Femtomol Single-DNA Molecules Analysis by Electro Field Strength in a Microfluidic Chip Using TIRFM

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Received October 25, 2004

Key Words : Single-molecule detection, Microchip, TIRFM

Microchip techniques such as the micro total analysis system (μ-TAS)[1] or lab-on-a-chip[2] have been developed, practically in the microfluidics fields. Microfluidics has enabled many developments in chemical analysis of biomolecules including high-speed separation, high-throughput, parallel assays, and microscale sample preparation.[3] Recently, microchip electrophoresis (ME), which uses various microfluidic chips, has become a powerful and effective analytical technique for DNA analysis, due to its analytical throughput, speed, small reagent volume, automation, miniaturization, and high resolution.[4] The most significant advantage of ME in DNA analysis is its high speed compared to conventional slab gel electrophoresis. However, although ME provides a fast analysis, a major limitation of ME analysis is a small sample volume that causes low concentration sensitivity.

Single-molecule detection (SMD) techniques with a high sensitivity have recently attracted plenty of attention in the field of life science.[5] The observation and manipulation of single biomolecules allow their dynamic behaviors to be studied to provide insight into molecular genetics,[6-8] biochip assembly,[9-12] biosensor design,[13-15] DNA biophysics[16-30] and basic separation theories of capillary electrophoresis and liquid chromatography.[31-37] Although the major advantage of SMD is the ability to observe species that present at low concentrations in biomaterials, a recent SMD study demands an ultra-sensitive sample detection system in solution. However, the major problem to overcome when individual biomolecule in an aqueous solution is the huge amount of background noise, which can be caused by Raman scattering from water molecules, incident light, luminescence from the objective lens, and dust.[5]

Total internal reflection microscopy (TIRFM) is an optical technique used to observe single molecule fluorescence. After single fluorophores in an aqueous solution were first observed in 1995 using TIRFM and conventional inverted fluorescence microscopy,[23] some biophysicists and chemists have used the TIRFM technique for many years, while others are just beginning to explore the boundaries of this versatile mechanism for studying phenomena occurring at interfaces.

In our TIRFM application of a microchip, the evanescent field layer (EFL) was formed when the incident beam was totally reflected by the interface between the run buffer solution and the surface of microchip. We applied the microchip to an EFL, which can detect fluorescently labeled DNA molecules, and which is based on the principle of the TIRFM. The EFL is not restricted to the diffraction limit of light,[5,30] thus it could be localized close to the microchip surface, which resulted in the penetration depth (~150 nm) being several-fold shorter than the wavelength of light (Figure 1). Therefore, the illumination was restricted to a complex fluorescent DNA sample either bound to the microchip inner surface or located in the microchip, thereby reducing the background light. This effect leads to images of high contrast having an excellent signal-to-noise. We demonstrated the detection of different size single-DNA molecules (*i.e.*, 48.5 kb DNA and 5.4 kb DNA) by TIRFM

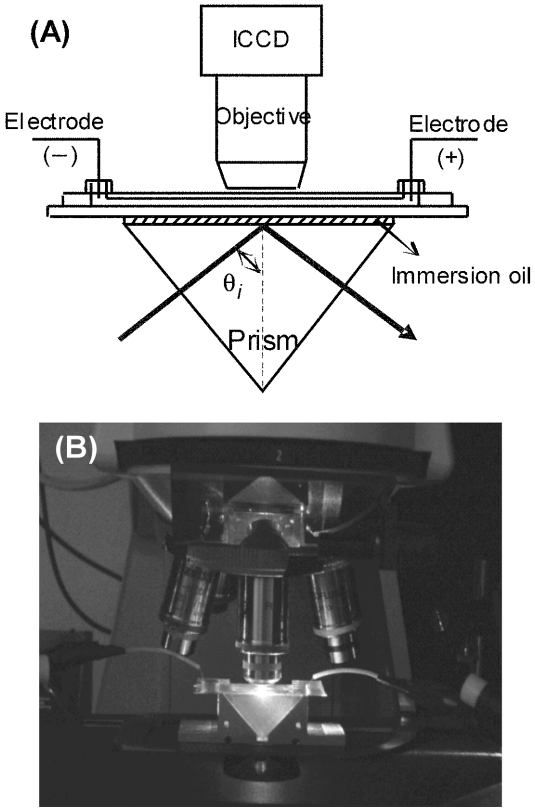

Figure 1. (A) Schematic diagram and (B) picture of TIRFM experiment setup at the PDMS/glass microfluidic chip for single-DNA molecule monitoring within the evanescent field layer.

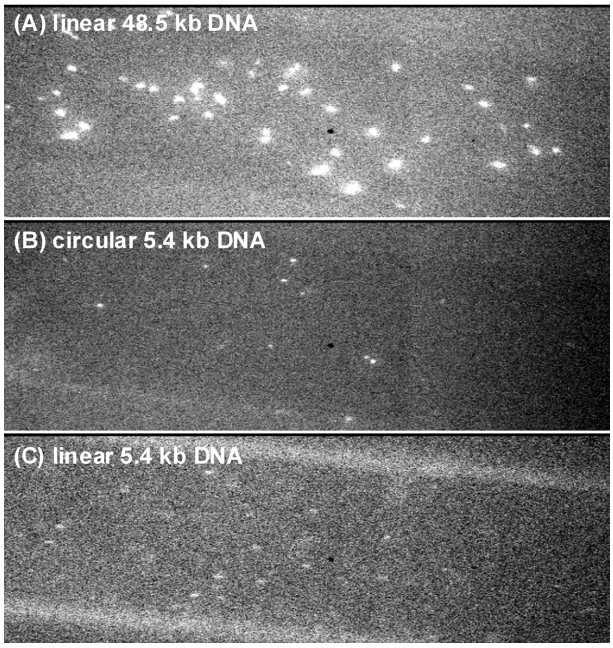

Figure 2. Video images showing the shape of (A) 48.5 kb DNA, (B) circular 5.4 kb DNA molecules and (C) linear 5.4 kb DNA molecules in the PDMS/glass microchip. Run buffer solution, 0.25% $C_{16}E_6$ in 10 mM Gly-Gly buffer (pH 8.2); applied electric field, 40 v/cm. DNA sample concentration, 100 fM. ICCD exposure time, 10 ms; frame rate, 5 Hz; laser power, 50 mW.

and the manipulation by the electric field strength in a PDMS/glass microchip (Figure 2). The video images of flowing individual DNA molecules in the mixture of 48.5 kb DNA and circular 5.4 kb DNA (Figure 3) were also well distinguished in the microchip as well as in each pure single-DNA molecules (Figure 2). The migration velocities of different size DNA molecules as a function of the electric field in the PDMS/glass microchip are shown in Figure 4. The TIRFM technique was successfully applied to follow real-time dynamic behavior of the individual DNA molecules as the ultra-sensitivity of femtomol (fM) concentration level in the microchip. The migration velocity was recorded for 50 molecules from each data set. Over an electric field strength of 25 V/cm, the velocities of both linear type DNA molecules (linear 5.4 kb DNA and λ-DNA) were consistently increased as field strength increased. However,

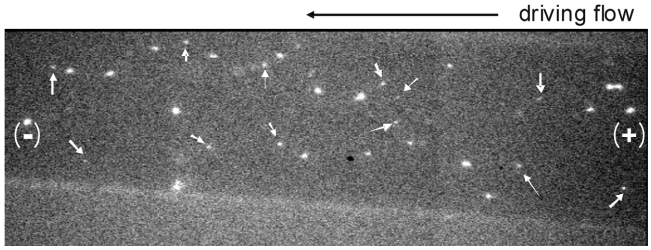

Figure 3. Video images of the DNA mixture sample (circular 5.4 kb DNA molecules and 48.5 kb DNA molecules) at the PDMS/glass microchip. Experiment conditions were the same as those in Figure 2. *Arrows indicate the individual circular 5.4 kb DNA molecules.

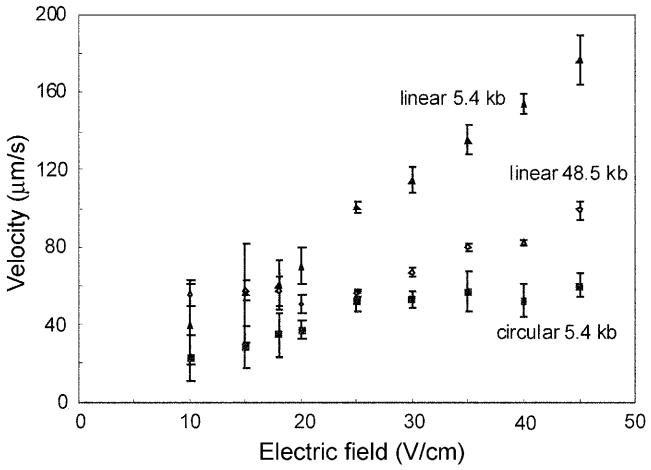

Figure 4. The migration velocities of the different size DNA molecules as a function of the electric field in the PDMS/glass microchip. The experiment conditions were the same as those in Figure 2. Solid triangles: linear 5.4 kb DNA. Solid squares: circular 5.4 kb DNA. Open circles: 48.5 kb DNA.

the velocity of circular DNA molecules was not in proportion to the field strength. Because the liner DNA molecules have a stretched-form under a high electric field ($\geq$ 25 V/cm) and the molecules have a weak steric hindrance at the sieving medium, while the circular 5.4-kb DNA molecules still have only the circular form. So, the DNA molecule is faster than the circular DNA molecule above 30 V/cm and the buffer condition. This phenomena indicated that the shape of DNA molecules have an influence on the migration velocity of DNA in the microchip. The calculated migration velocity of the linear 5.4 kb DNA molecule in the microchip showed the highest velocity in the range of 20-45 V/cm. It was possible to distinguish 5.4 kb DNA from 48.5 kb DNA on the basis of electrophoresis at the single-molecule level with almost no doubt.

To summarize, we demonstrated a single-DNA molecule detection method in a microfluidic chip by TIRFM that allows single DNA molecules of different sizes to be screened at a time based on their migration velocity. Since the measurement only depends on being able to follow a molecule for a few milliseconds in the solution, the different size DNA molecules can be analyzed within a few seconds and with an ultra-sensitive detection intensity as fM level.

Experimental Section

Chemicals. Gly-Gly, $C_{16}E_6$ and sodium hydroxide were A.C.S. grade purchased from Sigma Chemical Co (St. Louis, MO, USA). A 10 mM aqueous solution of Gly-Gly was prepared, and the pH was adjusted to 8.2 with the addition of 1 M NaOH. All buffer solutions were filtered through a 0.2-μm filter and left for photo-bleaching overnight with a UV-B lamp (G15T8E, 280-315 nm, Hansung Ultraviolet Co., Ltd., Korea). The run buffer was 0.25% $C_{16}E_6$, dissolved in the 10 mM Gly-Gly buffer (pH 8.2). The nonionic surfactant, $C_{16}E_6$ was used as a sieving matrix for the separation of DNA

fragments.[38]

DNA Samples Preparation. λ-DNA (48 502 bp), ΦX174-DNA (5 386 bp) and others DNA samples were obtained from Promega (Madison, WI, USA). All DNA samples were prepared in a 10 mM Gly-Gly buffer. DNA samples at a concentration of 200 pM were labeled with an intercalating dye YOYO-1 (Molecular Probes, Eugene, OR, USA) at a ratio of one dye molecule per five base pairs according to the manufacturer's instructions. DNA samples labeled with YOYO-1 were allowed to incubate for 5 min before further dilution and use. For the single-molecule imaging experiments, these DNA samples were further diluted to 1-100 fM, prior to the start of the experiment.

Microscope and CCD Camera for Microfluidic Chip. A Pentamax 512-EFT/1EIA intensified CCD (ICCD, Princeton Instruments, Princeton, NJ, USA) camera was mounted on top of a Zeiss Axioskop2 upright microscope with a Nikon 40 × Plan-fluor oil type (1.30 N.A.; Japan). The digitization rate of the camera was 5 MHz with software controller gain set at 3, and a hardware intensifier gain set at 80. The camera was operated in the external synchronous mode with the intensifier disabled open, and was also used in the frame-transfer mode. The excitation source was a wavelength tunable argon ion laser (out power 150 mW at 488 nm; model 35LAP431-220, Melles Griot, Irvin, CA, USA), which was used as the excitation beam. A Zeiss Filter set No. 09 was used. A Uniblitz mechanical shutter (model LS2Z2, Vincent Associates, Rochester, NY, USA) was used to block the laser beam when the camera was off in order to reduce photo-bleaching. The shutter was controlled by a model VMM-D1 shutter driver. The experimental timing was controlled with a DG535 four-channel digital delay/pulse generator (Stanford Research Systems, Inc., Sunnyvale, CA, USA). The ICCD camera was triggered at a time of 0-ms with 10-ms duration TTL pulse. The sampling frequency was 5 Hz, with the shutter driver set to 10-ms exposure and 190-ms delay. WinView/32 software (version 2.5.14.1, Downingtown, PA) was used for DNA image collection and data processing.

Evanescent Wave Excitation Geometry. The excitation geometry was similar to that previously described (Figure 1).[35,37] Briefly, the microchip was placed on the hypotenuse face of a right-angle prism (Melles Griot; BK7, A = B = C = 2.54 cm, refraction index (n) = 1.516). The chip and the prism were index-matched with a drop of immersion oil (Immersol™ 518F, Zeiss, n = 1.518). The laser beam was directed through the prism toward the microchip/run buffer interface. The angle of incidence θ_i was slightly greater than 69°.

PDMS/Glass Microchip Preparation for TIRFM. The microchip preparation for TIRFM is similar to that described in ref. 39. Briefly, the chip had a simple cross injector design composed of one PDMS plate (cover for microchannel, 0.26-mm thick) and one glass plate (No. 1 Corning cover glass, n = 1.523). Tygon hose (I.D. 2.4 mm, O.D. 5.5 mm; SAINT-GOBAIN, OH, USA) pieces were cut to orifice at the end of each channel and immobilized with UV epoxy for the sample and run buffer reservoirs. The channel dimension was 50-mm long, 50-μm wide and 50-μm deep. The reservoirs were 2.0 mm in diameter and 1.76-mm in total depth. A PDMS/glass microchip was placed on the prism with the index-matched with immersion oil (n = 1.518).

Acknowledgements. The authors wish to thank the research team of the Center for University-Wide Research Facilities (Chonbuk Natl. Univ.) for their assistance with instrumentation, and Ji-Young Lee at Iowa State University for fruitful discussions. This work was supported by the Korea Research Foundation Grant (KRF-2003-002-C00189).

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