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Thin-Film Microfabricated Nanofluidic Arrays for Size-Selective Protein Fractionation

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Abstract

Size-selective fractionation and quantitation of biostructures in the sub-hundred nanometer size range is an important research area. Unfortunately, current methods for size fractionation are complex, time consuming, or offer poor resolution. Using standard microfabrication technology, we developed a nanofluidic sieving system to address these limitations. Our setup consists of an array of parallel nanochannels with a height step in each channel, an injection reservoir, and a waste reservoir. The height steps can size fractionate a protein mixture as a solution flows through the nanochannels via capillary action. We tested this system with different sizes and concentrations of five proteins to understand protein size and height step effects on trapping. Our results clearly show size dependent trapping of proteins at nanometer-scale height steps in nanochannels. We also developed a model that predicts the observed size-dependent trapping of proteins. This work is a key step towards scalable nanofluidic methods for molecular fractionation.

1. Introduction

Various biostructures in the sub-hundred nanometer range are associated with human diseases.^{1–3} For example, hepatitis B virus causes liver disease,⁴ and lipoprotein size and concentration distribution is linked with cardiovascular disease.⁵ High density lipoprotein (HDL) and low density lipoprotein (LDL), with diameters in the 5 to 25 nm size range, are important in cardiovascular risk assessment. Additionally, aggregation in pharmaceutical protein formulations is a major issue that can render a formulation physically unstable.⁶ Therefore, size-selective fractionation and distribution studies of these molecules or particles are an important analytical research area.

There are several techniques available to separate biomolecules based on size; for example, size exclusion chromatography (SEC), field flow fractionation (FFF), membrane filtration, ultracentrifugation, and electrophoresis. However, these techniques all have limitations. SEC can reproducibly separate molecules based on size but requires a >10% difference in

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molecular weight for adequate resolution.⁷ FFF can separate molecules and particles in the size range of 1 nm to 50 μ m based on their interaction with an external applied field.⁸ However, FFF requires a complex setup and experienced personnel. Membrane filtration fractionates based on a single cutoff size but suffers from sample loss, fouling, and pore clogging.⁹ Ultracentrifugation, which separates molecules based on size or density, is slow (>24 h) and energy intensive.^{10, 11} Gradient gel electrophoresis can separate biomolecules with high resolution based on electrophoretic mobility, but this technique is time consuming (~18 h) and requires skilled personnel.¹² Thus, improved methods for size fractionation of biostructures, especially in the 5–100 nm size range, are needed to overcome the disadvantages of current approaches.

Nanofluidics studies the behavior and manipulation of fluids confined in 1–100 nm dimensions.¹³ In these small size scales, fluids exhibit phenomena different from those at macroscale or even microscale levels due to overlap of these dimensions with molecular sizes and the electric double layer formed on channel walls. Nanofluidics is a growing field of research because various biostructures including proteins, nucleic acids, and viruses have sizes comparable to nanofluidic dimensions. Ongoing developments in micro and nanofabrication, including nanoimprint lithography, sacrificial approaches, etching, and bonding methods, have furthered the field of nanofluidics by enabling the fabrication of controlled nanostructures.¹⁴ Nanochannels smaller than 5 nm in width have been made by using focused ion beam milling.¹⁵ There are many fields such as biophysics and separation science where nanofluidics is now being evaluated,¹⁶ for example in pre-concentration and separation of proteins and nucleic acids,^{17, 18} and single molecule DNA sequencing.¹⁹ There are several challenges in making nanoscale devices, such as the fabrication costs imposed by high-resolution methods like focused ion beam milling and e-beam lithography; and issues with precision in channel dimensions, particularly after bonding steps. These challenges are especially problematic for fabricating nanochannels with dimensions below 30 nm.

Here, we have developed a nanofluidic-based sieving system that provides size separation of structures such as proteins in the ~10 nm diameter range. Our system consists of an array of 200 parallel nanochannels having height steps from 100 nm down to 15–30 nm. These readily adjustable heights can be achieved using widely available, standard thin-film micromachining methods. Capillary action draws solutions through the nanochannels, with larger molecules being trapped at the height steps while smaller molecules reach the ends of the nanochannels. We have evaluated this system with five model proteins whose sizes approximate those of HDL and LDL. We have measured the effects of protein diameter and nanochannel step height on trapping behavior of proteins. Additionally the influence of protein concentration on trapping was studied. These data provide an understanding of the correlation between protein size and height step, information that we have compared to a predictive model of size-based nanosieving. Our new system offers advantages over current nanoscale fractionation methods in terms of device size and simplicity, reduction of reagent volumes, and assay speed.

2. Experimental

2.1 Material and Reagents

As shown in Table 1, five proteins of different diameters from 3.5–17 nm^{20–23} were obtained from Sigma-Aldrich, St. Louis, MO. Each protein was labeled with fluorescein isothiocyanate (FITC, Invitrogen, Eugene, OR) for fluorescence detection. A 4 mg/mL stock solution of FITC was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and stock solutions of 2–3 mg/mL of each protein were prepared in 100 mM bicarbonate buffer at pH 9. Then, 20 μ L of FITC stock solution was added to 250 μ L of protein stock solution and incubated in the dark for 24 h at room temperature. Next, excess dye was removed from the protein solution using centrifugal membrane filters (Millipore, Billerica, MA) with mass cutoffs of 10 kDa (Mb), 30 kDa (Hb), and 100 kDa (Fer, Ct, and Tg). Protein solutions were rinsed multiple times with 10 mM bicarbonate buffer (pH 8.3) in an Eppendorf microcentrifuge (Hauppauge, NY) at 10,000 rpm for 6–8 min until a clear filtrate was obtained. Finally, bicarbonate buffer was replaced with 100 mM Tris-HCl buffer (pH 8.3) containing 2 mM sodium azide to make up the final volume of protein stock solution. The final stock concentration of each protein was measured using a Nanodrop spectrophotometer (ND-1000, Wilmington, DE). Subsequently, 1.0, 0.5, 0.1, and 0.05 mg/mL solutions of each protein were prepared volumetrically from the stock solution in 100 mM Tris-HCl buffer (pH 8.3), containing 1 mM sodium dodecyl sulfate (SDS, Shelton Scientific, Peosta, IA) and 0.12% v/v Triton X-100 (Sigma-Aldrich) as surfactants. All solutions were stored at 4 °C and vortexed before use to make a uniform solution.

2.2 Device layout and fabrication

Device design and fabrication are adapted from Hamblin et al.²⁴ Briefly, devices are fabricated on a 4" Si wafer using UV photolithography, thin metal film deposition, plasma enhanced chemical vapor deposition (PECVD), and wet and dry etching. Each 4" Si wafer produces 49 devices; each device has dimensions of 1 cm² and consists of 200 parallel nanochannels with height steps. These nanochannels are connected to an injection and waste reservoir for solution loading and solvent evaporation, respectively, as shown in Figure 1. Figure 1A shows a top view photograph of a completed device. Figure 1B–C shows zoom photomicrographs of nanochannels, reservoirs, and the height steps. Figure 1D–F shows schematically the relatively simple operational principles of these nanosieving devices.

Our fabrication scheme is shown in Figure 2. The following steps are involved in the fabrication of height step nanodevices. A ~100 nm layer of silicon dioxide is deposited on a clean 4" Si <100> wafer (Nova Electronic Materials, Flower Mound, TX) using PECVD; then, a layer of Al is deposited using e-beam evaporation with the thickness equal to the intended height of the shorter segment (Figure 2A). A ~15 nm thick Cr protecting layer is next deposited via e-beam evaporation as shown in Figure 2B. Photoresist (AZ nLOF 2020, AZ Electronic Materials, Branchburg, NJ) is then spun and patterned using photolithography, followed by Cr removal using chrome mask etchant (OMG Cyantek, Fremont, CA) in preparation for the second layer of Al to be deposited to make the taller nanochannel segments (Figure 2C). A second layer of Al is deposited; the sum of the thicknesses of the first and second depositions is equal to the height of the taller

nanochannel segments (Figure 2D). After a Cr protective layer is deposited, Al is lifted off by dissolving the photoresist in N-methyl-2-pyrrolidone (Spectrum, New Brunswick, NJ) at 90 °C and then the Cr is again removed using chrome mask etchant. AZ 3330 photoresist is next spun on the wafer and patterned to cover the core nanochannel lines as shown in Figure 2E. Exposed Al is etched in Al etchant (Type A, Transene, Danvers, MA) at 50 °C until Al is removed (~2 min), followed by photoresist stripping with acetone and 2-propanol (Figure 2F). A 4 µm thick capping layer of SiO₂ is next deposited using PECVD to cover the core lines as illustrated in Figure 2G. AZ 3330 photoresist is spun on the substrate and patterned to define injection and waste reservoirs (Figure 2H). Next, exposed oxide is etched completely down to the Si wafer in buffered oxide etchant (BOE, Transene) to open the channel ends into the reservoirs (Figure 2I). Finally, Al core lines are etched in aqua regia at 130 °C overnight to remove the sacrificial cores and open up the nanochannels, which are then soaked in deionized water for 4–6 hr (Figure 2J). We made five sets of devices, having a height step from 100 nm down to either 15, 18, 19.5, 22 or 29 nm; all other channel dimensions (length and widths) were held constant.

We made two important modifications to our prior fabrication method²⁴ to increase reliability and reproducibility, enabling capillary flow in channels with height steps as small as 15 nm. First, we used a ~15 nm Cr layer to protect the Al layers from the photoresist developer (AZ300 MIF, AZ Electronic Materials) as shown in Figure 2B. AZ300 developer slowly etches Al, resulting in inconsistent height steps, as well as discontinuities in <20 nm height channels. Secondly, the short segment length and tall segment height were both decreased to ~30 µm and 100 nm, respectively, to reduce the hydraulic resistance difference between these segments. These improvements enabled fabrication of devices with short segment heights down to 15 nm that sustained capillary flow without any bubble formation. This smaller height range is especially important because it opens the possibility of separating proteins. In addition, we streamlined several fabrication steps, such as overnight water and Nanostrip (OMG Cyantek) treatment of nanochannels, which shortened the fabrication time by ~50%. Step heights were confirmed using atomic force microscopy (AFM) for all five device types as shown in the Electronic Supplementary Information in Figures S1–S2. Prior to trapping experiments, all devices were baked at 110 °C, 170 °C, and then 250 °C, for 15 min at each temperature. This helped to remove any water that had condensed on the channel surfaces during storage and also aided in preventing bubble formation during capillary flow. Additionally, the high temperature bake decreased the number of free surface silanol groups, which could interact with proteins and cause adsorption.²⁵ Our scalable thin-film fabrication approach provides for batch processing of ~50 chips per 10-cm-diameter wafer, such that individual devices are considered to be single use and disposable. However, we note that a Nanostrip soak after trapping experiments could potentially be implemented to clean out and reuse devices.

2.3 Device operation

Nanochannels with height steps can be used to separate proteins based on their size as illustrated schematically in Figure 1D–F. We studied the trapping behavior of five different size proteins in five different sets of devices to understand the height step and protein size correlation. Each device design was used to test four different concentrations (0.05, 0.1, 0.5,

and 1.0 mg/mL) of each protein, and each concentration was run on three different devices from the same wafer to assess reproducibility. For each experiment, 400–500 nL of protein solution was loaded into the injection reservoir using a 5 μ L syringe (Hamilton, Reno, NV), which caused filling of the nanochannels due to capillary action. Some protein was trapped at the height step, while protein that made it through the step accumulated at the exit. Solvent evaporation from the channel ends after capillary flow maintained some additional flow. Each trapping experiment was run for 1.5 min. The experimental setup used to record fluorescence signal included an upright Axio Scope.A1 microscope (Zeiss, Thornwood, NY) fitted with a 625 mW blue LED light source (470 nm center wavelength, MBLED, Thorlabs, Newton, NJ). A brightline filter cube (FITC-LP01-Clinical-OMF, Semrock, Rochester, NY) consisting of a longpass dichroic mirror with a cutoff wavelength of 515 nm was used to separate excitation and emission signal. Fluorescence was detected using a Photometrics CoolSNAP HQ2 (Tucson, AZ) cooled CCD camera at 1.67 Hz with a 600 ms exposure time. The entire detection system was controlled using Micromanager 1.3, an open source, free plug-in to ImageJ.

2.4 Data analysis

Statistical data analysis was performed on each fluorescence image. We measured the average fluorescence intensity by drawing the same size region of interest around the fluorescence signal at the height step and the nanochannel exit, using ImageJ software. For each image, background-subtracted intensity measurements were made on nine nanochannels. The same size region of interest was used for all nine channels in an image. The region of interest width was kept constant at 30 pixels ($\sim 20 \mu\text{m}$), while the region of interest length was set to encompass the longest fluorescence signal in each image (25 to 40 pixels). Then the ratio of fluorescence intensity at the height step (trapped, T) divided by the sum of the signals from the height step and exit (total, t) was measured (T/t) for each channel. From the triplicate data sets for each concentration, an average T/t ratio and pooled standard deviation were calculated. Triplicate data analysis also helped in the assessing the reproducibility between devices obtained from the same wafer.

3. Results and discussion

The effect of protein size and concentration on trapping behavior was explored using five different size proteins and five different height step devices. This correlation study provides useful insight into the trapping behavior of biomolecules in the 5–20 nm size range at nanosize openings, and can serve as a guide to devise nanofluidic devices for separating two or more proteins of different sizes.

3.1 Trapping patterns of different proteins in devices with the same height step

The five proteins are trapped with different ratios in devices with the same height step due to their different sizes and shapes. Figure 3 shows representative fluorescence images of the five proteins after loading at 0.1 mg/mL in devices with height steps from 100–15 nm. Figure 3A shows that the smallest protein (Mb) traversed the entire length of the nanochannels with minimal stoppage at the height step and significant accumulation at the exit. Figure 3B shows that Hb also largely passed through the height step, although a small

proportion of the total Hb was trapped. Increasing amounts of Ct and Fer were trapped at the height step, and decreasing amounts passed through to the exit for these larger proteins as seen in Figure 3C–D. Finally, the largest protein (Tg) was almost completely trapped at the height step as shown in Figure 3E, with very little passing through to the exit. Overall, trapping at the 15 nm height step increased as the size of protein increased, even though the height step was larger than the protein diameter for all except Tg.

3.2 Trapping patterns of the same protein in devices with different height steps

We also investigated the effect of changing the height step on trapping behavior of a given protein. Figure 4 shows fluorescence images taken for trapping of Tg (0.1 mg/mL) in five different height step devices. In the 15 nm step devices (Figure 4A), Tg was almost completely stopped at the height step. In devices with 18 nm and 19.5 nm steps, some Tg reached the ends of the channels while some was trapped at the height steps as shown in Figure 4B–C. In nanochannels with 22 nm and 29 nm height steps (Figure 4D–E), Tg largely passed through to the end of the channels. A clear change in trapping behavior is observed as the height step goes from smaller than the protein diameter to considerably larger than the diameter.

3.3 T/t ratio as a function of protein concentration

Average T/t ratios obtained for all five proteins in five height step devices were plotted as a function of protein concentration as shown in Figure 5. These data show that trapping behavior of the proteins was largely independent of concentration, except in few instances for Hb, Ct and Tg, usually at the highest concentration (1 mg/mL). This could be a function of a particular protein's propensity to stick to itself or to the channel surface. Alternatively, concentration-dependent trapping could also possibly be explained by the "keystone effect" (see Figure S3 in the Electronic Supplementary Information), wherein a group of particles acts as a single larger particle that can block the passage of individual particles.^{26, 27} The formation of a "keystone" will depend on various factors such as protein size, concentration, and interactions with the walls or other proteins. Although the overall effect of concentration on the T/t ratio was relatively modest, to avoid this complicating factor, it may best to operate our devices at or below concentrations of 0.5 mg/mL. Alternatively, we have previously shown that clogging at height steps can be addressed by applying an oscillating voltage along the channels.²⁷

3.4 T/t ratio versus protein size

The T/t ratio for different step heights was plotted against protein size as shown in Figure 6. Figure 6A shows that the T/t ratio followed a general upward trend as protein size increased from 3.5 nm to 17 nm in 100–15 nm devices. In Figure 6B, the smaller proteins (Mb and Hb) show a low T/t ratio for 100–18 nm height steps, and higher T/t ratios were observed for Ct, Fer and Tg. Figure 6C shows a similar trend to Figure 6B for trapping as a function of protein size. In Figure 6D the T/t ratio is low for all data points except for 1 mg/mL Tg. Finally, Figure 6E shows a T/t ratio that is independent of protein size in 100–29 nm devices, as approximately the same (low) T/t ratio was obtained for all protein sizes and concentrations. Importantly, a clear protein size dependent change in trapping is observed as the height step is altered. This height step and protein size correlation demonstrates that size-

specific protein trapping can be accomplished in these nanofluidic systems. Moreover, this correlation study provides valuable information for developing and evaluating a model to predict the trapping of proteins at different height steps as described below.

3.5 Trapping model

We derived a model to predict the trapping of a rigid spherical particle at a nanoscale height step, leveraging an earlier study by Giddings et al.²⁸ on the equilibrium distribution of rigid spheres in a porous network. An equilibrium partition constant, K , across both sides of an opening for a rigid spherical particle approaching a gap between two parallel plates can be given as

$$K = \frac{A_{\text{available}}}{A_{\text{pore}}} \quad (1)$$

where $A_{\text{available}}$ is the area available to the particle inside the pore, and A_{pore} is the overall area of the pore. For a slit opening and a spherical particle,

$$A_{\text{available}} = W(H - D) \quad (2)$$

where W is the slit opening width and H is the height of the slit.

$$A_{\text{pore}} = WH \quad (3)$$

where D is the particle diameter. Substituting (3) and (2) into (1) yields

$$K = \frac{H - D}{H} \quad (4)$$

These equations apply to a slit geometry; however, our devices have a step, rather than a slit geometry (a slit has a step from both the top and bottom surfaces). For the step model A_{pore} is further reduced because the bottom wall constrains the lower position of the particle on either side of the height step (see Figure 1E–F), effectively reducing the pore height by the radius of the particle. Therefore, the effective area of the pore for a step geometry is given by

$$A_{\text{available}} = W(H - D) \quad (5)$$

Combining equations (1), (3) and (5) yields the partition constant for a nanochannel with a height step,

$$K = \frac{H - D}{H} \quad (6)$$

Now, the particle partitioning at the nanoscale opening can be viewed having as an entropic energy barrier (E_b) given as

(7)

where k_B is the Boltzmann constant and T is the temperature in Kelvin. The probability for a particle to pass the barrier (P_p) can be given as

(8)

Substituting equation (7) into equation (8) yields

(9)

The trapped particles (T) are given by

(10)

Finally, substituting equations (6) and (10) into equation (9) yields

(11)

Equation (11) gives a theoretical prediction of the correlation between the fraction of trapped particles and particle size (for a given step height). This model can be used to predict the trapping of spherical particles in our nanosieving devices if H and D are known, as they are for our devices and proteins. Moreover, the measured T/t ratio can be used to determine D for an unknown nanostructure for a given height step. We plotted equation (11) along with our experimental data in Figure 6. This model fits quite well with the results obtained in our nanosieving systems for smaller height steps (15–20 nm), with somewhat greater deviations at larger height steps (22 and 29 nm). There are several possible reasons for the observed differences between theoretical and experimental data. The proteins used in these experiments are not exactly spherical and are somewhat deformable compared to rigid spheres. There is also some interaction among protein molecules, and between proteins and the channel walls, which could affect protein trapping. Finally, unlike with Giddings' theory, in our experiments the proteins have a directional velocity component towards the nanosieve opening due to capillary flow, which should increase the chance of a protein passing through the opening, compared to diffusion alone. We also note that flow eddies that might form at the height steps are not a significant source of protein trapping at height steps in our devices.

4. Conclusion

We have developed and tested a nanosieving system for size-selective protein fractionation. We modified our previous fabrication procedures to make devices more reproducibly and with channel heights down to 15 nm. These improvements enabled us to study the effects of protein size and concentration on distribution between a height step and the nanochannel

exit. The protein distribution at a nanoscale opening clearly depends on protein diameter and opening size: as the protein diameter increased from 3.5 to 17 nm for devices having the same height step, an increase in the T/t ratio was obtained, until height steps were much larger than the protein diameter. For some proteins, the highest concentrations led to an increase in the T/t ratio relative to lower concentrations. We also developed a model, predicting the trapping behavior of rigid spherical particles at height steps, which provided good correlation with our experimental data.

These results lay the experimental and theoretical foundation for separating proteins of different sizes in nanosieving systems. In particular, multiple height step trapping systems could be used to separate several proteins simultaneously, based on their size. Such a refined nanosieving system that additionally has a suitable surface coating to limit nonspecific adsorption could be used to separate HDL and LDL fractions²⁹ to better diagnose and treat cardiovascular disease. Moreover, these nanosieving systems could be explored in preconcentration of analytes such as viruses and proteins;^{18, 30} removal of captured analytes could be achieved, for example, with use of a backflow or an applied voltage²⁷ from the channel exits. Finally, our approach has promise for separating or selectively isolating protein aggregates that occur in some pharmaceutical formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Vinayavekhin N, Homan EA, Saghatelian A. ACS Chem. Biol. 2010; 5:91–103. [PubMed: 20020774]
2. Vlahou A, Fountoulakis M. J. Chromatogr. B. 2005; 814:11–19.
3. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, Gonzales PA, Star RA, Knepper MA. Nephrology. 2005; 10:283–290. [PubMed: 15958043]
4. Perrillo R, Tamburro C, Regenstein F, Balart L, Bodenheimer H, Silva M, Schiff E, Bodicky C, Miller B, Denham C, Brodeur C, Roach K, Albrecht J. Gastroenterology. 1995; 109:908–916. [PubMed: 7657121]
5. Levine GN, Keaney JF Jr, Vita JA. New Engl. J. Med. 1995; 332:512–521. [PubMed: 7830734]
6. Filipe V, Hawe A, Jiskoot W. Pharm. Res. 2010; 27:796–810. [PubMed: 20204471]
7. Skoog, DA.; Holler, FJ.; Crouch, SR. Principles of Instrumental Analysis. 6th edn.. Belmont, CA: Thomson Brooks/Cole; 2007. p. 844–848.
8. Baalousha M, Stolpe B, Lead JR. J. Chromatogr. A. 2011; 1218:4078–4103. [PubMed: 21621214]
9. Ersahin ME, Ozgun H, Dereli RK, Ozturk I, Roest K, van Lier JB. Bioresource Technol. 2012; 122:196–206.
10. Edelstein C, Pfaffinger D, Scanu AM. J. Lipid Res. 1984; 25:630–637. [PubMed: 6747466]
11. Krauss RM, Burke DJ. J. Lipid Res. 1982; 23:97–104. [PubMed: 7057116]
12. Warnick GR, McNamara JR, Boggess CN, Clendenen F, Williams PT, Landolt CC. Clin. Lab. Med. 2006; 26:803–846. [PubMed: 17110241]

13. Prakash S, Piruska A, Gatimu EN, Bohn PW, Sweedler JV, Shannon MA. *IEEE Sens. J.* 2008; 8:441–450.
14. Duan C, Wang W, Xie Q. *Biomicrofluidics.* 2013; 7:026501.
15. Menard LD, Ramsey JM. *Nano Lett.* 2011; 11:512–517. [PubMed: 21171628]
16. Eijkel JCT, van den Berg A. *Microfluid. Nanofluid.* 2005; 1:249–267.
17. Han J, Craighead HG. *Science.* 2000; 288:1026–1029. [PubMed: 10807568]
18. Wang Y-C, Stevens AL, Han J. *Anal. Chem.* 2005; 77:4293–4299. [PubMed: 16013838]
19. Tegenfeldt JO, Prinz C, Cao H, Chou S, Reisner WW, Riehn R, Wang YM, Cox EC, Sturm JC, Silberzan P, Austin RH. *Proc. Natl. Acad. Sci., USA.* 2004; 101:10979–10983. [PubMed: 15252203]
20. Papadopoulos S, Jürgens KD, Gros G. *Biophys. J.* 2000; 79:2084–2094. [PubMed: 11023912]
21. Stewart MW, Laker MF, Dyer RG, Game F, Mitcheson J, Winocour PH, Alberti KG. *Arterioscl. Throm. Vas.* 1993; 13:1046–1052.
22. Bruns RR, Palade GE. *J. Cell Biol.* 1968; 37:277–299. [PubMed: 5656395]
23. Kahook MY, Liu L, Ruzycki P, Mandava N, Carpenter JF, Petrash JM, Ammar DA. *Retina.* 2010; 30:887–892. [PubMed: 20458261]
24. Hamblin MN, Xuan J, Maynes D, Tolley HD, Belnap DM, Woolley AT, Lee ML, Hawkins AR. *Lab Chip.* 2010; 10:173–178. [PubMed: 20066244]
25. Bergna, HE.; Roberts, WO. *Colloidal Silica: Fundamentals and Applications.* CRC Press; 2005.
26. Pumera M. *Talanta.* 2005; 66:1048–1062. [PubMed: 18970090]
27. Xuan J, Hamblin MN, Stout JM, Tolley HD, Maynes RD, Woolley AT, Hawkins AR, Lee ML. *J. Chromatogr. A.* 2011; 1218:9102–9110. [PubMed: 22055518]
28. Giddings JC, Kucera E, Russell CP, Myers MN. *J. Phys. Chem.* 1968; 72:4397–4408.
29. Xuan, J. Ph.D. Dissertation. Brigham Young University; 2013. Size-based Separation of Bioparticles Using Planar Nanofluidic Devices.
30. Connelly JT, Kondapalli S, Skoupi M, Parker JSL, Kirby BJ, Baeumner AJ. *Anal. Bioanal. Chem.* 2012; 402:315–323. [PubMed: 21909662]

Figure 1.

Overview of device design and operation. (A) Top view photograph of a nanodevice having 1 cm² dimensions. (B) Magnified photo of channels. (C) Zoom view photo showing height steps and exit reservoir. (D) Top view schematic showing operation of device including solution injection, flow and trapping of protein in nanochannels. (E–F) Side view schematics; channel lengths are not drawn to scale. (E) A ~10 nm protein passes through a 100–15 nm height step and accumulates at the exit. (F) A ~17 nm protein traps at a 100–15 nm height step.

Figure 2.

Fabrication of dual height nanochannels. (A) A ~100 nm thick oxide layer is deposited on a 4" Si wafer followed by an Al layer. (B) A ~15 nm thick Cr protective layer is deposited. (C) Photoresist is coated and patterned using photolithography and the exposed Cr is removed. (D) A second Al layer is deposited to make the tall segment. (E) After depositing a 15 nm Cr protective layer, Al is lifted off and Cr is removed. Then photoresist is coated and patterned. (F) Exposed Al is removed followed by photoresist stripping. (G) A capping layer of ~4 μm thick oxide is deposited to cover core lines. (H) Photoresist is coated and patterned. (I) Exposed oxide is etched to open the ends. (J) Aluminum lines are etched using aqua regia, and the hollow channels are then filled with water.

Figure 3.

Images showing protein trapping at a height step of 100-15 nm as protein diameter increases from (A–E). All protein concentrations are 0.1 mg/mL. (A) Mb, (B) Hb, (C) Ct, (D) Fer, and (E) Tg.

Figure 4.

Images showing a change in trapping pattern of Tg as height step is increased. (A) 15 nm, (B) 18 nm, (C) 19.5 nm, (D) 22 nm, and (E) 29 nm.

Figure 5.

Plots of variation of trapped/total ratio with protein concentration in (A) 100-15 nm, (B) 100-18 nm, (C) 100-19.5 nm, (D) 100-22 nm, and (E) 100-29 nm devices. 1 mg/mL data are not shown in (E) because the length of the plug of fluorescent material that accumulated at the exit extended back beyond the height step. The error bars indicate plus or minus one standard deviation of the mean.

Figure 6.

Plots of variation of trapped/total ratio with protein diameter in (A) 100-15 nm, (B) 100-18 nm, (C) 100-19.5 nm, (D) 100-22 nm, and (E) 100-29 nm devices. The solid lines plot the predicted T/t ratio as a function of protein diameter, as derived in equation (11). The error bars indicate plus or minus one standard deviation of the mean.

Table 1

Proteins of different sizes used in nanosieving experiments.

Protein	Abbreviation	Molecular weight (kDa)	Diameter (nm)
Myoglobin	Mb	17	3.5
Hemoglobin	Hb	64.5	5.5
Catalase	Ct	247.5	10–11
Ferritin	Fer	450	11–12
Thyroglobulin	Tg	669	17