

Quantitative measurements of the strength of adhesion of human neutrophils to a substratum in a microfluidic device: A novel inflammation diagnostic tool

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ABSTRACT

We describe a quantitative assay of the strength of adhesion of activated and nonactivated human neutrophils to a substratum, which is carried out in a custom-made microfluidic device. The strength of adhesion is quantified by the fraction of cells remaining adherent (ACF) after a given time of exposure to shear stress in test microchannels. The microfluidic device is made of two layers of poly-(dimethylsiloxane) with integrated membrane valves. This construction allows concurrent testing of two different populations of cells. The test microchannels have a tapered profile, exposing cells to nearly an order of magnitude range of shear stress. High throughput scans allow reliable quantitative assessment of the ACF. Adhesion of untreated neutrophils and fMLP activated cells was reliably assessed in a series of experiments with a fibrinogen-coated glass substratum. The proposed device and assay could be used to assess the state of activation of neutrophils in blood with a potential application to diagnostics of inflammation.

Keywords: microfluidic, adhesion, neutrophil, inflammation

1 INTRODUCTION

Over two-thirds of leukocytes circulating in blood are neutrophils, which are the primary cells involved in inflammation. Stimulated by early inflammatory cues, neutrophils undergo a process of activation that involves changes in conformation of adhesion molecules on their surface. The propensity of neutrophils to adhere to a substratum and the strength of the adhesion are indicators of their state of activation [1].

Here we describe the design and operation of a high-throughput microfluidic device (Figure 1), in which the strength of adhesion of cells to a substratum is quantitatively assessed by applying controlled shear stress and recording the dynamics of detachment of cells from the substratum. The device was used to measure the strength of adhesion of human neutrophils to a substratum. The measurements were performed concurrently for neutrophils activated with fMLP (formyl-Met-Leu-Phe) and non-activated neutrophils that were loaded in the two isolated compartments of the device. The strength of adhesion was quantified by the fraction of cells remaining adhered to the substratum after exposure to a given shear stress for a given time. The adherent cell fraction (ACF) was evaluated by

direct counting of cells in different areas of the test channels before their exposure to flow and after different durations of flow exposure. Cells were counted using a custom-made image recognition code and a computer-controlled video-microscopy system.

The capacity of the device to detect differences between populations of activated and non-activated neutrophils could be used to evaluate the level of activation of neutrophils extracted from patient blood, with a potential application to diagnostics of inflammation.

2 MICROFLUIDIC DEVICE DESIGN

The microfluidic device (Figure 1) has two layers of channels, the flow layer and the control layer, which are separated by thin membranes in the areas where they overlap. The flow layer has three inlets, three outlets and eight identical long tapered channels (test channels), in which the strength of adhesion of cells to the substratum is measured. Because of the tapered profile, the mean flow velocity and shear stress in the test channels grow along the flow direction (positive x -direction).

The microfluidic device has eight integrated membrane valves (blue rectangles in Figure 1a), which are connected through channels in the control layer. The valves allow partitioning of the device for independent loading of different cell populations into the two compartments. The presence of four identical test channels in each compartment allows analyzing a large number of cells from each population and collecting large data samples in a single test. The compartmentalization of the device could also be used for in-situ coating with different molecules.

The segments of the flow layer channels lying under the membrane valves have rounded profiles that are essential for tight sealing of the channels by the valves [2]. In contrast, the profiles of the test channels are rectangular for better cross-channel (y -direction) uniformity of stress at the substratum. The test channels have a length $L = 12$ mm, a uniform depth $h = 35$ μm and a width, w , that varies linearly from $w_1 = 1800$ μm at the beginning to $w_2 = 225$ μm at the end as $w = w_1 - (w_1 - w_2) \cdot x/L$, where x is the distance from the beginning of the channel (Figure 2a). The maximum value for the Reynolds number in the test channels (measured at $w = 225$ μm) was ~ 0.1 , suggesting that the flow was laminar and non-linear effects in the flow were negligible.

We computed the three-dimensional field of flow velocity in a test channel using FemLab (COMSOL Inc., Los Angeles, CA). We then numerically calculate the distribution of shear stress at the substratum, τ_s . At all positions along the channel the distributions of the surface stress across the channel, $\tau_s(y)$, are nearly uniform over most of the channel width. In particular, near the end of the channel (P2 in Figure 2a), $\tau_s(y)$ is within 10% of its maximal value over 75% of the channel width (Figure 2c); near the beginning of the channel (P1 in Figure 2a), $\tau_s(y)$ is within 10% of the maximum over 92% of the channel width (Figure 2b).

The dependence of the shear stress at the substratum in the middle of the channel (along the dashed line in Figure 2a), τ_{mid} , on the position along the x -axis is shown in Figure 2d. The characteristic shear stress at the substratum increases about 8-fold between the beginning and end of the channel (where $w = 1800$ and $225 \mu\text{m}$, respectively). Therefore, the test channels allow testing the strength of cellular adhesion to the substratum in almost an order-of-magnitude wide, continuous range of shear stress.

2.1 Device Fabrication

The microfluidic device is made of two layers of polydimethylsiloxane (PDMS) created using two different master molds. Both molds are fabricated lithographically with 8000 dpi photomasks. First, a $35 \mu\text{m}$ layer of UV-curable epoxy (SU8-2015 by MicroChem) is spin-coated onto a 4 in. silicon wafer, exposed to UV-light through a photomask and developed. Next, the wafer is coated with a $35 \mu\text{m}$ layer of a positive photoresist (SPR220-7.0 by Shipley) and exposed through another photomask, aligned with the SU8 pattern. After development, the SPR220 relief is rounded by baking for 15 min on a 115°C hot plate, which completes the first master mold. To make the master mold for the control channels, a $35 \mu\text{m}$ layer of SU8-2015 is spin-coated on another silicon wafer, exposed to UV-light through a third photomask, and developed.

A PDMS pre-polymer (5:1 mixture of base and curing agent of RTV615 by General Electric) is poured onto the control layer mold to a depth of ~ 5 mm and partially cured by baking at 85°C for 30 min. The PDMS cast is peeled off the wafer, cut into individual chips, and control ports are punched out. In parallel, the flow layer mold is spin-coated with a $75 \mu\text{m}$ layer of another PDMS pre-polymer (20:1 mixture of the base and curing agent). The PDMS is partially cured for 30 min at 85°C , and the 5 mm thick chips are aligned with the photoresist relief on the mold. After 2 hr of baking at 85°C , a monolith of completely cured PDMS with integrated membrane valves is formed [2]. The chips are reversibly bonded to 1 mm thick glass slides (FisherBrand, 50×75 mm) by baking at 85°C for 2 hr.

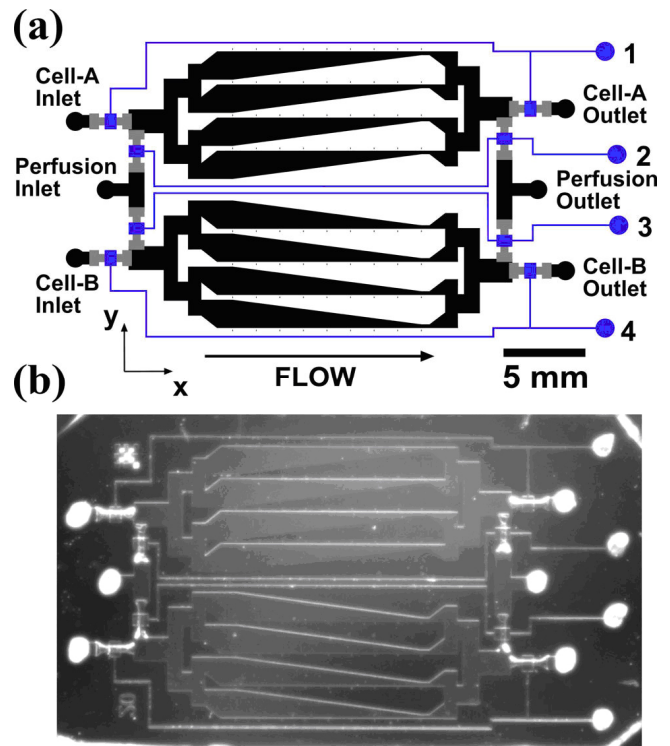


Figure 1: Drawing of the microchannel network.

(a) Rectangular and rounded segments of microchannels in the flow layer are shown in black and gray, respectively; channels in the control layer are in blue. Membrane valves are blue rectangles. (b) Photograph of the device. Bright rounded spots are the inlets and outlets of the device.

3 RESULTS AND DISCUSSION

When cells are exposed to the shear stress, there is a notable reduction in the number of fMLP activated cells and a major reduction in the number of untreated cells. The results on dependence of the ACF on the shear stress and on the time of exposure to the stress for incubation times $t_{inc} = 7$ and 12 min are summarized in Figure 3, in which the mean values of ACF are plotted in twelve separate regions along the test channels vs. the mean values of τ_{mid} in the regions. The experiments had a high throughput, with $\sim 3 \times 10^4$ cells from each group analyzed in each experiment. The total number of cells in identical regions of four test channels (with a homogeneous cell population) varied from ~ 8000 at the beginning of the channels to ~ 550 at the end of the channels. We performed five experiments with $t_{inc} = 7$ min and four experiments with $t_{inc} = 12$ min. For both incubation times and both cell groups, the rate of reduction of ACF after first 10 min of the exposure to flow was found to be minimal, and thus no data was acquired past that time point. In all experiments, $\sim 5\%$ of the reduction of ACF after the first 2 min was due to residual erythrocytes, which were initially present on the substratum.

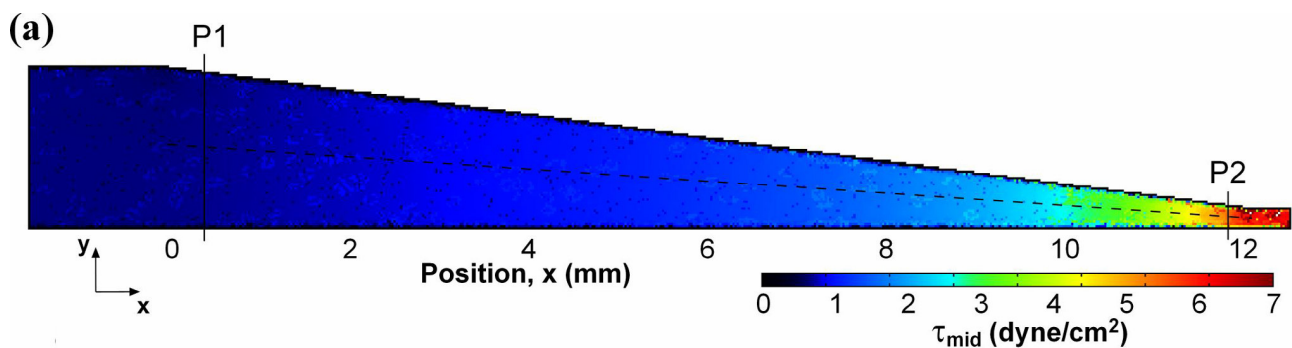
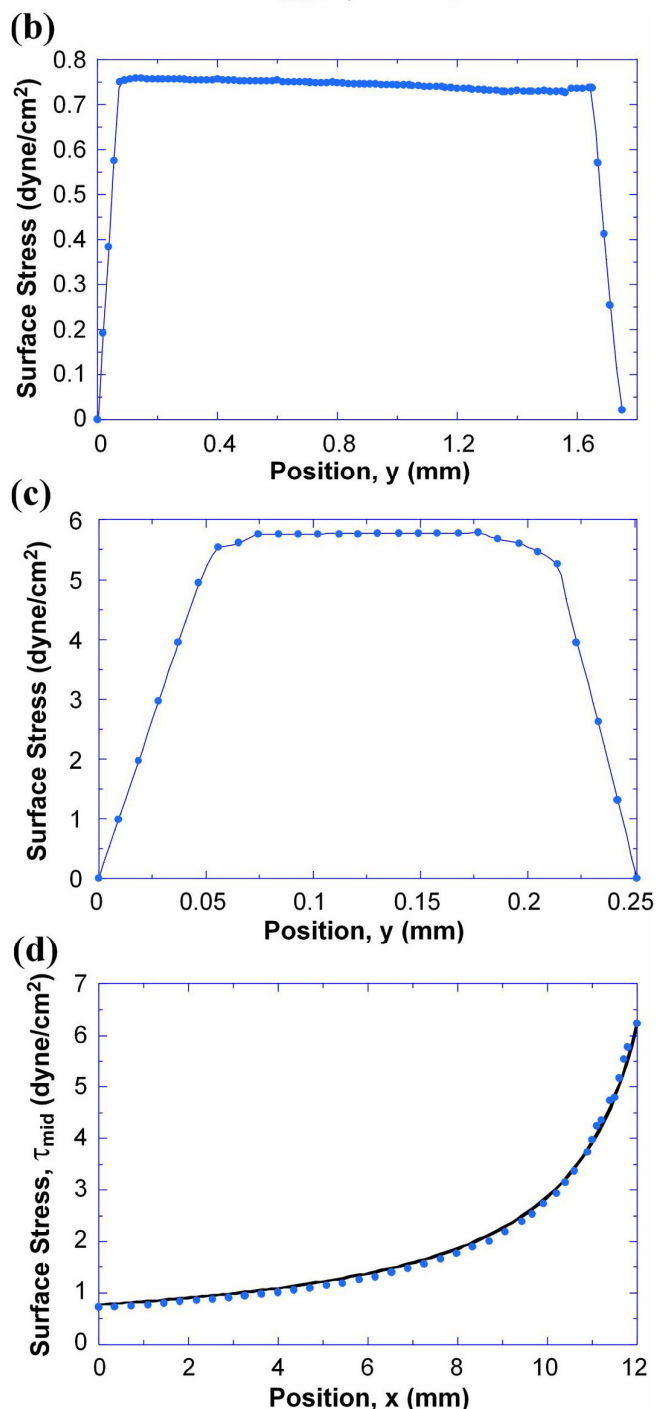


Figure 2: Shear stress at the bottom of a test channel. (a) Distribution of $\tau_s(x, y)$ in the channel (linear scale). (b) Distribution of the surface stress, $\tau_s(y)$, across the channel across line P1. (c) $\tau_s(y)$ across line P2. (d) Surface stress in the middle of the channel along x [dashed line in (a)]. Continuous line is a curve $\tau_{mid} \propto 1/w(x)$, where $w(x)$ is the channel at x .

The most striking result of the experiments with $t_{inc} = 7$ min (Figure 3a) is a major difference in the strength of adhesion between cells activated with fMLP and untreated cells. For example, after 2 min of exposure, only ~15-20% of the fMLP-activated cells detached from the substratum, whereas the fraction of untreated cells that detached from the substratum was ~70-75%. The difference in ACF between the two groups of cells after 2 min was three-fold on average. The rate of reduction of ACF substantially decreased with the time of exposure to stress, t_{str} . So, the last 6 min of the exposure (from $t_{str} = 4$ min to 10 min) accounted for only 19% and 3% of the ultimate reduction in ACF for the fMLP-activated and untreated cells, respectively (an average between all channel sections). For the fMLP-activated cells, the ACF was practically independent of the stress at $t_{str} = 2$ and 4 min, but became increasingly dependent on it at extended exposure times, decreasing by as much as ~10% between $\tau_{mid} = 0.72$ and 6.2 dyne/cm² at $t_{str} = 8$ and 10 min (Figure 3a; the reduction of the ACF was estimated by the slope of straight lines fitted to the data points). In contrast, the ACF of the untreated cells consistently exhibited substantial dependence on stress, decreasing by ~30% between $\tau_{mid} = 0.72$ and 6.2 dyne/cm² at all time points.

The dependence of ACF on the stress and the time of exposure to it with $t_{inc} = 12$ min (Figure 3b) had the same general trends as with $t_{inc} = 7$ min: the ACF was higher for the fMLP-activated than for untreated cells, and it decreased with the magnitude of stress and the time of exposure to it. Remarkably, however, the ACF of the untreated cells was consistently about twice higher than with $t_{inc} = 7$ min at the corresponding time points, substantially reducing the disparity between the untreated and fMLP-activated cells. Further, the reduction in the ACF during the last 6 min of exposure to stress accounted for a



larger portion of the total reduction as compared with $t_{inc} = 7$ min; it was 30% and 13% of the total reduction, for the fMLP-activated and untreated cells, respectively. In addition, the dependence of the ACF of the untreated cells on stress at $t_{inc} = 12$ min substantially diminished compared with $t_{inc} = 7$ min, and the ACF decreased by an average of only $\sim 8\%$ between $\tau_{mid} = 0.72$ and 6.2 dyne/cm². In contrast, for the fMLP-activated cells, the dependence of the ACF on τ_{mid} became stronger, with the ACF decreasing by $\sim 15\%$ on average between $\tau_{mid} = 0.72$ and 6.2 dyne/cm².

The proposed neutrophil adhesion assay has several advantages over the assays used before to distinguish between activated and non-activated neutrophils based on their strength of adhesion to a substratum [3]. In the proposed assay, the hydrodynamic stress applied to cells is precisely controlled and exactly reproducible. The strength of cellular adhesion is quantified by a straightforward parameter, ACF, the fraction of cells remaining adhered to the substratum after exposure to a given shear stress for a given time, which is calculated by direct counting of cells before and after the stress exposure. The comparison between the two groups of cells is based on the analysis of a large number of cells (3×10^4 from each group), and all cells loaded into the device are individually accounted for. The three-fold difference in the ACF between the fMLP-activated and untreated neutrophils is detected after 7 min of incubation, 2 min of exposure to the flow, and 1.5 min of the video-microscopy scanning (2 min curves in Figure 3a). This minimal version of the assay, providing quantitative results on the strength of adhesion of both activated and non-activated cells and a clear differentiation between them, is finished in ~ 11 min from the moment of loading of cells into the device without any intervention by the operator.

Existing clinical tests of the inflammatory response usually rely on measurements of the concentrations of soluble signaling molecules, such as C-reactive protein (CRP) and to lesser extent Interleukins 1 and 6 (IL1, IL6), usually with ELISA type assays [4]. The difference in concentrations between a normal versus an acute inflammatory state can be very small. For example, for CRP it corresponds to the levels <1 mg/L and >3 mg/L, respectively, whereas the dynamic range of physiological concentrations for CRP is from 0.01 mg/L to 250 mg/L. Therefore, a reliable test based on CRP may require multiple assays over a period of time. In the proposed neutrophil adhesion assay the physiological response of neutrophils to an inflammatory cue (fMLP) is measured before their programmed respiratory burst, which is the root cause of tissue damage and the ensuing inflammation cycle. Thus, we speculate that the proposed assay can potentially be used for alternative diagnostic tests and allow earlier detection of gradual changes in the state of inflammation (especially low-grade inflammation).

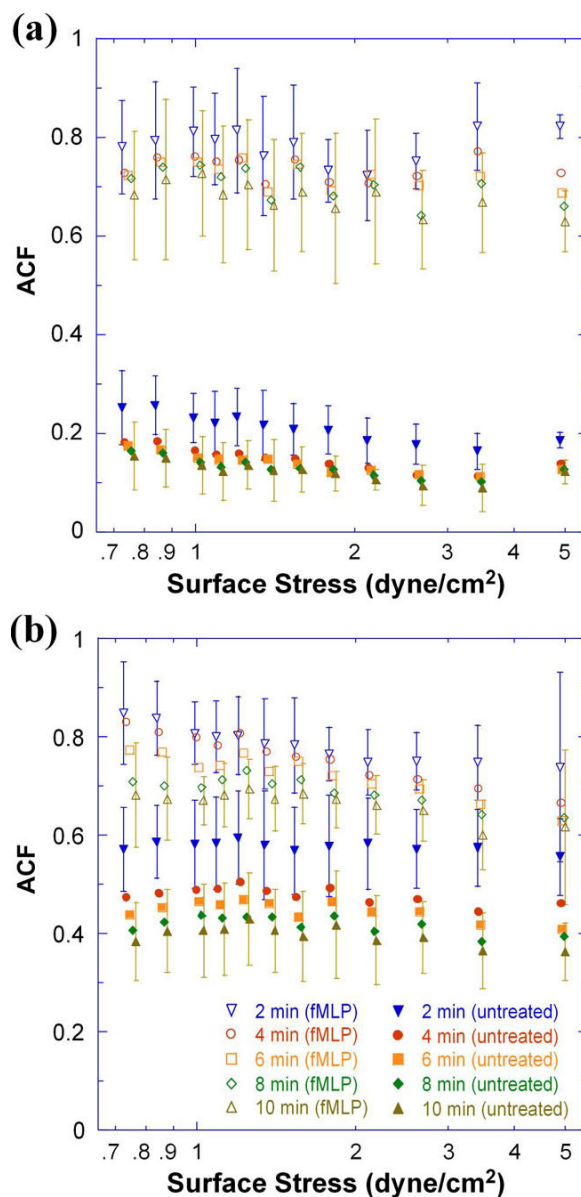


Figure 3. Results of adhesion assays. Averages of five experiments with cell incubation time $t_{inc} = 7$ min (a) and four experiments with cell incubation time 12 min (b). Plots show fractions of cells remaining adherent, ACF, in twelve different sections of the test channels, as functions of the mean surface stress in the middle the channel, τ_{mid} , in the sections. Filled symbols are untreated cells; open symbols are fMLP-activated.

REFERENCES

- [1] G. Schmid-Schonbein, "Analysis of Inflammation," *Annu Rev Biomed Eng*, 8, 93-131, 2006.
- [2] M.A. Unger, et al., *Science* 288, 113-116, 2000.
- [3] E. Dejana and M. Corada, "Methods in cell Biology," Academic Press, 69, 2002.
- [4] J. Danesh, et al., *New England Journal of Medicine*, 350, 1387-1397, 2004.