# NANOFLUIDIC MOLECULAR FILTERS FOR EFFICIENT PROTEIN SEPARATION AND PRECONCENTRATION

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## ABSTRACT

A regular nanofluidic channels, as thin as 20nm, have been fabricated and used for protein separation and preconcentration. The nanochannels were fabricated by the substrate-bonding method, and the regularity of the nanochannel has been confirmed by the cross-section SEM imaging. A nanofilter array device, with an array of 60-120nm thin nanofilters, was used for separating small biomolecules (short double-stranded DNA and SDS-coated proteins) based on the size. The sieving of biomolecules (which are comparable or smaller than the nanofilter gap size) is achieved by steric hindrance of biomolecules within nanochannels. The efficiency of this gel-free separation device is comparable to the capillary gel electrophoresis, and can be made better by further optimization. In addition, we have developed a novel biomolecule concentrator using a 40nm nanofluidic channel as a perm-selective membrane. More than a million time preconcentration of dilute protein or peptide solution was demonstrated, which was enabled by the stability of the device.

**Keywords:** Nanofluidic channels, preconcentration, separation, protein, Ogston sieving

## INTRODUCTION

Current proteomic research requires very efficient sample preparation tools that can decrease the complexity of common biomolecule samples. It is well known that typical biosamples such as blood serum contains more than 10,000 different proteins with concentrations that differ by more than $10^9$ times. Biomolecule separation and preconcentration are essential sample preparation steps in advanced biomolecule analysis, but current techniques using polymeric sieving materials and membranes are difficult to integrate with other detectors and sensors. Also, the lack of chemical amplification strategy (such as PCR) critically demands the sample preconcentration strategy for proteomic samples, since most information-rich targets (biomarkers) are present at tiny concentrations. However, most current sample preconcentration techniques demonstrate up to ~1000 fold concentration enhancement, which is not enough to deal with the dynamic range of biosamples such as blood serum.

It is now possible to fabricate a regular nanofluidic channel with uniform thickness as small as 20nm. In such a nanofluidic channel, channel depth is comparable to the molecular size, as well as the Debye layer thickness of the system. Therefore, one can take advantage of unique molecular and ionic transport characteristics to build a truly integrated bioanalysis microsystems.

Micro/nanofluidic molecular sieving structures[1] have great potential for faster molecular separation. However, their use has been largely limited to big molecules such as viral DNA, since it is rather challenging to fabricate structures with near-molecular dimension. We present a novel separation method for proteins using nanofluidic filters, which is based on the steric hindrance effect of molecules[2]. To demonstrate this, we fabricated nanofluidic trap array devices consists of alternating thin (60~120nm) and thick (250~500nm) regions in a microfabricated channel. The device was fabricated using the standard photolithography and etching techniques, and filled with standard electrophoresis buffer without any gel or sieving matrix. We also developed a microfluidic protein preconcentration device that is based on the unique ion transport properties of nanofluidic filters. Using the test device, we have demonstrated more than a million-fold preconcentration of a protein solution within a microchannel. The preconcentration factor for protein in this device is comparable to that of PCR process for nucleic acids. This device will find many uses in various applications such as biomarker detection from serum and chemical-biological warfare agent detection.

## EXPERIMENTAL

Fabrication of nanochannel was done by firstly etching a very thin channel (20~100nm) on Si or glass substrate, and then bonding another, flat substrate on top of the first substrate, as previously described[3]. Both glass-Si anodic bonding and glass-glass fusion bonding technique were used for this work. Supporting pillars are sometimes required to prevent the channel collapsing during the fabrication process. We have characterized the maximum aspect ratio (width / depth) of the nanochannel that can be achieved by the standard anodic bonding and fusion bonding processes.


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After the bonding, the devices were cut in half, and the cross section of the channel was inspected by SEM imaging, to confirm the uniformity and the deformation of the nanochannel fabricated.

Figure 1 shows the schematic diagram of the nanofluidic molecular filter array device. A serial array of nanofluidic filter array was fabricated, with alternating deep (250-500nm) and shallow (60-120nm) regions. The period of the device was between 1-4μm. Photolithographic patterning was done by the stepper in the MTL (Microsystems Technology Laboratories) cleanroom of MIT. Short double-stranded DNA ladder samples (50-1000bp) were purchased and labeled with intercalating YOYO-1 dye (Molecular probes) following standard labeling procedures. Also fluorescently labeled proteins were purchased and treated with SDS (sodium dodecyl sulfate) for the SDS-protein complex separation. In both experiments, Tris-Borate-EDTA(TBE) buffer at 5X (~0.4M) concentration was used as the buffer in the device and reservoirs. For the electrophoresis operation, standard electrokinetic band launching procedures were used by controlling the voltages in the reservoirs. The device was loaded on an inverted microscope (IX-71, Olympus) and the molecular motion was observed by cooled CCD camera (Sensicam QE, Cooke), using mercury arc lamp (or Ar-ion laser) as the excitation light source.

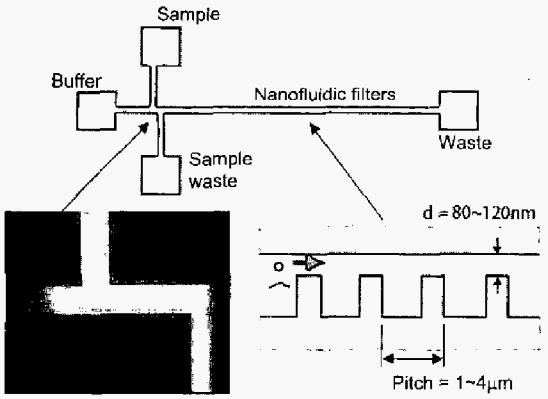


**Figure 1** Schematic diagram of nanofluidic filter array devices for biomolecule separation

Figure 2 shows the schematic diagram of nanofluidic biomolecule concentrator. It consists of two microchannels bridged by a nanochannel (40nm thin). At moderate buffer concentrations (10mM phosphate buffer, for example), Debye layer within the 40nm nanofluidic channel is not negligible, and the nanochannel acts as a perm-selective membrane. When an electric field $E_n$ is applied to the nanochannel, perm-selective ion current through the nanochannel generates an extended space charge region (or induced space charge layer), which is essentially a non-electroneutral charge-screening layer just like a Debye layer[4, 5]. Therefore, when an additional tangential electric field ($E_T$) is applied, negatively charged biomolecules can be brought in and trapped in front of this boundary.

For the concentration experiment, green fluorescent protein (GFP) was used as test molecules. Also, synthetic peptide molecules labeled with a fluorescent dye was used for testing concentration devices. 10mM phosphate buffer was used for the most time as a buffer, but several different buffer systems were tried. Experiments were done on an inverted microscope using cooled CCD camera as a detector.

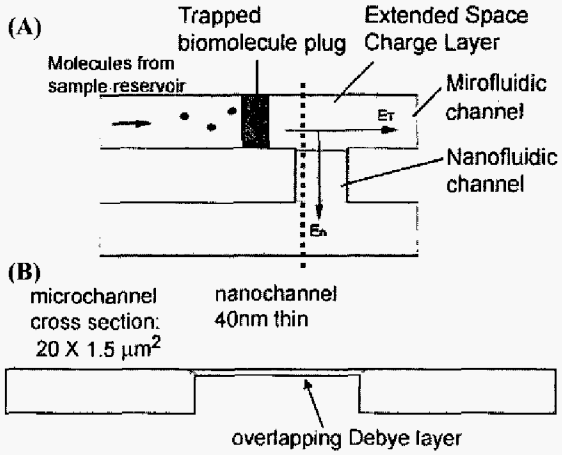


**Figure 2**: Schematic diagram of nanofluidic biomolecule concentrator. **a)** Top view. An electric field $E_n$ is applied across the nanochannel, to create extended space charge layer (SCL). SCL will be an energy barrier for anionic biomolecules, and they will get trapped before the SCL. **b)** Cross section diagram along the dotted line

## RESULTS AND DISCUSSION

***Nanofluidic channel fabrication*** The method we used allows good control on the dimension of nanochannel without the need for nanolithography techniques, simply by making 'thin' channels instead of 'narrow' channels. Also, both Si-glass anodic bonding technique and glass-glass fusion bonding

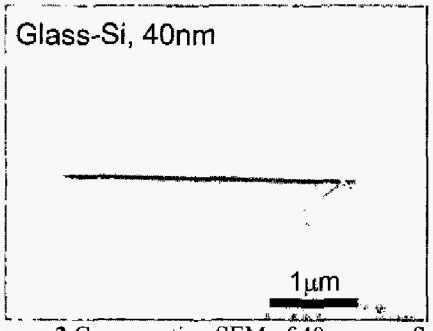


**Figure 3** Cross-section SEM of 40nm nanofluidic channel

technique can be used for this strategy. We have characterized the bonding conditions for both cases, and found out that the substrate-bonding method of nanochannel fabrication can generate nanochannels as thin as 20nm, with the aspect ratio of 250 (with anodic bonding) ~ 1000 (glass-glass fusion bonding).

The cross section of the nanochannel revealed no defects, deformations or collapse (Figure 3), and the measured channel thickness (from the cross-section SEM images) was in agreement with the channel thickness measured before the bonding process. AFM imaging of the etched surface showed that the surface roughness of the RIE- or wet-etched surface is typically in the order of 1~3nm[6].

***Protein and DNA separation using Nanofluidic Filter Array Device*** Using the nanofluidic filter devices, we have separated small double-stranded DNA (dsDNA) molecules (50-1000bp, Figure 4) as well as SDS-coated protein molecules (Figure 5). The separation speed obtained by the device was comparable to that of capillary gel electrophoresis with liquid sieving matrix, which is the current state of the art. However, the column length of the nanofluidic filter array device was much shorter (5~10mm typically).

The separation efficiency in this device critically depends on the number of nanofluidic filters per unit length. In the control experiment using the flat, 60nm thin nanofluidic channel (no shallow or deep regions), no separation was observed over a wide driving electric field values. When the period of the device was decreased from 4μm to 1μm (4-fold increase in the number density of nanofilters), the separation speed increased almost by ~10 fold (Figure 3 and 4 was obtained from 1μm period devices). The period of the nanofilter structure could be further decreased, perhaps by e-beam or other nanolithography techniques, to obtain even faster separation and shorter channel length.

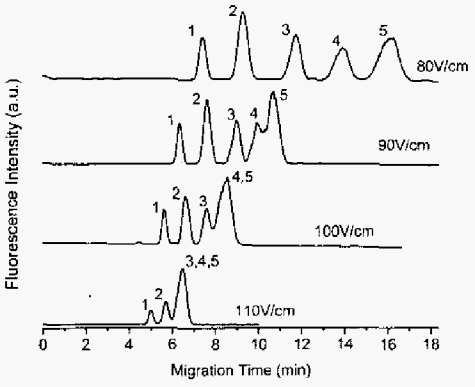


**Figure 4** Separation result of short, double stranded DNA molecules. Band assignment: (1) 50bp; (2) 150bp; (3) 300bp; (4) 500bp; (5) 766bp.

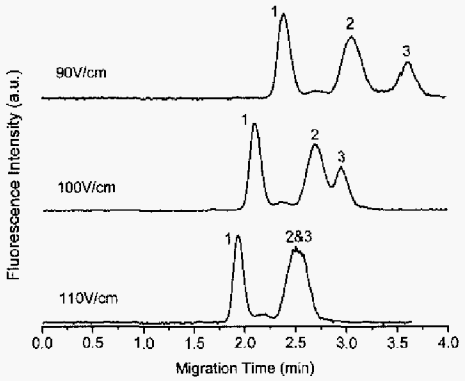


**Figure 5** Separation of SDS-coated proteins. Band assignment: (1) cholera toxin subunit B, 11.4kD; (2) PHA-L, 120kD; (3) low density human lipoprotein ,179kD

Some dsDNA molecules and SDS-coated protein molecules used in this work are expected to have molecular size smaller than the size of the nanofilter, which was 60-80nm in this work. Still, size-based separation of these molecules was achieved by electrophoresis. The mechanism of separation was attributed to the Ogston sieving mechanism, which is based on the steric hindrance of biomolecules within the nanopores (nanochannels in this case). The possibility of hydrodynamic chromatography is excluded since the ionic strength of the buffer is quite high (0.4M). Also, separation resolution deteriorated when the electric field was increased, which suggests that a non-electrical force is responsible for sieving effects.

***Nanofluidic Protein Preconcentration*** The protein preconcentration is achieved when the molecules are driven in by applying tangential electric field ($E_T$), as well as the field in the nanochannel ($E_n$), as shown in Figure 2. Figure 6a shows the preconcentrated GFP molecules at the boundary of the extended space charge region. Proteins were driven in by electrokinetic flow and collected at the concentrator. The operation of the device can be maintained stably for more than 3 hours, which allows very high (~$10^6$) concentration factor. In our experiment, 33pM GFP solution was concentrated to a very localized sample bolus (shown in Figure 6a and 6d) with local concentration much higher than ~3.3μM. The quantification of the local concentration was done by measuring and comparing the fluorescence signal from the sample bolus with the fluorescence signal of GFP solution of known concentration in the channel.

Concentrated sample plug can be released by simply turning off the $E_n$, and the resulting plug can be mobilized either by electrokinetic or pressure-driven flow. The throughput of the nanofluidic concentration is not limited by the nanochannel dimension, since the

nanochannel is only providing the energy barrier for molecular trapping. A larger volume concentration device, with the microchannel cross-section area of 50μm×50μm, has been recently demonstrated in the lab. While the concentration process became more efficient when the ionic strength of the buffer was decreased, it was still possible to maintain efficient concentration even at 10mM phosphate buffer condition. In addition, different pH conditions (pH 5~9) or different buffers have been used successfully in the device.

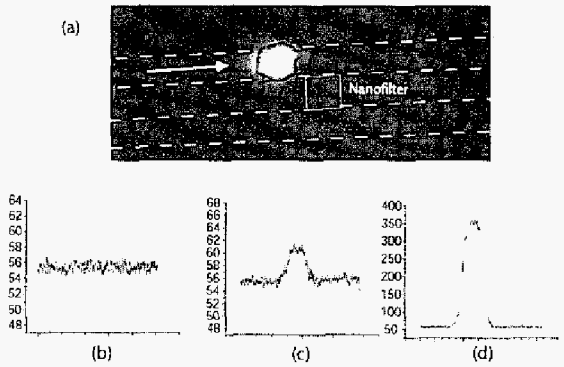


**Figure 6**: (a) Fluorescence image of focused proteins (GFP) in the channel. This preconcentration was obtained from initial 33pM GFP solution, after 50 mins of sample collection. The microchannel (20μm wide, 1μm deep) is shown by a broken line, while nanofilter is drawn by a solid line (40nm deep).(b) Channel fluorescence signal profile at the initial concentration (33pM GFP solution), which is below noise floor. (c) Channel fluorescence signal profile at the concentration of 0.33μM GFP, which is barely detectable by the detection setup. (d) The fluorescence signal profile of the concentrated GFP in the channel (figure (a)). The concentration of the plug is much higher than that of (c).

## CONCLUSION

These nanofluidic separation and preconcentration tools for molecular analysis have many advantages over conventional random, nanoporous filtration / sieving materials. Aside from impressive separation efficiency and preconcentration factors, solid-state nanofluidic filters are much more robust than polymer membranes, both chemically and mechanically. The uniformity of the nanofluidic channels allows stable operation, repeatability, and the possibility for modeling and optimization. Integration with other biomolecule detection systems, such as antibody-based biosensors or mass spectrometry, would be easier since there is no liquid sieving gel or membranes. It is expected that these nanofluidic tools could be essential in the development of microanalysis systems for proteomic samples.

One technical issue with these nanofluidic devices is the limitation in sample throughput. This is especially an issue for nanofluidic protein separation device, where nano-confinement of protein molecules was achieved by fabricating very thin channels. While this strategy allows very tight control on the channel thickness, it also limits the molecular throughput of the separation device. One possible solution for this would be the fabrication massively parallel, vertical nanofluidic filters, which is currently actively sought in our group. Once a massively-parallel, solid-state nanofluidic filter membrane is realized, it could be applied to many diverse applications, from molecular sorting / biosample preparation to the ion-selective membranes for batteries and fuel cells.

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