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Fast Separation of DNA Fragments in On-chip Electrophoresis Microcapillary

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Abstract: Separation of DNA fragments is performed in on-chip electrophoresis microcapillary fabricated on a photosensitive glass. Since the UV-irradiated part of the photosensitive glass has the etching rate 20 times higher than the other parts, we can obtain the channel structure with the high aspect ratio, which is valuable to the high-sensitivity detection of the signal. The confocal laser scanning microscopy (CLSM) is used to observe and detect the fluorescent sample plug in the on-chip electrophoresis microcapillary. The CLSM is a powerful tool to observe and detect the sample inside the channel since it can capture the fluorescence images and can vary the laser focal plane depth. In this study, Hydroethylcellurose (HEC) polymer solution is using in the electrophoresis as sieving matrix. Therefore the on-chip electrophoresis microcapillary with the short separation channel is able to separate DNA fragments.

Keywords: On-chip electrophoresis microcapillary, Photosensitive glass, Confocal laser scanning microscopy, Hydroethylcellurose

1. Introduction

Micro Total Analysis System (μTAS) and Lab-On-a-Chip (LOC), which were suggested in the early 1990s with application of the microfabrication technology, have made remarkable progress during the past decade.[1] These concepts are integrating and miniaturizing biochemical, biological and chemical analytical equipments or an environmental monitoring system on a chip size. The integrated or miniaturized capillary electrophoresis (CE) device, which is one of the concrete devices with these concepts, has been fabricated on glass[2),3)], Si[4] and plastic substrate[5] using micromachining technology. Electrophoretic techniques are widely used originally for separation of biological molecules and are becoming increasingly important in the era of proteomic analysis and biotechnology-derived drugs. The potential advantages of such CE device are to save sample and reagent consumption, to increase sample throughput, to reduce analytical time, and to decrease power consumption and the total cost. The microfabricated CE devices have been demonstrated for analysis of various substances including small drug molecules, amino acids, peptides, and oligonucleotides.

DNA analysis using electrophoresis technique is important for DNA sequencing process included performing separation of oligonucleotides, restriction fragments[6),7)], sequencing mixtures[8], PCR products[9] and genotyping samples.[10] Generally, DNA analysis uses the gel such as polyacrylamide and agarose for matrix material in electrophoretic separation. The slab-gel electrophoresis is one of the general DNA analysis methods. However, for electrophoretic separation high voltage is not able to apply it since the increasing temperature denatures the gel requiring a long analytical time. Moreover the slab-gel analysis is troubled to detect due to need the complicated step: transfer of the DNA fragments from the gel to a filter, hybridization of target DNA with probe DNA, and the detection of probe DNA based on the radio activity or fluorescence. On the other hand, capillary gel electrophoresis (CGE) is performed in the capillary injected gel and is possible to shorten analysis time since high voltage can be applied because of effect of dissipation of heat.[11] But CGE is somewhat less practical in routine analysis, since a gel-filled capillary has some technological problems, such as a short lifetime and difficulties in reproduction and maintenance.[12] Using the polymeric materials such as liner acrylamide (LAA)[13] and hydroxyethylcellulose (HEC)[14], DNA analysis is performed in CE. CE using the polymer solution surpasses in the experimental handling because of no necessity for polymerization treatment in the capillary. Also polymer matrixes are loaded and replaced easily than that in the gels.

In this study, DNA analysis using the polymer

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solution is performed in the on-chip microcapillary fabricated on photosensitive glass substrate. This glass contains small amounts of rare metal (Au or Ag) ions and Ce^{3+} in addition to more usual glass constituents.[15] Since the UV-irradiated part of the glass has etching rate 20 times higher than the other parts, we can fabricate the channel with high aspect-ratio structure. This high aspect-ratio structure is valuable to the high-sensitivity detection of the fluorescent signal. Also, the glass makes possible to etch the UV-exposed parts without etching masks. Thus the fabrication process is simple comparing with conventional SiO_2 microfabrication process. As a sieving matrix, HEC is used in electrophoretic separation for DNA analysis in the device. The confocal laser scanning microscopy (CLSM) in fluorescence mode (FV300 Fluoview OLYMPUS JAPAN CO., LTD, Japan) was used to detect the sample plugs and to observe the movement in the channel. The CLSM has the pinhole that can eliminate the noise from background light and can obtain the fluorescent signals only from the point excited by focused laser on chip pass through it. Therefore, the CLSM is able to perform the low noise and high sensitive observation. By scanning the excitation laser to an X-Y plane laterally, the fluorescent images on the laser focal plane can be obtained. Therefore the CLSM is useful to investigate the microfluidic manipulation for small sample volume. By understanding the phenomena in the microchannel, the microfluidic devices in the electrokinetic condition can be operated more completely and all potential applications of the devices will be brought out. Using these devices and equipments, fast separation of 100-bp DNA ladder fragments and was performed within about 60 seconds.

2. Experimental Section

Chip Fabrication. **Figure 1** shows the channel design with a straight channel on 2 cm x 2 cm substrate used in this study. The simple design consisting of two straight channels and four reservoirs with the 3 mm diameter was used. Each part was named as assigned in **Fig.1**. Each channel length was 7.5 mm from sample inlet to the cross point, 4.0 mm from buffer inlet to the cross point, 11.0 mm from the cross point to waste, respectively. The fabrication process of on-chip microcapillary is following: first, the glasses were cut into 2 cm x 2 cm square pieces and exposed by UV light ($\lambda = 365$ nm, 1.8 J/cm^3) through the UV mask pattern. Subsequently, they were heated in an electric furnace at 505°C for 90 min, then at 535°C for 120 min. After the heat treatment, they were wet-etched in 5% HF solution using a stirrer. Varying wet-etching time can control the channel-depth without widening the channel width because the substrate material has vertical high etch rate selectivity. The channel structure used in this study was 72.6 μm in depth and 52.3 μm in width by 8 minutes HF wet etching as shown in **Fig.2**. Polysilazane coating process was used in order to smooth the channel surface and to attach a cover glass onto the substrate. This solution makes SiO_2 layer by combining with H_2O and O_2, by low-temperature heat treatment. The smoothness of channel surface is very important because it affects the dispersion of sample plug with respect to the separation efficiency, thus this solution is very useful for coating in the microfluidic system. The fabricated chip was dipped into the diluted polysilazane solution with cyclohexane and annealed at 95°C for 4 hours to smooth the channel surface. In order to adhere the cover glass, the chip was dipped into the solution again, and then the cover glass (0.18 mm thickness) drilled at reservoir position was placed on the chip. After that, the microcapillary was annealed the same step as describe before. The channel surface was treated with the H-jerten's method to coat with the acrylamide which prevents from adsorbing samples and suppresses to electroosmosis flow (EOF).[16] Briefly to explain the process, all channels on the chip were sequentially rinsed with water, 1 M sodium hydroxide, and methanol, respectively. The channels were treated with compound solution, prepared in methacryloxypropyltrimethoxysilane (MPTS) adding acetate

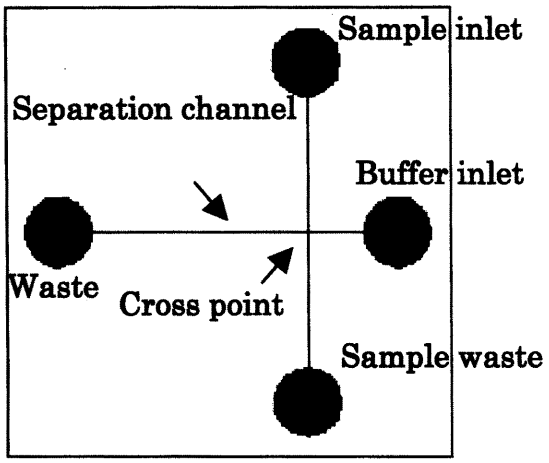

Fig.1 Design of on-chip microcapillary with simple composition in 2 cm x 2 cm chip.

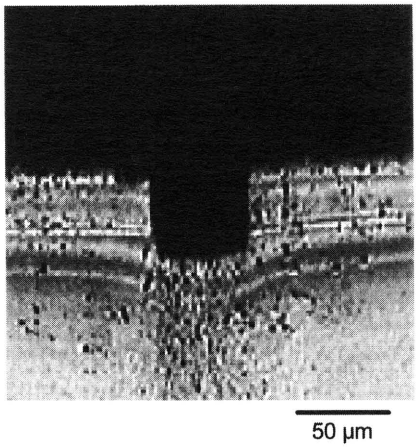

Fig.2 A cross sectional view of the channel. The channel depth is 72.6 μm and the width is 52.3 μm in 8 min. HF wet-etch.

acid. After 1 hour, polymer solution, prepared in 340 μL of 10% acrylamide solution adding 50 μL of 1.34% N, N, N',N',-tetramethylethylenediamine (TEMED) and 50 μmL of 1.34% ammonium persulfate (APS) to polymerize, was pulled through the channel for 30 min. The channels were then rinsed with water to remove the non-polymerized solution.

Reagents and Buffers. The 5 x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA, pH 8.4, Nippon Gene) was used as a running buffer and was adjusted with NaOH to pH 9.1. For the preparation of the polymer solution, the running buffer and HEC (Wako Pure Chemicals Industries, Ltd.) as sieving matrix was mixed using a stirrer overnight. The 100-bp fluorescein molecular ruler (DNA ladder; Bio-Rad Laboratories) which contained 10 bands, 100- to 1000-bp in exact 100 bp increments, was used as the DNA ladder and prepared 100 μL in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), 200 μg/ml concentration. The DNA ladder was stored at 4°C in the dark.

Apparatus. A CLSM equipped with an argon ion laser (MELLES GRIOT LASER GROUP: 10 mW) was used. Excitation from the laser operating at 488 nm was reflected by a dichroic mirror and then focused on the channel through a 20 x 0.5 N.A. or 10 x 0.3 N.A. achromatic objective. Fluorescence emission from the fluorescent sample was collected by the same objective and passed through a band-pass filter (510 nm) to filter spectrally that discriminate against Rayleigh and Raman scattered light, and spatially filtered using a 60 μm pinhole to reject out-of-focus light. To apply high voltage, three high voltage supplies (Keithley 248 High Voltage supply) were arranged.

Chip Operation and Observation. In this study, " pinched valve " injection method was used.[19] At the beginning all the channels were filled with the HEC polymer solution. Pt electrodes were inserted in each reservoir. The voltage was applied to the sample waste reservoir and the other reservoirs were connected to ground when the sample was loaded to the cross point. During the static state in loading, the objective was focused on the cross point and the laser was scanned to an X-Y plane laterally to observe the state of sample loading. The X-Y image series of fluorescent image were obtained using Fluoview software (OLYMPUS JAPAN CO., LTD, Japan) to construct by stacking these scanning images. In the observation of the sample plug in separation channel the irradiated laser light was focused on the each detection point and the adjusted vertical position inside the channel, then scanning field was determined along the channel area on the position. Also, to obtain the electropherogram, the laser was adjusted to irradiate at a point of the channel center in order to detect the flowing sample plug. The voltage was switched in the separation voltage state, in which the waste reservoir was set to the high voltage, the sample inlet and sample waste reservoirs were adjusted a certain voltage to pull sample plug, and the buffer inlet reservoir was connected to ground. The moving fluorescent sample plug was scanned as a two-dimensional image or as an electropherogram.

3. Results and Discussion

The X-Y scanning image of DNA ladder fragments plug using CLSM is shown in **Fig.3**. The scanning was performed at 5 mm from the cross point and the applied electric field was 333 V/cm. The plug shape was distorted because the plug on the side of sample waste reservoir was flowed into the separation channel faster than that on the other side when the applied voltage changed from the pinched valve injection mode. DNA ladder plug was separated to the short DNA ladder fragments (100-400 bp) and the long DNA ladder fragments (500 bp or more) in 5 mm separation distance because of the electrophoretic velocity difference of each fragment. Detecting the separation of such distorted plug was difficult extremely using CCD camera or photomultiplier tube. However, using the CLSM it was not hard to obtain the electropherogram from the plug because the system was able to detect the fluorescent signal from a point on the channel.

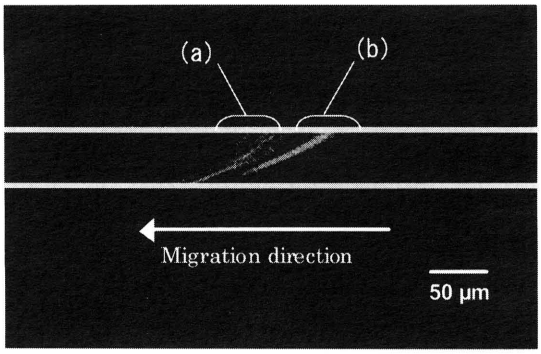

Fig.3 An X-Y scanning image of separated sample plugs using the CLSM. Scanning point is 5 mm downstream from the cross point. Electric field strength is 333 V/cm and HEC concentration is 1.0%. The sample plug is separated to (a) the short DNA fragments (from 100-bp to 400-bp) and (b) the long DNA fragments (500-bp or more).

Figure 4 shows the separation of 100 bp fluorescein molecular ruler in the on-chip electrophoresis microcapillary. The separation was performed in 1.0% HEC in the TBE running buffer at an electric field strength of 333 V/cm and the detection point was 8 mm from the cross point. In **Fig.4**, the peaks of 100-, 200-, 300- and 400-bp fragments were resolved for about 40 seconds completely, and the peaks of 500-, 600- and 700-bp fragments were found, although they were not resolved completely. On the other hand, the peaks of fragments over 800-bp were overlapped and herd to resolve in this experiment. The resolution was calculated using the equation[20],

$$R = (2ln2)^{1/2} \frac{t_2 - t_1}{hw_1 + hw_2} \quad (1)$$

where t is the migration time of the n-th peak and hw is the full width at half-maximum of the n-th peak. According to the equation, the resolution of 100-200 bp peaks was the highest value of 3.68 in the electropherogram. The separation in this experiment was performed with analytical time about 45 seconds and in 8 mm separation distance. Therefore, fast separation of short DNA fragments was performed in on-chip microcapillary. But the resolutions were decreased as the base pair numbers were increased, and the resolution of 500-600 bp was 1.13.

Figure 5 shows the separation of the same DNA ladder fragments sample as that of **Fig.4**. Applied electric field strength was 233 V/cm and the other experimental conditions were same as that in **Fig.4**. According to ref. 5, applying high electric field strength to long DNA fragments separation is not suitable because of the phenomenon of biased reptation with stretching. In contrast to this, the separations of long DNA ladder fragments (400-bp or more) in **Fig.5** were improved in comparison with that in **Fig.4** to decrease applied voltage. But the resolutions of the short DNA fragments were decreased to 1.01 at 100-200 bp resolution. The analytical time was extended since the electrophoretic velocity was delayed by decreasing applied field strength. Accordingly, the peaks were broadened by increasing the dispersion due to diffusion in the electropherogram.

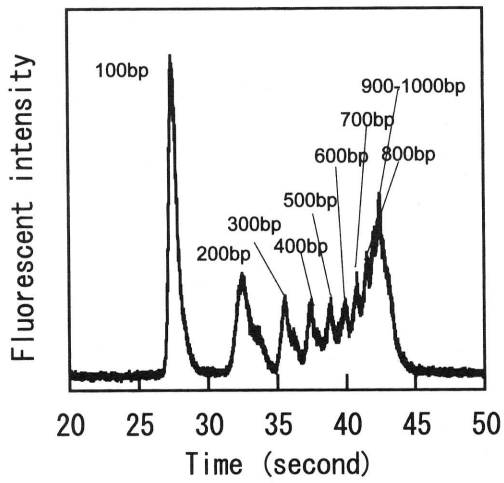

Fig.4 Separation of FITC-labeled DNA ladder in 1.0% HEC sieving matrix. Separation length is 8 mm and electric field strength is 333 V/cm. The sample concentration was 200 μg/ml.

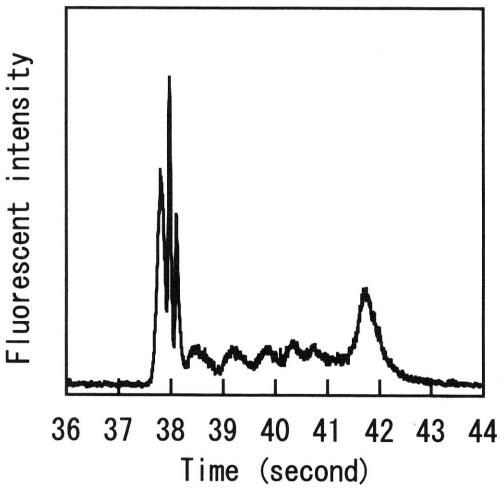

Fig.5 Separation of FITC-labeled DNA ladder in 1.0% HEC sieving matrix. Separation length is 8 mm and electric field strength is 233 V/cm.

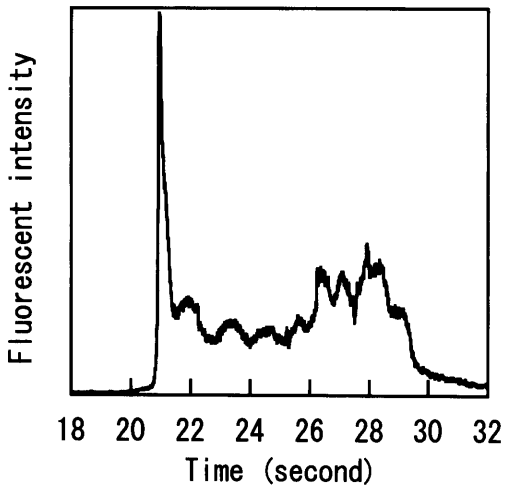

Fig.6 Separation of FITC-labeled DNA ladder in 0.5% HEC sieving matrix. Separation length is 8 mm and electric field strength is 333 V/cm.

Figure 6 shows the electropherogram obtained using 0.5% HEC concentration. The whole plug migration speed was increased as HEC concentration decrease. As a result, the analytical time was shortened comparing with that in **Fig.4**. However, the resolutions deteriorated on the whole peaks in the electropherogram, that is due to a poor separation although it was found the each peak with respect to each base pair fragment. This reason was considered that the diffusion was increased since the HEC viscosity was brought down by the decrease of HEC concentration and the sieving effect by HEC polymer solution was declined.

4. Summary

Separation of DNA fragments was performed using on-chip electrophoresis microcapillary. The device was fabricated on the photosensitive glass with simpler process than quartz glass conventional fabrication process on the glass. Using the HEC polymer solution as sieving matrix, the on-chip electrophoresis microcapillary with the short separation channel was able to separate DNA fragments. The short DNA fragments in DNA ladder was separated within 40 seconds in 8 mm separation distance. Also, by adjusting the applied electric field strength, it was performed to separate the long DNA fragments. However, to separate all of the DNA ladder fragments well, it needs that further improvements of the device and reconsideration of appropriate experimental condition, for example, lengthening the channel length and changing HEC concentration and applied voltage. Using the CLSM to detect the separation of DNA fragments was so useful because of low noise, high sensitive and one point detection which no depended on the plug shape in the separation channel.

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