Reagentless mechanical cell lysis by nanoscale barbs in microchannels for sample preparation

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A highly effective, reagentless, mechanical cell lysis device integrated in microfluidic channels is reported. Sample preparation, specifically cell lysis, is a critical element in 'lab-on-chip' applications. However, traditional methods of cell lysis require purification steps or complicated fabrication steps that a simple mechanical method of lysis may avoid. A simple and effective mechanical cell lysis system is designed, microfabricated, and characterized to quantify the efficiency of cell lysis and biomolecule accessibility. The device functionality is based on a microfluidic filter region with nanostructured barbs created using a modified deep reactive ion etching process. Mechanical lysis is characterized by using a membrane impermeable dye. Three main mechanisms of micro-mechanical lysis are described. Quantitative measurements of accessible protein as compared to a chemically lysed sample are acquired with optical absorption measurements at 280 and 414 nm. At a flow rate of 300 μL min^{-1} within the filter region total protein and hemoglobin accessibilities of 4.8% and 7.5% are observed respectively as compared to 1.9% and 3.2% for a filter without nanostructured barbs.

Introduction

Sample preparation is a critical element in biochemical analyses and traditionally requires extensive manual labor. Integration of sample preparation processes in a lab on a chip format is essential for complete automation of molecular diagnostics.[1] As a significant element of sample preparation, intracellular biomolecules must be accessed for reaction and detection, and this requires a method of cell membrane disruption. Applications that require cell lysis include pathogen detection, immunoassays for point of care diagnostics, and mRNA transcriptome determinination. Typically, off-chip cell lysis for nucleic acid extraction requires numerous steps including adding lytic agents (detergents, enzymes), washing, centrifugation, and elution, which are not readily miniaturized. Many of these purification steps are required because polymerases used for the polymerase chain reaction (PCR) are inhibited by chemicals used in lysis protocols.[2] Also, protein-based assays such as the coomassie concentration assay are affected by the presence of detergents such as sodium dodecyl sulfate (SDS).[3] Other methods have been explored off-chip that address these problems including thermal lysis, sonication, bead milling, and freeze-thawing; however, these methods require additional actuators that add to the complexity of the sample preparation component of a microsystem. Therefore, micro total analysis systems (μTAS) could benefit greatly from an easily integrated and non-polluting lysis method that does not interfere with a range of possible detection steps which follow for different analytes.

Several methods of cell lysis have been successfully applied to microsystems. On chip electronic lysis of *Escherichia coli* has been demonstrated[4,5] and the subsequent analysis of intracellular nucleic acids was performed.[5] Thermal lysis of cells as a first step in PCR has also been demonstrated on-chip for DNA amplification and sizing.[6] Chemical lysis has also been employed on chip,[7,8] however, only once[8] to our knowledge was an intracellular protein subsequently quantified on chip.

All of the above methods for on-chip cell lysis have been successful for accessing a range of different analytes. For example, thermal lysis is functional for extracting nucleic acids for further amplification, however, it is not ideal for extraction of proteins for immunodetection since the proteins may have denatured to a non-native state not recognized by antibodies. Also, many of these microsystems, like electronic-based lysis chips, require more time consuming extra fabrication steps and external components like a high voltage power supply. Chemical-based lysis using non-ionic, less denaturing detergents is useful, but requires more reagents, extra reservoirs and integrated micro-pumps which increases device complexity and may not be suitable for portable or disposable diagnostic applications. A simple mechanical method is a reagentless alternative, however, it is known that just subjecting cells to shear and frictional forces induced by entering contractions may not be enough to rupture membranes.[9,10] Adding sharp nanostructure to contraction sidewalls and increasing volumetric flow rates is implemented in our device to concentrate and amplify frictional forces and penetrate the cell membrane.

Mechanical lysis using nanostructured filter-like contractions (nano-knives) in microfluidic channels with pressure driven cell flow is a useful alternative to previously discussed techniques, and can provide non-adulterated cell lysates that can be used for both nucleic acid and protein based assays. No additional reagents or power sources are required and creating nanostructures on the filter posts does not increase device fabrication complexity or cost over fabrication of a simple microchannel in silicon. We show the effective extraction of intracellular protein from defibrinated sheep blood using our device and present an optical characterization of protein extraction efficiencies. Using this quantitative technique the extraction of more than twice the amount of protein made available by a smooth-walled filter is demonstrated.

Experimental

Materials

Both HL-60 and a whole blood product are used for lysis experiments. The HL-60 cell line was obtained from American Type Culture Collection (Manassas, VA). Defibrinated sheep

blood was obtained from HemoStat Laboratories (Dixon, CA). Phosphate buffered saline (PBS), Trypan blue, Bovine Serum Albumin (BSA), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein diacetate (FDA) was purchased from Molecular Probes Inc (Eugene, OR).

Device design

The sample preparation microsystem is designed to contain mechanical sorting, mechanical lysis, and affinity purification regions and the mechanical lysis region is seen in Fig. 1(a). A sudden expansion was designed after the lysis filter in order to reduce the speed of cell debris after lysis so that it would be observable. Design of the mechanical lysis region is schematically represented in Fig. 1(b) and consists of a semicircular nanostructured filter with 3 μm wide posts. The depth at the lysis region is 20 μm and width is 2 mm so even a reasonable flow rate of 40 μl min^{-1} leads to a high average fluid velocity of 1.67 cm s^{-1} in this region.

To reduce non-specific adsorption of cell lysate proteins and other biomolecules onto the surfaces of the device 1 mg ml^{-1} BSA in PBS is flowed in and allowed to adsorb and block surfaces for 1 h before experiments are conducted. A flow-through device with little surface fouling is beneficial for large continuous flow samples.

Microfabrication

The sample preparation device is microfabricated using a two mask bulk etching and Pyrex wafer bonding process. First, a silicon oxide layer (0.5 μm) is grown on n-type silicon wafers using wet thermal oxidation. This oxide is patterned and etched with the designed microfluidic channel, filter, and collection geometries. Photoresist (3.0 μm thick) is then spun on and patterned with fluid access ports. A deep reactive ion etch (DRIE) is then performed to etch through the wafers to form the patterned high aspect ratio backside access ports. Next the photoresist layer is stripped and channel and filter geometries are etched into the wafer to a depth of 20 μm using the silicon oxide mask and a modified DRIE process. This etching step formed the sharp nanostructure for mechanical lysis, which we term nano-knives- (Fig. 2), the winding biomolecule collection chamber, and connecting microchannels. Next, the wafers are cleaned in piranha to remove any excess polymer left from the DRIE process. Any remaining silicon dioxide is then removed from the wafers by washing in HF. Immediately after the HF washing, thin thermal expansion coefficient matched Pyrex wafers are anodically bonded to the bulk-etched silicon wafers forming enclosed channels with backside access ports in the silicon. The lysis filter of the completed device is seen in Fig. 1(a). Tubing for a syringe pump is then attached to the backside with epoxy.

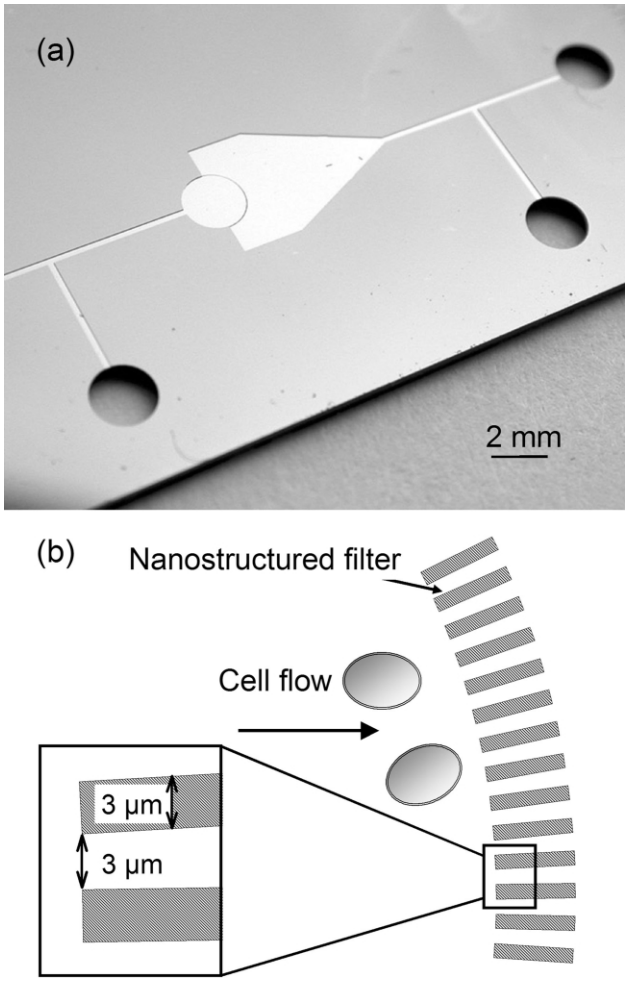

Fig. 1 (a) The nanostructured mechanical filter is shown in a photograph. (b) A schematic is shown of the mechanical lysis portion of the device that consists of a semicircular array of nano-knives.

The nano-knives are fabricated using a modified DRIE recipe that increases the "scalloping" effect. A normal DRIE recipe for silicon micromachining will form high aspect ratio low surface roughness structures using successive etch and passivation steps with a Teflon-like polymer. In the modified recipe the etch step is more isotropic enhancing the scalloped effect. A modified recipe[11] consists of successive 9 s etch steps with SF_6 and 7 s passivation steps with a Teflon-like polymer. The top plasma RF coil is set at 600 W and the platen coil to direct ions is set at 120 W. With this increased scalloping effect sharp nano-knife tips are formed at corners as two orthogonal scallops meet (Fig. 2). The distance between tips in the upper region of the pillar is characterized as 0.34 ± 0.03 μm from SEM images, while the radius of curvature at the tips is less than 25 nm. A schematic diagram of the nano-knives is shown in Fig. 2c.

A control device with smooth sidewalls (50 nm peak to trough viewed by SEM) is also fabricated as a control for lysis experiments. The recipe consisted of a 6 s etch step with SF_6 and a 4.6 s passivation step with C_4F_8. The coil and platen were maintained at the same power as for the modified recipe.

Cell lysis measurements

The amount of cell lysis for different flow rates is quantified using two techniques. First, a Trypan viability assay is conducted on HL-60 cells passed through the device with several flow rates. HL-60 is a human leukemia cell line that is easily grown in suspension. For cells that have ruptured membranes Trypan blue cannot be excluded from the cell cytoplasm and these lysed cells can be counted using a standard hemocytometer and transmission microscope. The cell lysate is dropped and wicked in the hemocytometer entry port. Cells are counted in an etched grid of known area with a cover slip on top to create a known volume. Lysed cells and cellular debris appear blue while intact cells will remain white and are easily distinguished. Live cell concentration per volume is calculated and compared to the initial live cell concentration counted the same way. A percentage lysis is determined from this ratio and reported.

The second technique involves measuring the optical absorption of released proteins at different wavelengths and gives a better measure of how accessible biomolecules have become for subsequent assays. This technique has been used previously at 414 nm for detection of haemolysis in red blood cells.[12] The cells we used for this technique are red blood cells from defibrinated sheep blood since the heme group of hemoglobin

absorbs well at 414 nm. Free total protein and nucleic acid concentration are also measured with absorbance at 280 nm where tyrosine and tryptophan amino acid residues absorb strongly and nucleic acids also absorb. The linear regime of protein concentration to absorbance is characterized by starting with a 5% debfibrinated sheep blood lysed solution, measuring absorbance and then diluting the solution 2:1 and repeating absorption measurements. This is repeated until halving the concentration resulted in halving the absorbance value for 3 consecutive measurements.

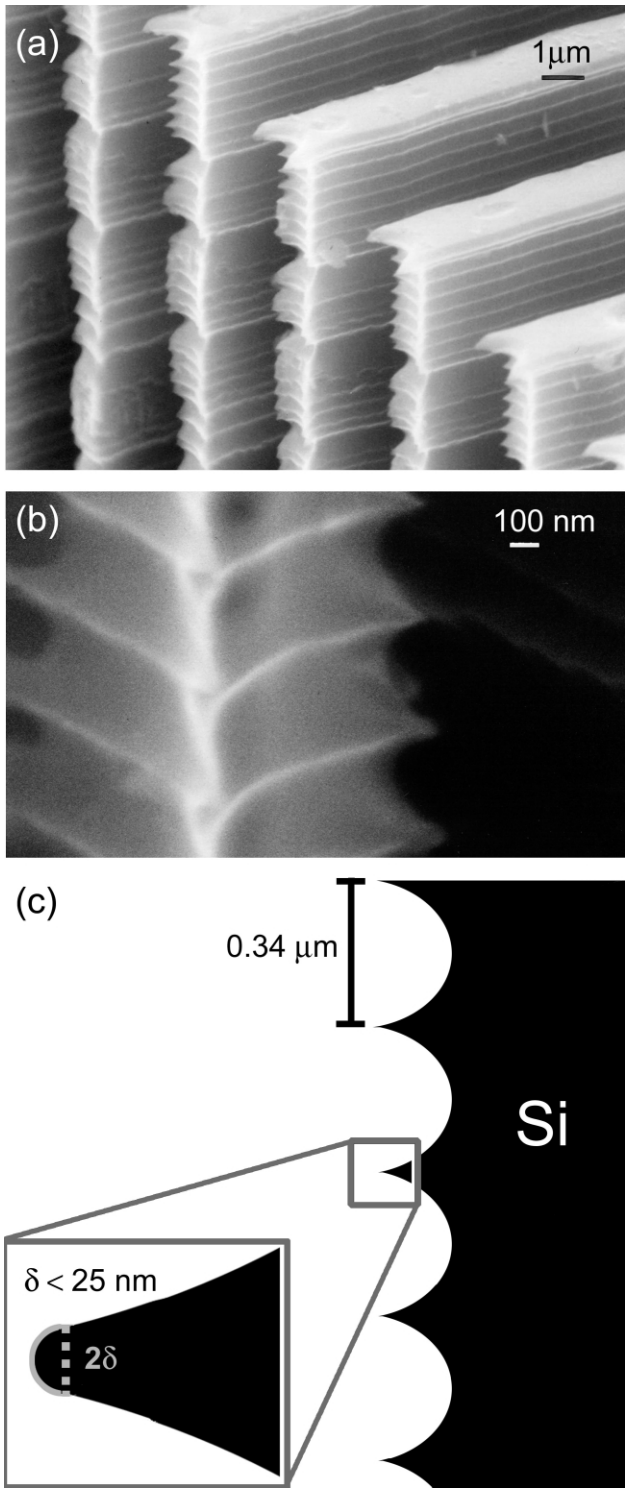

Fig. 2 (a–b) SEMs of the nano-knives are shown. Sharp protrusions are clearly seen as orthogonal scallops meet at corners during the DRIE process. (c) A schematic drawing of the nano-knives is shown describing the geometry. Distance between barbs is ~0.34 μm and radius of curvature of tips is below 25 nm.

In order to determine a relative scale of protein and nucleic acid accessibility and thus cell lysis, first, an absorption measurement is conducted using a chemically lysed sample with 3% Triton X-100 by volume in 5% defibrinated sheep blood in PBS. Since Triton X-100 also absorbs light at 280 nm the spectrophotometer must be blanked with a 3% Triton X-100 solution in PBS. The chemically lysed sample is then agitated for 5 min and afterwards centrifuged for 10 min at 6 000 *g* and 4 °C. The supernatant then contains any free protein made available from the lysed cells. The supernatant of all samples is characterized in the linear regime by diluting it 20:1 in PBS after centrifugation. Absorbance measurements were taken of this chemically lysed sample as a standard to compare to mechanically lysed samples. Another sample of 5% defibrinated sheep blood solution was also centrifuged without any treatments and optical absorbance is measured to account for any lysis occurring during centrifugation. This was considered the zero point for cell lysis and protein release.

Mechanical lysis is then conducted on 5% diluted sheep blood in PBS with different flow rates. The same device is used for each flow rate and after an aliquot of lysate is collected at that flow rate a wash with PBS is conducted to flush the device. After each volume of mechanically lysed cells is collected it is also centrifuged for 10 min at 6 000 *g* and 4 °C. Absorbance measurements are then conducted on the supernatant which has been diluted 20:1 with PBS in a 500 μl quartz cuvette with a path length of 10 mm. This is then converted to the amount of free biomolecules made available as a fraction of a chemically lysed sample, subtracting the baseline absorbance of the control sample. We can define this fraction since concentration of biomolecules scales linearly with absorbance according to the Beer–Lambert Law and the linear regime has been characterized. Both nanostructured and smooth-walled control devices are tested with the same procedure.

Finally, cell lysis is optically observed under a fluorescent microscope as a qualitative measurement. HL-60 cells are first stained with fluorescein diacetate (FDA), 10 μM in DMSO diluted with PBS. The cell solution, at ~10^7 cells per ml is incubated for 30 min with FDA and then washed with PBS. FDA is uncharged and cell membrane permeable; however, once it enters the cell esterases cleave the acetate groups to form charged fluorescein, which is maintained within the charged lipid bilayer of the cell. When the cell membrane is ruptured by mechanical lysis fluorescein diffusion from the cell region is observable. After initializing flow of the FDA stained cells and observing cell flow and lysis fluorescent images are taken at different time steps and spatial regions of the device to document the characteristic mechanisms of mechanical cell lysis.

Experimental results and discussion

Fluorescent images of cell lysis are seen in Fig. 3. Cellular debris and whole cells leaking fluorescein are visible immediately following the nano-knife filter. An image taken 10 min later and 500 μm downstream shows aggregation of fluorescein into 40 μm and smaller debris clumps and higher background fluorescence in the solution. Since with mechanical lysis there is no surfactant to homogenize cellular lipids, coalescence of ruptured cell debris with fluorescein is observed. Fluorescein is an amphiphilic molecule that is assumed to partition within the aggregating lipids to a larger extent than with the aqueous solution.[13] Even further downstream at 3 mm, a brighter fluorescent background is observed with smaller fluorescent debris clumps that seem to follow specific streamlines. We are surprised by this result since a random coalescence should lead to randomly distributed debris clumps, however, this phenomena may be due to the structured arrangement of the

lysis filter that affects the downstream fluid velocity profiles. To investigate whether re-coalescence of biomolecules from cell lysate into micelles inhibits the ability to use mechanical lysis for efficient extraction required a more quantitative assay to determine biomolecule accessibility, such as the optical assay that we conducted.

Three mechanisms of mechanical lysis are observed using fluorescence imaging. The method that occurred most often and is not readily observed in a still picture was a quick flow of cells through the mechanical filter, appearing as a smear because of a high fluid velocity. Further downstream after fluid velocity is decreased these cells are observed to rapidly leak fluorescein, indicating membrane rupture. The other two methods observed repeatedly and shown in characteristic images (Fig. 4) are visible even at high fluid velocities seen in the filter region. These mechanisms are defined by the cell, first, flowing into and being pierced by nano-knives of the filter entrance. Then a portion of cell slowly elongates into the gap between micro-knives and, finally, disassociates from the main cell body. The mechanisms differ in that the amount of protrusions into the filter varies from a single protrusion to several protrusions for larger cells.

Since qualitative images of cell lysis using FDA do not indicate the amount of free biomolecules available for lysis, two quantitative methods are conducted to characterize mechanical cell lysis. Using the Trypan blue assay with HL-60 cells described in the experimental section results in 99 ± 0.8 % cell lysis above 140 μl min^{-1} with 8 samples of lysate tested. This result is based on the amount of cells excluding dye in a given volume subtracted from the starting number of cells in that volume assuming that all the cells and cell debris pass through the device. One issue with this result is that cells permeable to Trypan blue may not be permeable to larger biomolecules that are required to leave the cell in order to be accessible for assays. Therefore, an optical absorption assay is conducted on centrifuged lysate to quantify the amount of free protein as discussed in the experimental section.

Results of protein availability measurements using this absorption technique are shown in Fig. 5. The fraction of protein available compared to a chemically lysed standard is plotted for different flow rates at the filter region. Mechanical lysis with flow rates of 40, 80, 110, 200, and 300 μl min^{-1} are conducted using both nanostructured lysis filters and smooth-walled (control) devices. A clear trend of higher protein and biomolecule availability is seen at higher flow rates for both devices; however, the nanostructured device shows more than double the protein availability at 300 μl min^{-1} and gives consistently higher protein availability at all flow rates. Interestingly, a jump in protein accessibility is observed from 200 to 300 μl min^{-1} for the nanostructured device and may indicate a threshold force required to puncture intracellular membranes like the nuclear membrane with nano-knife protrusions. It may also be indicative of more pronounced homogenization of amphiphilic biomolecules that reduces coalescence and increases the amount of soluble biomolecules after centrifugation. The fraction of hemoglobin accessible is higher than the amount of total protein made available at all flow rates. This is most likely due to the fact that hemoglobin is a cytosolic protein while much of the total protein is present in cellular membranes that are centrifuged to the pellet and not present in the supernatant. Also, negative fractions of protein are observed for the smooth-walled control device at 110 μl min^{-1}. This indicates that less protein was present than in the supernatant of 5% sheep blood which was centrifuged without any treatment. This exception can be explained by an observed bubble (with enhanced red cell concentration around it) that flowed through the tubing of the device immediately after the lysate was collected, skewing the red cell debris concentration in the lysate.

Overall, we demonstrate that mechanical lysis using nanostructured filters can result in biomolecule accessibility for bioassays, with improvement over non-structured mechanical filters. Several novel mechanisms observed for mechanical lysis are described and free protein in the lysate is quantified using an optical absorbance technique at 280 nm. This is compared to the amount of hemoglobin made accessible using absorbance at 414 nm. At 300 μl min^{-1} flow rates, accessibilities of 7.5% for hemoglobin are observed compared to 3.2% for a filter without nanostructured barbs. Since protein accessibility is shown not to be an issue, and chemical pollution of the sample does not occur, as with lysis with detergents, mechanical lysis may be a useful alternative method for on-chip sample preparation. Also,

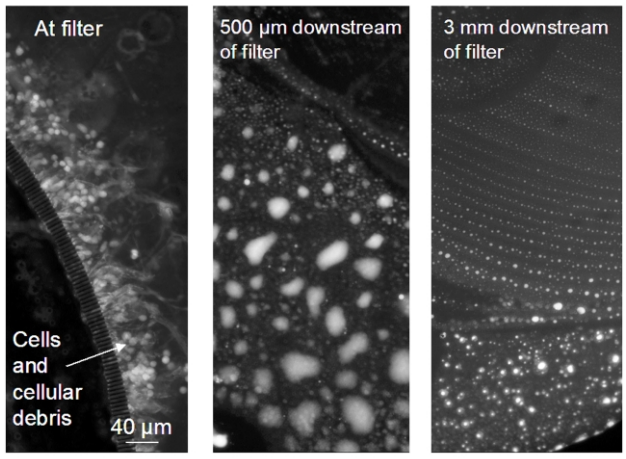

Fig. 3 Fluorescent images of cellular debris are shown at the filter (left), 500 μm downstream and 10 min after mechanical lysis initiated (middle), and 3 mm downstream at 10 min (right). After lysis begins, aggregation of cellular debris and fluorescein into larger clumps is seen (middle). An unexpected streamline pattern of smaller liposome-like fluorescent debris is seen further downstream. All pictures are to the same scale and are taken after stopping fluid flow.

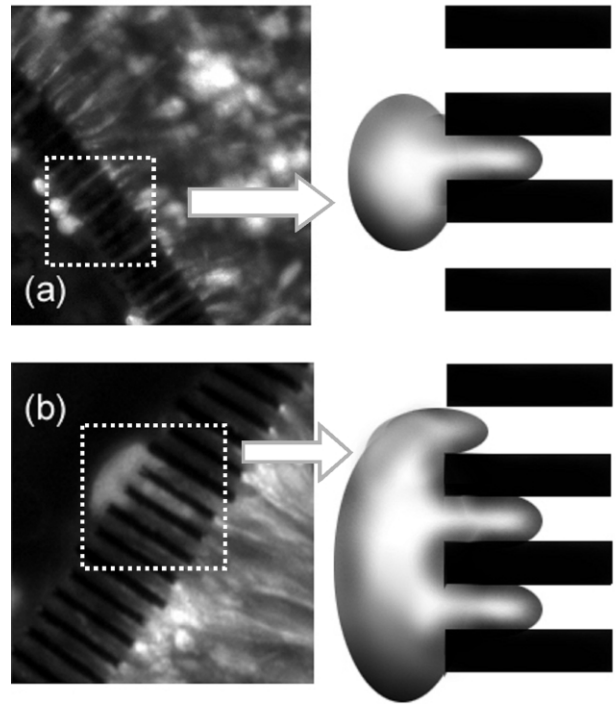

Fig. 4 Fluorescent pictures of two mechanisms of mechanical lysis at the nanostructured filter are shown and schematic drawings of these mechanisms are presented. Flow is from left to right. Cellular debris is visible fluorescing in the post-filter space. Single protrusions (a) and multiple protrusions (b) are observed repeatedly and representive pictures are shown here.

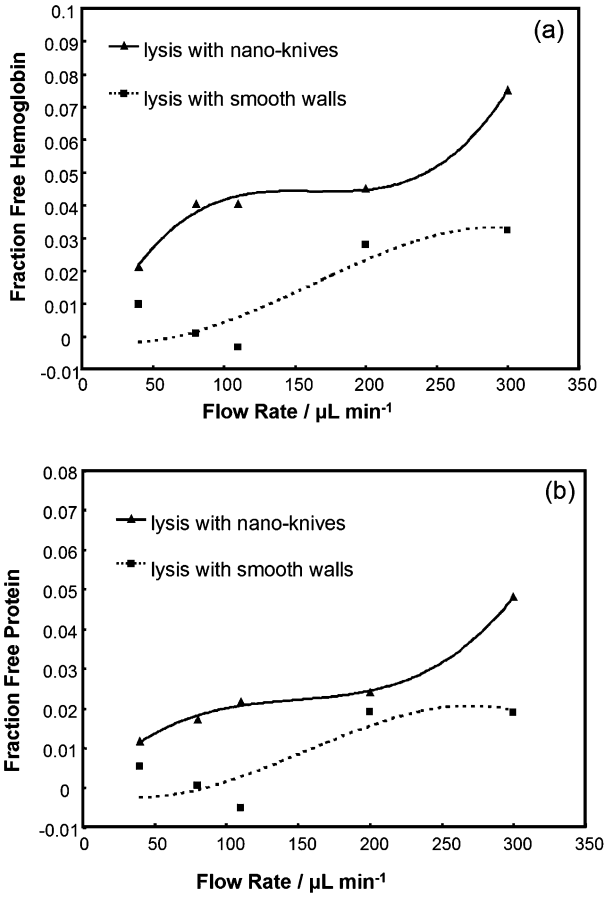

Fig. 5 The fraction of free hemoglobin (a) measured at 414 nm and free protein (b) measured at 280 nm absorbance are plotted as a function of volumetric flow rate for a single device. Results from a nano-knife modified device are compared to a smooth walled device. Curves are drawn to aid the eye. Negative values indicate less protein than in an unlysed centrifuged sample. Error of optical measurements is below 1%.

at lower flow rates there is the possibility of reversible poration with our mechanical filter since the main lysis mechanism observed is cell flow through with membrane permeabilization. Reversible mechanical poration would be valuable for gene delivery into cells without exposure to harsh chemicals or electric fields.

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