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A BIOMIMETIC METHOD FOR EXTRACTING LEUKOCYTES FROM BLOOD IN MICROFLUIDIC DEVICES

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Abstract - **The extraction of leukocytes from blood to be processed by micro-assay systems must be performed using methods which can be executed using minimal sample sizes and few preparatory steps. This demonstration proposes a biomimetic approach to performing this function using the adhesive rolling that is mediated by the interaction between a special class of proteins and ligands expressed by leukocytes but not red cells. The surfaces of microstructured flow channels made using standard microfabrication techniques can be coated with one or more of these adhesive proteins. These proteins facilitate the capture of leukocytes from blood samples sent through these channels. Captured cells are not immobilized but are restricted to slow rolling while blood passes rapidly through the channels. Eventually leukocytes become enriched and isolated as the rest of the blood is eluted through the channel. This study demonstrates the capture of HL-60 cells on E-selectin coated surfaces and quantifies their transit through two prospective designs for microstructured flow channels. Results show that white cells can be enriched greatly over initial density and that flow geometry strongly influences the transit of the captured cells.**

I. Introduction

The development of medical micro-assay systems (lab-on-a-chip) aims to shrink the functions of conventional laboratories to devices that integrate various sample processing steps on miniature microfluidic platforms. Such devices will yield several key advantages including the reduction of needed sample sizes to the scale of microliters and the acceleration of sample processing speeds, permitting fast turnaround and high throughput. This new technology will vastly improve medical diagnostics by providing low-cost substrates that can perform rapid blood tests not only in the clinical setting but also "in the field" and at the point of care [1]. To make these benefits possible, engineering efforts must focus on ways precisely manipulate small volumes of fluid, minimize the sample preparation and incubation steps needed for assays, and virtually eliminate the need for human intervention during assays. Existing laboratory techniques must either be adapted to this new paradigm or be replaced by new techniques more suitable for implementation in microfludic devices.

In assays using blood, one of the basic procedures is to separate and enrich leukocytes (white cells). This is important since red cells vastly outnumber white cells in whole blood by about a thousand fold, with red cells accounting for about 45% of blood volume, while all white cells constitute less than two-tenths of one percent. In conventional laboratories, the extraction of leukocytes is typically performed by centrifugation or by using Ficoll gradients [2]. These methods would probably prove impractical for microfluidic environments, in which inertial forces are less important than in the macroscopic world. Rather, to exploit the larger size of leukocytes compared to red cells, size-exclusion arrays can be implemented in microfluidic systems [3]. Such arrays, however, can incur damage to cells and can easily become clogged with debris. A more advanced technique uses paramagnetic microspheres that are functionalized with antibodies. The microspheres are targeted to specific leukocytes via these antibodies, allowing the cells to be removed from the rest of blood by a magnetic field. Implementation of magnetic cell separation in microdevices has been demonstrated with small "off-chip" magnets [4] as well as with "on board" electro-magnets integrated with the microfluidic device [5]. This approach, however, is still encumbered by the need for sample preparation and incubation steps to attach the magnetic spheres, and following separation, the extracted cells may still need to be "destained", or freed of the antibodies, requiring further processing. These fundamental requirements can limit throughput and sample processing speeds and even hurt the final yield.

An alternative method for separating cells mimics the physiological process by which blood vessels recruit leukocytes (and various other cell types) to particular sites. During the inflammatory response, blood vessels present a variety of adhesion proteins on their luminal surfaces to passing blood. These proteins, which include the selectin family (CD62) and a class of vascular adhesion molecules, do not affect red cells but capture other cell types from blood, resulting in substantial local enrichment of the leukocytes near the site of inflammation. Captured cells, however, are not fully immobilized but instead are permitted to roll slowly along the protein-presenting surfaces under fluid shear from passing blood flow [6]. This unusual interaction is made possible by the dependence of the protein-ligand affinity on mechanical tension [7]. Reproducing this phenomenon of protein-mediated cell rolling in a microfluidic device may provide a powerful method to extract leukocytes in a manner well suited to the

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requirements and limitations of micro-assay systems. Since most leukocytes constitutively express ligands for one or several of the adhesion proteins, no extensive sample preparation or incubation period would be needed for the cells. Additionally, captured cells would not be firmly immobilized by binding with the adhesion proteins and could be easily removed with gentle shearing or exposure to trypsin following extraction.

The goal of this project is therefore to demonstrate the use of protein-mediated, adhesive rolling to capture cells from blood. The implementation of this method is envisioned as a chromatographic column: blood is flowed through a microstructured fluidic channel whose surfaces are coated with one or several types of adhesion proteins. Leukocytes stick to the surfaces within the channel and transit the channel at much slower speeds via adhesive rolling, while the majority red cells elute through the channel at much higher speeds. The specific objective of this study is to observe the capture of HL-60 cell type, which serve here as a proxy for leukocytes, and measure the average transit speeds of cells through two different designs of microstructured channels with surfaces coated with human E-selectin.

II. Materials and Methods

Test structures. The "separation columns" consisted of microfluidic flow channels with microstructures to maximize surface area for capture of passing cells. Two different microstructures were studied: a regular array of 25 micron square posts (referred to as the "squares" geometry), spaced 25 microns apart; and alternating arrays of thinner posts, spaced 30 microns but with successive rows offset 15 microns in the cross-stream direction (referred to as the "offset" geometry). Test structures were fabricated in silicon using a single deep reactive ion etching (DRIE) step. Each consisted of a 10mm long by 500μm wide by 40μm deep trench containing microstructure and reservoirs with through substrates access holes at either end of the channel. Surfaces were treated with pirrahna to clean and to promote the growth of native silicon dioxide. Flow structures were then sealed with anodic bonding of pyrex coverslide.

Protein immobilization. Human E-selectin IgG chimera (Glycotech, Inc., Rockville, MD) was chosen as the adhesion protein in this demonstration because it was easy to obtain and binds to the most common leukocyte cell lines. The chimera protein is actually a recombinant protein consisting of the extracellular segment—which includes the ligand-binding lectin domain—of native human E-selectin and the Fc tail of IgG. This molecule is water soluble and adsorbs easily onto surfaces. The procedure for adsorbing selectin proteins on the microfabricated surfaces was based on established protocols [8]. The E-selectin IgG chimera can be dissolved in sterile phosphate buffered saline (PBS) at concentrations of 1 μg/mL. This solution was perfused into the microfabricated channels using a specially designed interconnecting fixture (which adapts Luer interconnects to the access holes on the flow devices) and incubated in the channels at room temperature for 2 hours. Following this step, the test structure was washed with several volumes of a 3% solution of fetal bovine serum (FBS) in PBS. The device was also allowed to incubate for 1 hour in this solution to cover all of the remaining sites on the device surface. Non-specific cell binding to the Fc segment of the chimera protein was block by the incubation of 2μg/mL of anti-Fc $F(ab')_2$ antibody (Biodesign, Inc., Saco, Maine) for 30 minutes prior to testing. This same concentration of the anti-Fc antibody was also added to the cell suspensions used in the testing.

Cell lines. The cell line used in this study was the HL-60 myeloid, neutrophil-like cell line. The HL-60 cells were maintained at 37°C with 5% CO_2 in air and at densities ranging between 300,000 and 1.5 million per mL in RPMI-1600 media with 10% fetal bovine serum (FBS) and sodium pyruvate. During testing, cells suspensions of 100,000 cells per mL were prepared in pure RPMI-1600 media and left at room temperature conditions for the duration of the flow experiments.

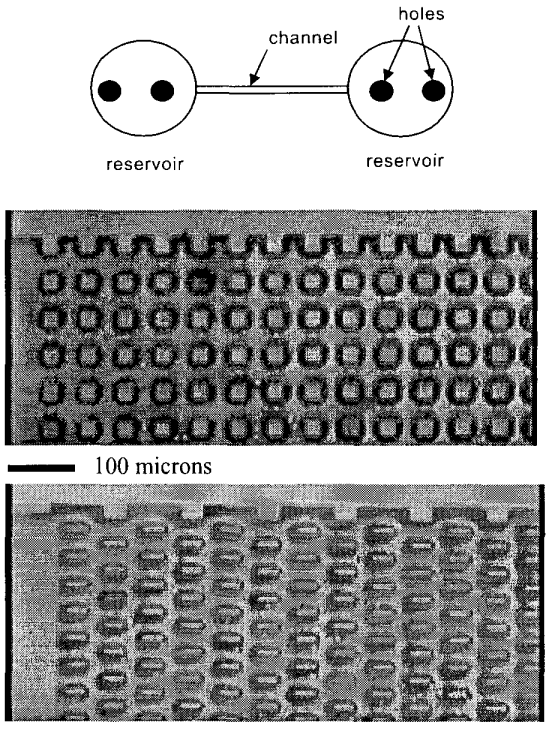

Fig 1. Test structures used for this demonstrates were etched in silicon, and native oxide formed on the exposed surfaces. The basic template is shown schematically (*top*). Within the channels, two designs for microstructure were prepared: a array of 25 x 25μm square posts (middle) and alternating rows of thinner posts (10 x 30 μm), with successive rows offset 15 μm. All views are from the top. Nominally, fluid flow is in the horizontal direction, and transit along this direction is quantified in this study.

Flow testing procedure. Suspensions of HL-60, at 100,000 per mL were pumped through the test structures by a syringe pump, which delivered a flow rate necessary to sustain average free stream velocities of 700 to 1400 μm/s in between posts. This corresponded to shear stress of 1 to 2 dynes/cm^2 (0.1-0.2 Pa). The flow was viewed under a microscope at 80x magnification and video of the flows and cell capture and transit was recorded and captured to computer for processing. Average transit speeds of the cells were determined by dividing the transit distance through the video frame, which corresponded to about 600 microns, by the total transit time. The average transit speeds were recorded only for "semi-dilute" condition, characterized by sparse distribution of cells in the channel and involving no interaction between cells on any give post.

III. Results

A. "Squares" flow structure

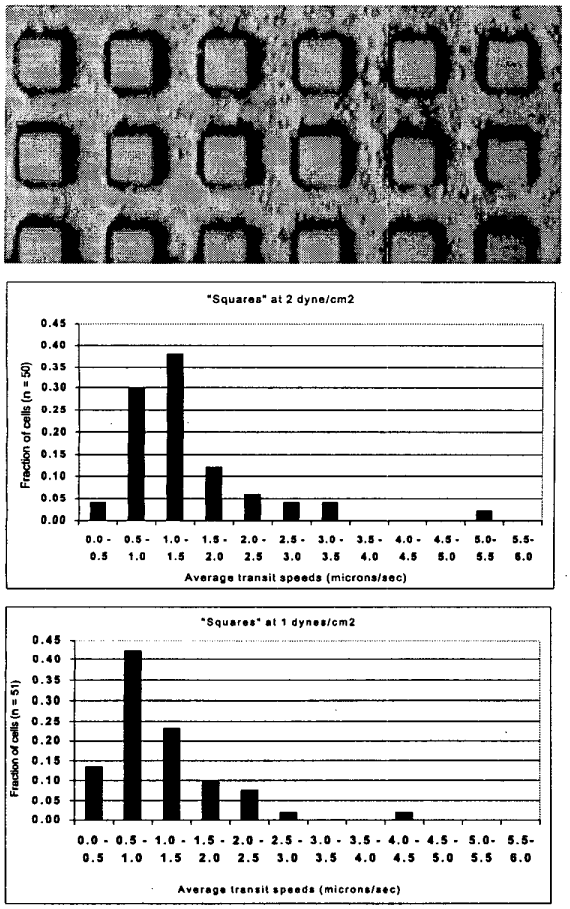

Fig. 2: *Top:* Red blood cells flow freely, while leukocytes adhere to surfaces coated with E-selectin. The leukocytes roll along the surfaces and slowly transit the channel (*middle, bottom*).

Through the "squares" lattice, transit speeds were restricted to an average of 1.4μm/s under shear stress of 2 dynes/cm^2 and free stream flow speed of 1400μm/s, while at 1 dyne/cm^2 and free stream flow speed of 700μm/s, average transit speeds were 1.1μm/s. Local cell concentration increased to a steady state level of on average 19.1 million/mL. Cell transit was greatly slowed in the stagnation region behind the posts; cells often lingered for some time in the region before being carried to the next set of posts.

B. "Offset" flow structure

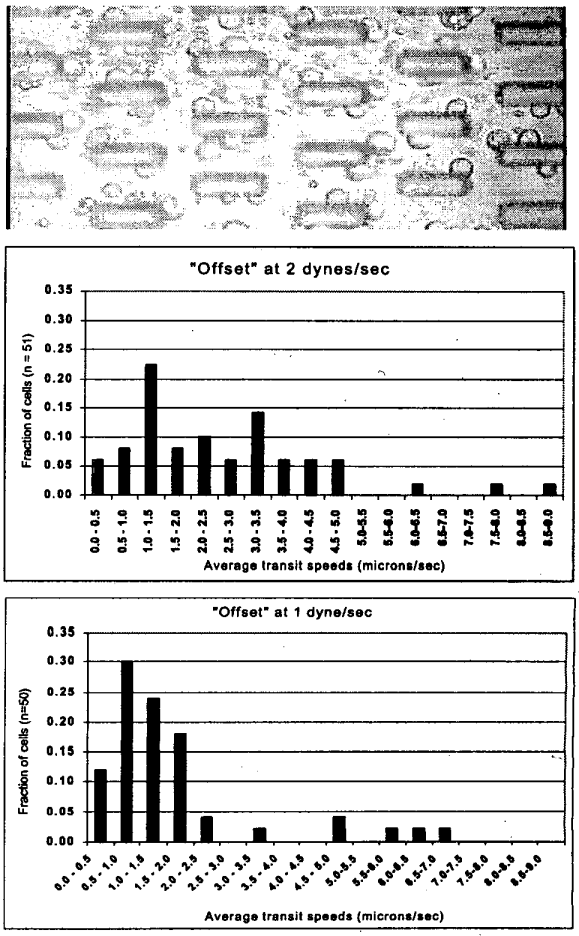

Fig. 3: *Top:* Again, red blood cells flow freely around flow structures, while leukocytes adhere to the surfaces (*middle, bottom*).

Cells transited somewhat faster through the "offset" geometry. Many cells were observed to hop from one set of posts to another. There was also an absence of stagnation regions where cells would be slowed. Through the "offset" lattice, transit speed was 3.8μm/s under shear stress of 2 dynes/cm^2 and free stream flow speed of 1400μm/s, while at 1 dyne/cm^2 and free stream flow speed of 700μm/s, average

transit speeds were 1.5μm/s. Local cell concentration increased to a steady state level of on average 15.0 million/mL.

IV. Discussion

In conventional clinical laboratories, the availability of large spaces permits the use of many sample preparation steps for a single function, and the use of large sample sizes compensates for processes that result in low yield. In micro-assay systems, however, the benefits of small sample sizes, rapid sample processing, miniaturized volumes, and automation can only be realized by implementing assay methods which rely on minimal sample processing and maintain high yields. These requirements may be fulfilled by adapting existing techniques to function with smaller volumes and consolidating process steps to be realizable in microfluidic environments. Alternatively, new methods can be developed to specifically meet the requirements of micro-assay systems. This study demonstrated the use of adhesion proteins to capture leukocytes cells from blood and to locally enrich their numbers.

The realization of this technique is proposed as a chromatographic fluidic channel, which contain microstructures whose surfaces are coated with one or several of a variety of adhesion proteins, including selectins and vascular cell adhesion molecules. Blood samples flow through these channels, allowing protein-laden surfaces to capture leukocytes from the passing flow. Because the protein-ligand interaction does not firmly immobilize cells, captured cells would roll along the surfaces under fluid shear but at speeds much slower then the free stream fluid flow. This would permit the rapid elution of the unaffected red cells, while captured leukocytes would remain on the channel surfaces. As more sample flows through the structure, more cells are captured by the microstructure, locally enriching the leukocyte population many fold from their original density in blood.

In the current demonstration, two designs for microstructured flow channels were studied for their ability to capture cells and mediate adhesive rolling motion using E-selectin IgG chimera and HL-60 cells. The purpose of including microstructure within the separation channels is to greatly increase the surface area to incoming flow and maximize the opportunity for cells to be captured. Observation and measurement of cell transits reveal that a large stagnation area behind each 25 x 25μm square post (in "squares" configuration) relative to the direction of fluid flow results in cells being substantially slowed in that region. In the "offset" geometry, however, the thinner cross-section presented to incoming flow resulted in a stagnation regions smaller then the cells, allowing the cells to be carried through the channel at higher speeds. Additionally, because of the thin 10μm cross-section presented against the flow, a cell that rolls behind the post is still exposed to shear. Moreover, this shear acts perpendicular and away from the surface of attachment, pulling the cell away from the trailing face of the post. Thus, many cells were observed to "hop" from one post to another, further increasing overall transit speeds. These results confirm that flow geometry is an important factor influencing the rolling speeds of adherent cells, and that the different shapes can be used to explicitly engineer the way adherent cells travel. Providing large stagnation areas, for example, seems to be an effective way to ensure that cells are slowed. Such structures then would be more effective at capturing and concentrating cells.

There were several limitations to the current study. Quantification of cell transit was performed under "semi-dilute" conditions, in which cells were sparsely distributed in the channel. The influence of closely packed cells on local shears and overall transit speed has not been explicitly addressed, nor have the transit speeds been quantified for cells captured when a high density of red cells are present in the free stream flow, as would be the case for realistic processing conditions. The present method of quantification observes cell transit through only 0.6 mm of channel length. Some captured cells may completely break free of the flow structures and be carried with the free stream. These cells could eventually be recaptured further downstream or may elute through the channel completely. These cells could not be accounted for with current methods. Furthermore, it is not clear to what extent the differences in cell transit speeds are attributable to inherent differences among cells in the population or simply to differences in the surfaces on which the cells are initially captured or migrate to. For example, cells which are captured near a corner and making contact with two perpendicular surfaces will transit slower than cells which are captured on just one surface. Baseline quantification of cells rolling on a simple flat surface suggests that there is a large degree of scatter in rolling speeds among different cells. However, it is nevertheless possible that during their transit through the structured micro-channel, cells will move in and out of corner areas, traveling faster during a part of their transit and moving slower during another. If this is the case, then the distribution of transit speeds would be somewhat narrower if measured over longer distances.

Despite the shortcomings of the current study, the method of cell capture demonstrated here by using protein-mediated cell rolling promises several important advantages for microfluidic systems. Desired cells are captured from a rapidly flowing sample and remain adherent to protein-laden surfaces, rolling along at only a fraction of the free stream flow. Since the targeted cells naturally carry ligands for these proteins, no preparation steps are required to either select or tag the cells. This eliminates the need for providing antibodies, fluorochromes, or other reagents, and no incubation periods will need to be added to the sorting and separation process. Cells are captured and sorted simply by using fluid shear in a microfluidic channel, which serves as the "separation column". No special instruments will be needed to apply magnetic fields, electric potentials, or

special optical detection methods. Standard on-chip devices to pump microliters of fluid will be sufficient. Following cell separation, there will not be a need to provide process steps to "destain" targeted cells, to remove bound markers. This is due to the unusual property of the protein-ligand interactions, which allow cells to roll and therefore maintain motion instead of being firmly immobilized to a substrate. Gentle fluid shear can remove cells from the separation column, especially if supplemented with some trypsin, which disrupts the protein-ligand bond.

Beyond mere capture from flowing blood, the adhesion proteins may also provide a means to select specific types of leukocytes and to sort mixed populations as well. With the variety of proteins that mediate adhesive rolling, not all cells are equally affected by a particular protein [6]. Even among those cells that do interact with a specific adhesion molecule, different cells have different affinities for that protein and will roll at different speeds under the same shear [9, 10]]. Furthermore, the phenomenon of rolling adhesion is not limited to differentiated leukocytes; blood progenitor cells and even some blood-borne cancer cells are also affected by these adhesion molecules, and are localized to particular tissue sites by the rolling adhesion mechanism [11, 12]. This means that there are a wide variety of cells for which this technique of sorting can apply to. Further studies will be required to address the effectiveness of adhesion-mediated cell rolling for sorting different cell types based on differences in rolling speed.

Future work will build upon the initial demonstrations reported here. Techniques for characterizing flow and transit of cells will be refined to include a much longer segment of channel. Different cell lines should be used in flow experiment to obtain a broader understanding of the transits of leukocyte cells in general. Additionally, representation of realistic flow conditions should include blood cells and suspensions of mixed cell populations. New designs for channel microstructures should also be studied.

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