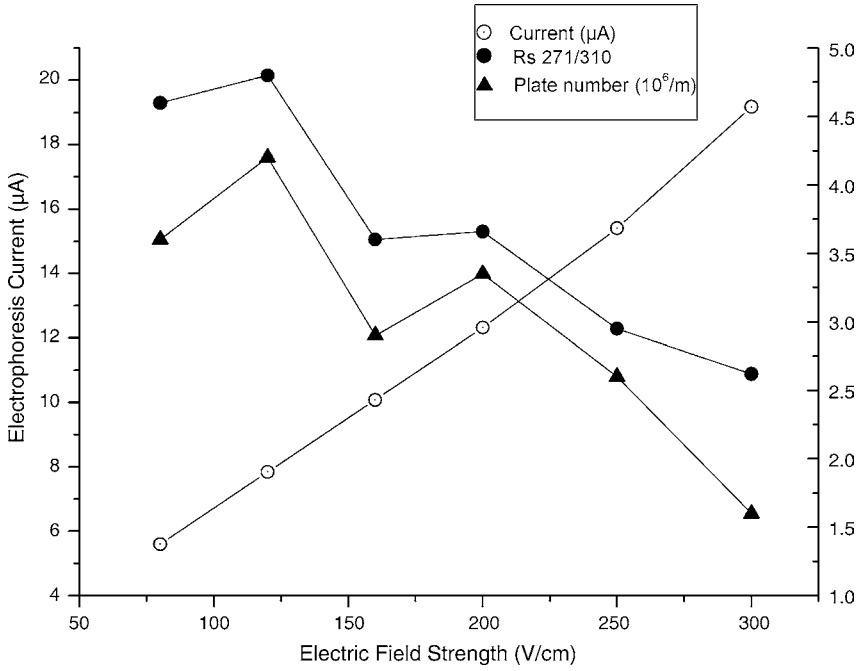

Fig. 3. Resolution (R_s, 271/310), plate number of 310 bp fragment and electrophoresis current against applied voltage. *Conditions*: 2.5%HPMC solution and 1.0 μmol/L SYTOX in running buffer.

3.2.2. Effect of applied electric field strength

The sieving buffer containing 2.5% HPMC-50 was used in studying the influence of applied field strength. Fig. 3 showed the resolution (R_s, 271/310), plate number of the 310 bp fragment and electrophoresis current against applied field strength. Lower field strength gives higher resolution, while higher field strength facilitates fast separation but increases electrophoresis current. The resolution was obviously distorted when applied field strength was higher than 200 V/cm, probably due to the increase of Joule heat. One hundred and twenty volts per centimeter applied field strength was adopted when resolution and running time were all taken in account.

3.2.3. Effect of intercalating dye concentration

The migration times of φX-174/HaeШ digests increased gradually with increased concentration of dye in 2.5% HPMC solution (data not shown). It could be explained that intercalating dye changes the charge-to-mass ratio of DNA fragments and their interactions with sieving matrix. The peak areas of DNA fragments increased with higher dye concentration and kept stable when the dye concentration was more than 1.0 μmol/L. Therefore, 1.0 μmol/L was selected as optimal SYTOX concentration.

3.3. Determination of clinical samples

PCR products mixed with φX-174/HaeШ digests were analyzed under optimized conditions. Sieving buffer containing 2.5% HPMC, 120 V/cm applied field strength and 1.0 μmol/L SYTOX presented in running buffer. The microchip electrophoresis showed high separation efficiency, PCR products and DNA marker were clearly baseline separated within 500 s and expected PCR products were all separated with fragments of the φX-174/HaeШ digests (Fig. 4). Specimens were considered positive if amplification products with expected sizes were detected. Except one case, expected triplex PCR products of the clinically confirmed SARS patients were all detected by the microchip electrophoresis, while 12 out of 18 cases were detected by agarose gel electrophoresis. Expected duplex PCR products were detected in 25 and 29 out of the 36 hepatitis B patients with microchip and agarose gel electrophoresis, respectively.

The total detection rates of SARS and HBV virus infection obtained by microchip electrophoresis were 94.4% (17/18) and 80.6% (29/36), which were enhanced by 27.8 and 11.1% compared with the agarose gel electrophoresis, respectively. Microchip electrophoresis showed higher sensitivity in the detection of SARS virus. We ascribed the reason to sample collection; the samples of SARS virus infection were all collected at day 10, when the virus was presented in body fluids at high levels, while that of hepatitis B virus infection were collected during intervals of antivirus therapy. Therefore, the level of hepatitis B virus in blood can be very low during the recovery phase. Microchip electrophoresis showed much higher sensitivity and less time consumption than agarose gel electrophoresis. The relatively low sensitivity of gel electrophoresis, which might cause false negative seems to be disadvantage for PCR products detection. Electroosmotic flow in the channel of PMMA chip was minimized using HPMC–TBE buffer as a dynamic coating, which was

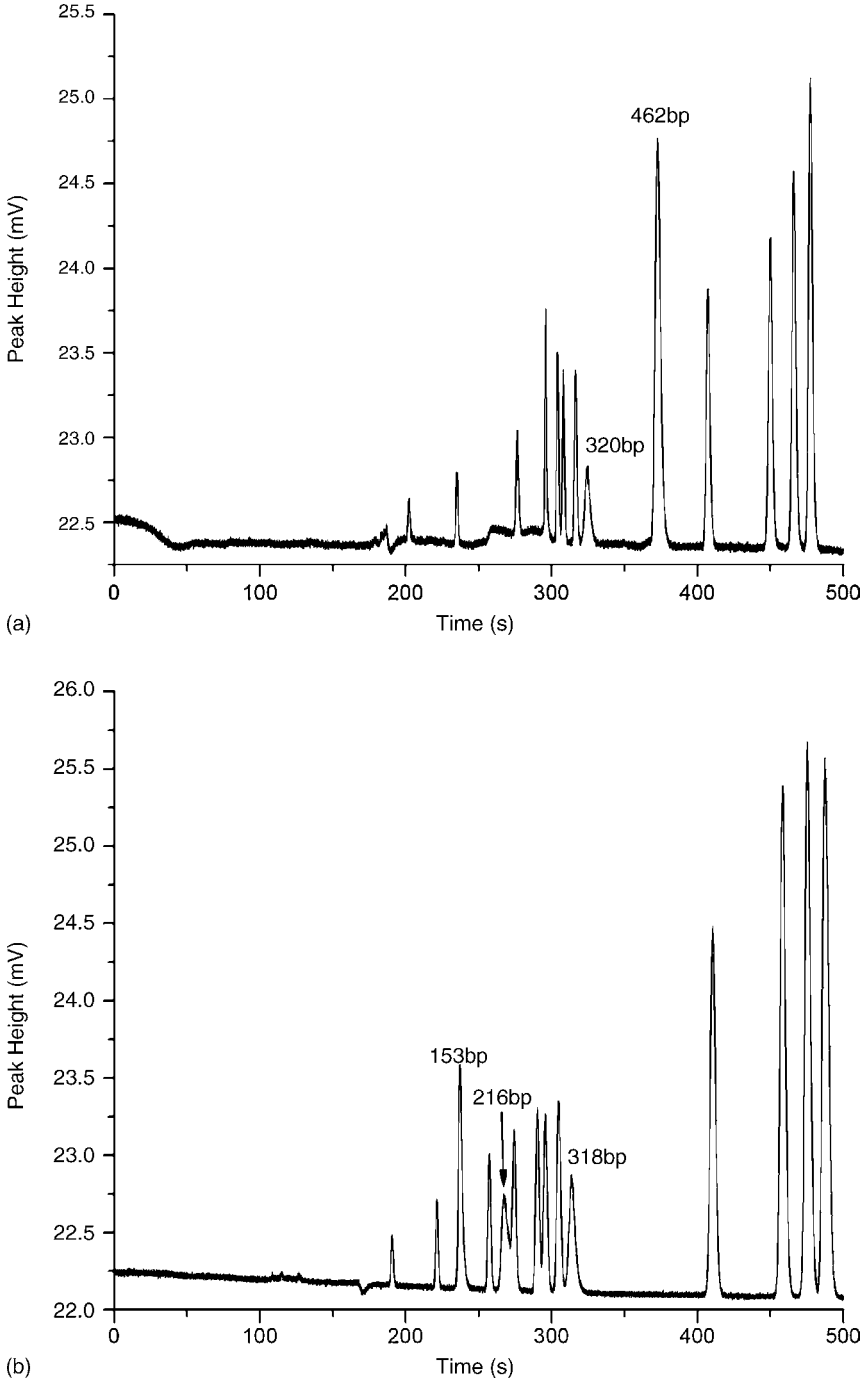

Fig. 4. Electropherogram of positive case analyzed against φX-174/HaeIII digests. (a) HBV positive case, duplex PCR fragments with expected size (320 and 462 bp) were detected. (b) SARS positive case, triplex PCR fragments with expected size (153, 216 and 318 bp) were detected. *Separation conditions*: sieving buffer containing 2.5% HPMC, 120 V/cm applied field strength and 1.0 μmol/L SYTOX presented in running buffer.

stable for multiple injection/separation operations and provided reproducible results. Separation time was therefore decreased with the absence of electroosmotic flow. Since DNA was less adsorptive to the surface of the micro-channel of PMMA chip, peaks with good form were obtained. The high resolution of microchip electrophoresis enables rapid analysis of multiple PCR products and the high sensitivity ensured low level of virus copy could be detected. At the same time, experiments became convenient with the fast separation and simple operation.

4. Concluding remarks

In this work, a method for mass production of PMMA chip was introduced, and the PMMA microchip was used

for the detection of multiplex PCR products of clinical samples with optimized separation conditions. The experimental results showed that the microchip electrophoresis with high sensitivity and high resolution could significantly improve the detection rate.

Our results suggested that PMMA chip could improve the ability of laboratories to analysis of multiple DNA fragments. The PMMA chip, which allows mass production and fast separation, has the potential for clinical application, especially in analysis of multiple DNA fragments.

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