Biomolecule Separation by Steric Hindrance using Nanofluidic Filters

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Abstract—**Micro/nanofluidics technologies can be used to solve toughest challenges in the biomolecule analysis. It is now possible to fabricate nanofluidic channels with the dimension of 30-500nm, and these nanofluidic structures have been formerly used to separate large DNA molecules where molecular dimension is larger than the nanofluidic filter gap size. In this work, we demonstrate separation of biomolecules (DNA and proteins) that are *smaller* than the nanofluidic filter gap size. This is possible due to the steric hindrance effect of the biomolecules; the entropy of biomolecules has to be decreased for the molecules to enter the nanofluidic filter, which leads to the free energy barrier for the molecular transport. Double stranded DNA molecules as small as 100bp (~34nm extended length), as well as SDS-coated proteins have been separated in a nanofluidic channel that has the filter gap thickness between 60-120nm. This result clearly shows the potential of using nanofluidic filters as a sieving medium for smaller biomolecules such as proteins. Compared with traditional random nanoporous materials such as gel or polymer monolith, nanofluidic channels can be made precisely to have a pre-determined 'pore' size and shape, which allows characterization and optimization of biomolecule separation process.**

Keywords—n**anofluidics, biomolecule separation, DNA, protein, steric hindrance**

I. Introduction

Efficient separation and purification techniques are very important in modern genomics and proteomics, mainly because most biomolecule samples are highly diverse and complex. Decreasing the sample complexity of the 'real' biosamples, such as blood serum or urine, is crucial for increasing sensitivity of downstream detection tools. More importantly, most biomolecule detection tools (such as mass spectrometry and antibody-based biosensors) have a limited dynamic range of detection, while the concentrations of biomolecules in a typical blood serum varies up to many orders of magnitude.

Biomolecule separation has benefited immensely from the advent of microfluidic biomolecule separation systems. However, the realization of truly integrated micro total analysis systems (μTAS) has been elusive, mainly because of the difficulties in integrating different components of biomolecule analysis on a microchip format. Especially, biomolecules separation and sieving is still largely done by polymeric liquid or solid gels (nanoporous materials), which are technically challenging to be coupled with MEMS fabrication techniques that are used to make microchips.

Recently, micro/nanofluidic molecular sieving and separation systems, using MEMS-fabricated regular structures, drew much attention. Compared with nanoporous materials or membranes, regular molecular sieving structures are mechanically and chemically more robust, can be integrated into standard MEMS processes easily, and could be precisely engineered to have better separation efficiencies. Since the first introduction of the idea[1], regular micro/nanofluidic structures have been used for separating large DNA molecules and particles with far greater speed and efficiency[2, 3]. However, separation of smaller biomolecules (such as proteins and small DNA) has not been achieved yet, mainly due to the difficulty in fabricating nanofluidic sieving structures (or filters) with sizes comparable to biomolecules.

In this work, we present the use of nanofluidic molecular filters for separating smaller biomolecules. These molecules are smaller than the gap size of the nanofluidic filters, but they still get separated in the nanofluidic filter devices due to the steric hindrance mechanism (Fig. 1). This clearly demonstrates the potential of nanofluidic molecular filter devices, even with relatively large (60-120nm) filter gap sizes, for separating various smaller biomolecules, including proteins.

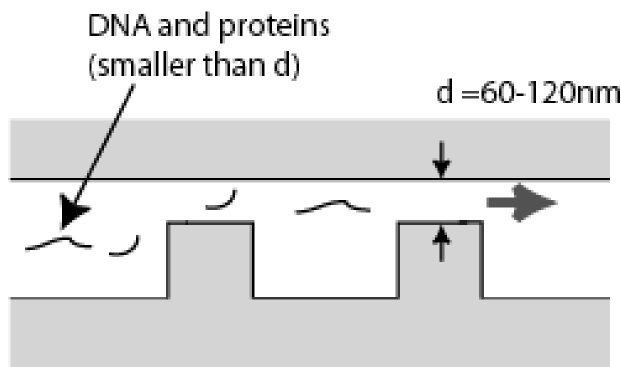

Fig. 1. Cross-sectional schematic picture of the nanofluidic trap array. When the sizes of DNA molecules are comparable to the thin channel depth, they are hindered from entering the trap with steric hindrance effect and their mobilities become size-dependent due to the confinement entropy change. Smaller DNA molecules migrate faster and have higher mobilities since they are favored to enter the trap due to their smaller size.

II. Methodology

1) *Device Fabrication*: The nanofluidic filter devices were fabricated on Si substrate, by standard

photolithography and etching techniques. After defining the nanofluidic filters and channels using photolithography, a thermal oxide is grown to prevent electrical leakage between the fluid and Si. Finally anodic bonding technique is used to complete the fabrication. Further details on device fabrication can be found elsewhere[4]. Since we are confining the molecules in the vertical direction, no high-resolution lithography techniques are required to make a nanofluidic channel. The final device will have nanofluidic filters (60-120nm thickness), in between the 'thick" regions (500-1000nm thickness). The period of the structure was 4μm, and each period contains one entropic trap (transition from thick to thin regions). The total length of the device was 3cm (separation channel), but detection was done at different distances from the band-launching site. The loading and launching the biomolecule sample was achieved in the standard double T-junction channel, by controlling the potential at each reservoir (Fig. 2).

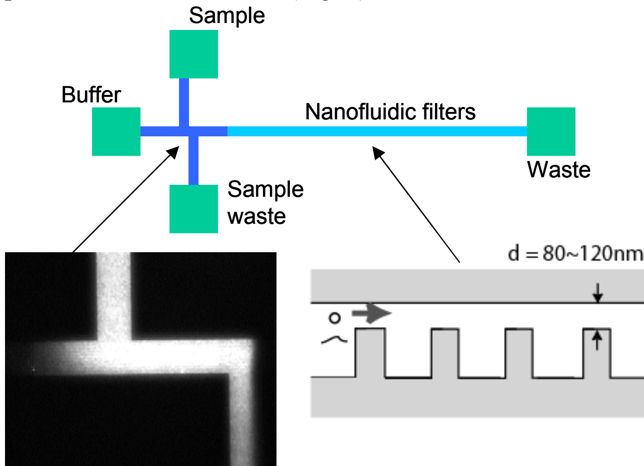

Fig. 2. Schematic diagram of the nanofluidic separation device.

2) *Sample Preparation*: Double stranded DNA molecules (sized between 100-1500bp) were purchased (New England Biolab), and were labeled by YOYO-1 dye (Molecular Probes) using the method suggested by the manufacturer of the dye. The dye to basepair ratio was 10:1. As a buffer for DNA electrophoresis, we used 5X Tris-Borate-EDTA (TBE) buffer. For protein separation experiment, fluorescently (FITC) labeled proteins were purchased from Molecular Probes, and were denatured in a buffer solution with 2% sodium dodecyl sulfate (SDS). As a final electrophoresis buffer, we used 5X TBE buffer with 0.1% SDS.

3) *Electrophoresis:* DNA or protein samples were first loaded into the sample reservoir, and the well-known microfluidic sample injection method was used to load and launch the separation. The injection volume was estimated to be about 10pL ($1\times50\times200\mu m^3$), and the amount of DNA molecules injected per each experiment were about $\sim10^{-18}$ moles.

In both DNA and SDS-coated proteins, molecules carry negative charges, which prevented them from adhering to the nanofluidic filter walls that is also negatively charged. This electrostatic repulsion effectively prevented non-specific binding of biomolecules on the nanofluidic channel surface.

4) *Optical Detection and Data Analysis*: Molecules in the nanofluidic channel can be imaged through the Pyrex glass bonded to the Si substrate. Inverted microscope was used for fluorescence microscopy. Ar-ion laser light at 488nm wavelength was coupled to the microscope for laser-induced-fluorescence (LIF) detection of biomolecules. Fluorescence light from the biomolecules were detected by a cooled CCD camera (Sensicam QE, Cooke Corp.) and processed using a imaging software (IPLab, Scanalytics) to produce electropherograms.

III. Results

A. DNA separation

Fig. 3 shows the separation result of 100bp double stranded DNA ladder sample, which were obtained at the 1cm away from the launching position. At the field as low as 26.67V/cm, all 12 peaks were baseline-resolved without ambiguity. (100, 500, and 1000bp DNA bands have higher intensity for easy peak assignment.) The separation resolution decreased as we increase the separation potential. The period of the device (the length of one thick and one thin region) was 4μm. The nanofluidic filter was 80nm thick in this case, and some of the smaller DNA molecules in this sample were smaller than 80nm. For example, 100bp double-stranded DNA molecules are only ~34nm long even when fully extended. Similar separation results were obtained using the device with 120nm filter gap size.

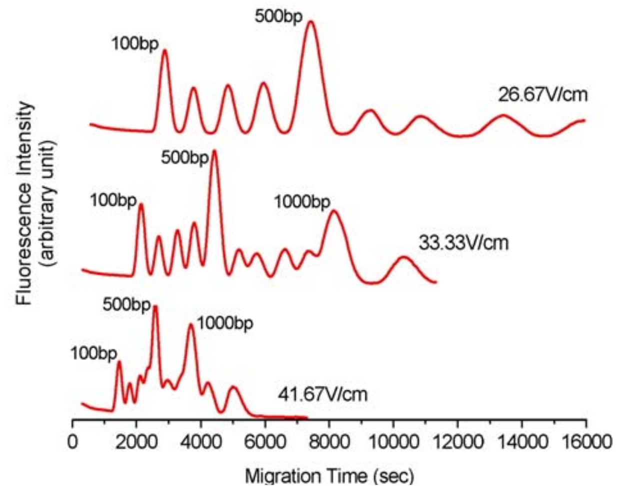

Fig. 3. Electropherograms for the 100bp DNA ladder by the nanofluidic filter device. The thick region of the device was 500nm, whiel the nanofilter gap was 80nm. These separation results were obtained at the 1cm from the launching site. All the peaks existing in the sample were assigned unequivocally, and a baseline separation was achieved in the case of 26.67V/cm.

We conducted experiments with many different DNA molecules and at different fields. The separation result was summarized in Fig. 4. The mobility data of DNA molecules with various lengths at low electric field values fits well into an exponential function, represented as a straight line in Fig. 4. At higher electric field, data starts to deviate from the exponential behavior.

We also conducted the separation experiment with DNA molecules in a nanofluidic channel with uniform thickness of 60nm. We did not observe any separation in these experiments at various field values (20-60V/cm).

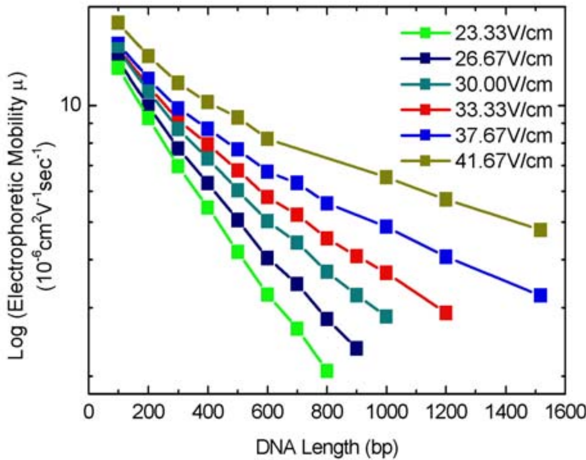

Fig. 4. Logarithmic plot of DNA mobility versus the DNA length at different field values. All of these data were obtained from the same device with 80nm nanofluidic filter.

B. Protein Separation

Fig. 5 shows the electropherogram of separating protein molecules (SDS-denatured). Two fluoresecently labeled proteins were denatured with SDS, and were separated in the nanofluidic filter devices with 80nm gap size. As in the DNA separation case, the separation resolution improves as the driving electric field is decreased. We also ran a control electrophoresis experiment with two SDS-coated proteins in a flat channel as a control experiment, where we did not see any separation. This confirms that the elctrophoretic mobility of SDS-coated proteins is the same, as in the case of double stranded DNA.

IV. Discussion

A. Sieving mechanism

It is clear that the separation observed was not due to the inherent differences in the electrophoretic mobility in free solution (CE mobility), as confirmed by the result of no separation in the flat channel CE experiment. (It is also well known that the electrophoretic mobility of double stranded DNA molecules (longer than ~20bp) is independent of the length of the strand.) The interaction between negatively charged biomolecules (DNA, SDS-coated proteins) and surface Debye layer is not a factor in this case, due to a relatively high buffer ionic strength.

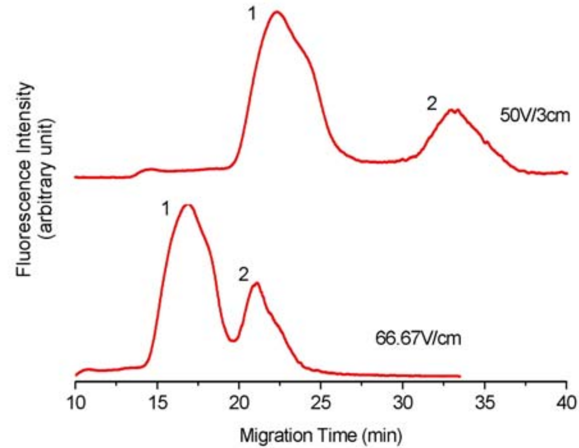

Fig. 5. Electropherograms for the two fluorescently labeled protein denatured with SDS. The thick region of the device was 500nm, while the nanofilter gap was 80nm. (4μm trap period) These separation results were obtained at the 1cm downstream from the launching site. The peak 1 represents Ovalbumin (45kD), and the peak 2 represents Human low-density lipoprotein (179kD).

The separation results are also not due to the separation mechanism called as "hydrodynamic chromatography"[5], where larger molecules are transported faster due to the Poiseiulle-flow profile in the channel. In electrophoresis in microchannel, the electroosmotic flow profile would be that of a plug (flat) flow. Also, hydrodynamic chromatography would make larger molecule elute first, rather than later.

Another possibility is the separation due to the dielectrophoretic force[6] at the field gradient. Due to the structure of the nanofluidic filters, there exist an electric field gradient in the nanofluidic filter device, especially in the region near the nanofilter entrance. If this were the case, however, the separation (and filtration) should have been improved as one increase the field intensity, since then the dielectrophoretic force would have become stronger. From the experimental data, it is clear that the separation selectivity become smaller as we increase the field intensity (The slope in Fig. 4 decreases as with increasing field.).

Indeed, the fact that increased field decreases the separation selectivity strongly suggests that there must be a different, non-electrical sieving mechanism in effect (in competition with electrical driving force), such as mechanical (or Ogston-like) sieving[7]. This was also the case for the entropic trapping of longer DNA molecules, as previously reported[8].

We believe that the sieving mechanism here is indeed

due to the confinement (entropy loss) caused by the nanofluidic filters. The entropy loss involves an increase in the free energy of the molecule, which would lead to the free energy barrier for the molecular transport. In the case of long, rod-like polymer such as double stranded DNA molecules, the main entropy that is affected would be rotational degree of freedom. This was studied theoretically by Giddings[9], where he calculated the partition coefficient for a molecule between confined space and free space, assuming near equilibrium condition. Even the molecules smaller than the nanofluidic filter gap size will be sieved in the nanofluidic filters, since their degree of freedom will be still reduced (near the wall) within the nanofluidic filters. In our case, 100bp DNA (~34nm extended length) and protein samples were smaller (shorter) than the gap size of the nanofluidic filter (80nm), but they were still separated by the filter devices. This is significant, since it opens up the possibility of using nanofluidic filters for the separation of smaller molecules such as proteins and carbohydrates.

B. Speed, Resolution, Integration, Optimization

The separation obtained in this work is still much slower than capillary CE using polymeric sieving matrices. It has to be noted that the 80nm filter gap size was not optimized in any way for this separation application, and there is a lot more room to increase separation efficiency and resolution, by carefully designing and modeling the nanofluidic filter structures. Simply decreasing the filter gap size would enhance the sieving effect for smaller molecules. More importantly, this device does not use any buffer sieving agents or matrix, which is difficult to be integrated into a compact, total bioanalytic microsystem. It would permit the use of any buffer with comparable ionic strength, which would facilitate the integration of different analysis components in one system without isolating the different chemistry. For example, the multidimensional separation of protein requires isolation of two heterogeneous buffer systems, which can be achieved by incorporating complex microfluidic valve systems[10]. In addition, nanofluidic filter devices could be coupled to the mass spectrometry more easily, since the separation is achieved through the molecular interaction with the structure, not with buffer additives that has to be removed from the sample afterwards for on-line MS detection.

Another important advantage of this device is the ability to fine tune the device structure for a specific separation application. This is especially important for certain applications, such as carbohydrate separation. The careful designing of nanofluidic filter could eliminate the need for tedious, time-consuming separation characterization process required for developing techniques with nanoporous sieving material for this important biomolecule group.

V. Conclusion

It is demonstrated that the nanofluidic filter of sub-100nm filter gap size can be used for the separation of smaller biomolecules, such as short DNA and proteins. The separation mechanism is based on the entropic free energy barrier at the interface between thin and thick region of nanofluidic filter devices, and the separation selectivity can be explained by a theory similar to the Ogston sieving of gel electrophoresis. Molecular sieving of proteins was achieved without the need of polymeric solid or liquid gels. The new technique would be much easier for the integration into complex biomolecule analysis systems.

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