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Hydrodynamic guiding for addressing subsets of immobilized cells and molecules in microfluidic systems

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Abstract

Background: The interest in microfluidics and surface patterning is increasing as the use of these technologies in diverse biomedical applications is substantiated. Controlled molecular and cellular surface patterning is a costly and time-consuming process. Methods for keeping multiple separate experimental conditions on a patterned area are, therefore, needed to amplify the amount of biological information that can be retrieved from a patterned surface area. We describe, in three examples of biomedical applications, how this can be achieved in an open microfluidic system, by hydrodynamically guiding sample fluid over biological molecules and living cells immobilized on a surface.

Results: A microfluidic format of a standard assay for cell-membrane integrity showed a fast and dose-dependent toxicity of saponin on mammalian cells. A model of the interactions of human mononuclear leukocytes and endothelial cells was established. By contrast to static adhesion assays, cell-cell adhesion in this dynamic model depended on cytokine-mediated activation of both endothelial and blood cells. The microfluidic system allowed the use of unprocessed blood as sample material, and a specific and fast immunoassay for measuring the concentration of C-reactive protein in whole blood was demonstrated.

Conclusion: The use of hydrodynamic guiding made multiple and dynamic experimental conditions on a small surface area possible. The ability to change the direction of flow and produce two-dimensional grids can increase the number of reactions per surface area even further. The described microfluidic system is widely applicable, and can take advantage of surfaces produced by current and future techniques for patterning in the micro- and nanometer scale.

Background

Microfluidic technology holds promise for advances in analytical biochemistry, drug discovery and development, and cellular and tissue research and engineering. Handling of fluids in structures of the micrometer scale gives rise to fluid dynamics that are very different from those at the macroscale [1,2]. The fluid dynamics, the large ratio of surface area to volume, and the significant surface tension forces of microfluidic systems can be exploited to achieve fast and sensitive bioanalysis [1,3]. Moreover,

microfluidics is useful when only a minute amount of sample material is available for analysis. In drug discovery and development, microfluidic systems for high-throughput screening and target validation are being developed [4]. In tissue engineering, microfluidics is useful for patterning of cells in tissue architectures, and for local delivery of chemical agents [5]. Microfluidic systems can provide the flow velocities present in the vascular system, which is important for endothelial-cell morphology and function [6]. Microfluidic-based assays should, therefore, be better suited than the classical static assay systems for studying endothelial cells and their interactions with blood cells, cancer cells and circulating stem and progenitor cells.

Several techniques for patterning of surfaces with mammalian cells have been described [7]. There are two main approaches: (i) the cells can be exposed to whole surfaces altered only in certain regions [8–10], for example, printing of a cell substrate onto a surface will lead to cell adhesion only on the printed regions of the surface; and (ii) alternatively, the cells can be exposed to only a part of the surface, for example, by placing stamps with accessible chambers on the surface [11], or by laminar flow patterning in capillary systems [12], or by using a combination of these methods [13]. Controlled molecular and cellular patterning is, however, a costly and time-consuming process. Methods for keeping multiple separate experimental conditions on the patterned area would increase the number of tests or reactions, and thus amplify the amount of biological information that could be retrieved from a surface area. The present article describes how this can be achieved in a microfluidic system, by hydrodynamically guiding sample fluid over biological molecules and living cells immobilized on a surface.

The microfluidic system presented here consists of a reusable microfluidic chip connected to fluid pumps by fine capillaries, and a docking station which holds the chip and a slide with immobilized biological material (Figure 1A). The chip and the surface of the slide confine a flow chamber, in which lanes of the immobilized biological material are sequentially exposed to sample. Two guiding streams are used to obtain the desired flow trajectory and width of sample stream in the flow chamber (Figure 1B). The narrow tubing and small dimensions of the flow chamber eliminates turbulence phenomena. Mixing between guiding and sample fluids is, therefore, due only to diffusion, which is a relatively slow process. The docking station fits a standard microscope, allowing observation and recording of events occurring in the flow chamber.

Three biomedical applications of hydrodynamic guiding are demonstrated: (i) an assay for cell-membrane integrity on exposure to substances, (ii) a leukocyte-endothelial cell adhesion model, and (iii) an assay for measuring the concentration of C-reactive protein (CRP) in whole blood.

Results

Variation within and between lanes produced by hydrodynamic guiding

Fluorescein-labeled biotin was guided sequentially in ten lanes over immobilized streptavidin. The flow direction was then changed, and another ten lanes were guided over the same area, but perpendicularly to the first set of lanes. The even shape of the resulting two-dimensional grid, shown in Figure 2, illustrates the small variation within and between lanes produced by hydrodynamic guiding. The fluorescence intensity decreased slightly along the length of the lanes.

Cell-membrane integrity of mammalian cells on exposure to saponin

Saponin was guided over chinese hamster ovary (CHO) cells, and the cells were immediately stained to determine their viability. The ratio of dead to viable cells increased with increasing concentration of saponin, as shown in Figure 3. On exposure to saponin at a concentration of 0.013% (w/v) for 30 s (shear stress of 15 dyn/cm^2), 45% of the CHO cells died. Almost all CHO cells died when exposed to saponin at a concentration of 0.020% (w/v).

Adhesion of mononuclear blood cells to endothelial cells under laminar shear stress

Human umbilical vein endothelial cells (HUVEC) were incubated with tumor necrosis factor (TNF)-α for 6 hr and then stained for CD54 (ICAM-1), CD62E (E-selectin) and CD106 (VCAM-1) by secondary immunofluorescence. CD54, CD62E and CD106 were all induced by TNF-α, and were present in the plasma membrane and around the nuclei (Figure 4A). A primary antibody with an irrelevant specificity (isotype control) did not give staining of the cells. Mononuclear leukocytes from two individuals were stained to fluoresce green, given macrophage inflammatory protein (MIP)-1α and monocyte chemoattractant protein (MCP)-1 chemokines, and then guided over a confluent monolayer of TNF-α-activated HUVEC (shear stress of 0.75 dyn/cm^2). Mononuclear leukocytes not activated with MCP-1 and MIP-1α showed only very limited adhesion to TNF-α-activated HUVEC, whereas activation with these chemokines increased the number of adherent leukocytes 14–25 fold (Figure 4B). Adhesion of the leukocytes decreased with time of preincubation with the chemokines, and no or very few leukocytes adhered when preincubated for more than 40 min with the chemokines (Figure 4C). Chemokine-activated mononuclear leukocytes from either of the individuals neither adhered to

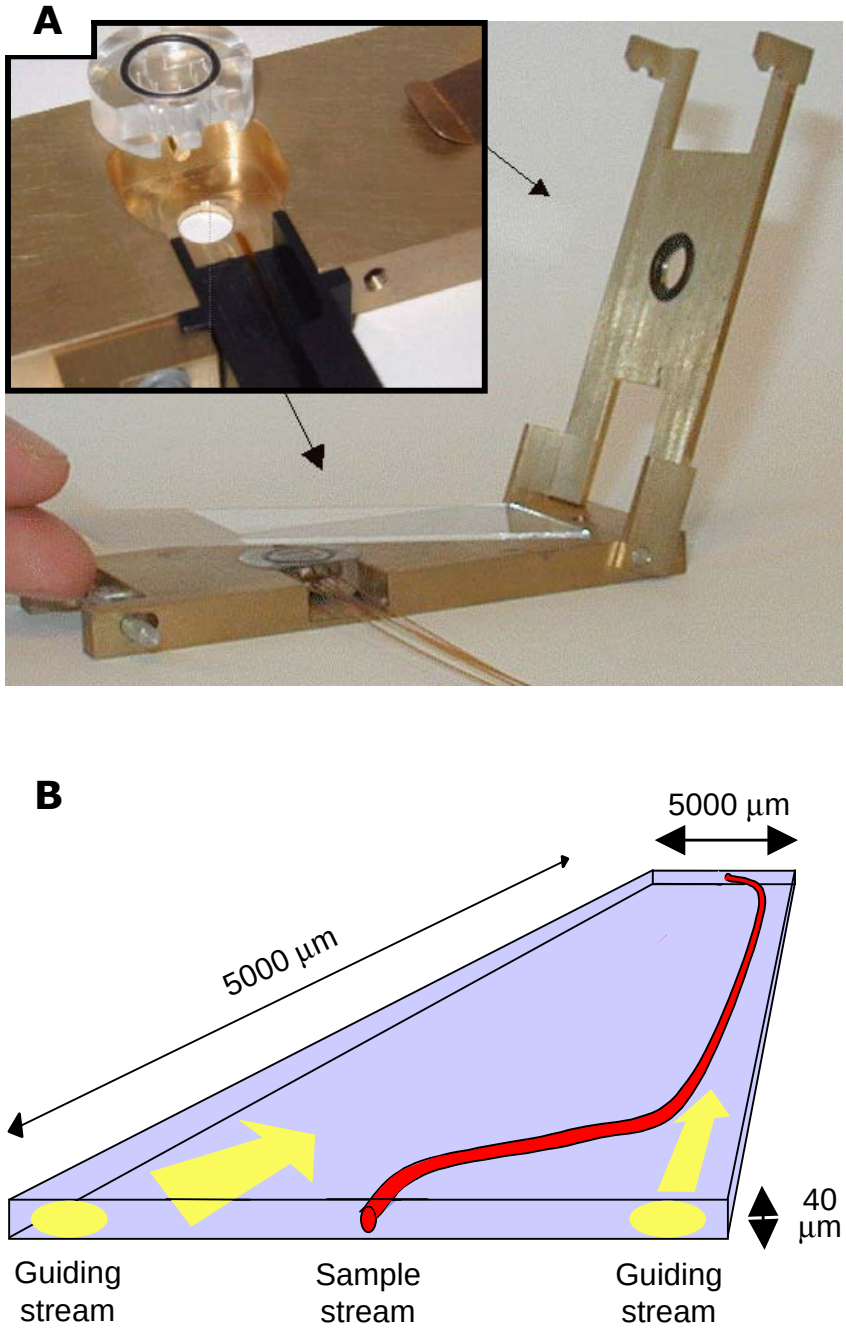

Figure 1
The microfluidic system. (A): The reusable microfluidic chip and a slide with immobilized biological material are inserted in the docking station. When closed, the docking station provides the mechanical force for sealing of the flow chamber between the chip and the slide. The loaded docking station is placed on the stage of a microscope, and the capillaries from the microchip are connected to a liquid-controlling unit (not shown). (B): Inside the flow chamber, lanes of the immobilized biological material are sequentially exposed to sample. Two guiding streams (arrows) are used to obtain the desired flow trajectory of the sample stream, and these form a sheath on both sides of the sample, confining it between the guiding streams and the roof and the floor of the flow chamber. The distance between the sample stream and the previous lane is fully adjustable, and overlapping lanes can be produced. Adjusting guiding and sample flows controls the width of the sample stream, which can be as narrow as 25 μm, given that a precise fluid-controlling unit is used, and as wide as the chip allows. In the present study, 50–500-μm-wide sample streams were positioned in the central 3000 μm of the chip with gaps of 50–500 μm, in order to obtain defined lanes of comparable shape (only the straight part of lanes was included in the analyses).

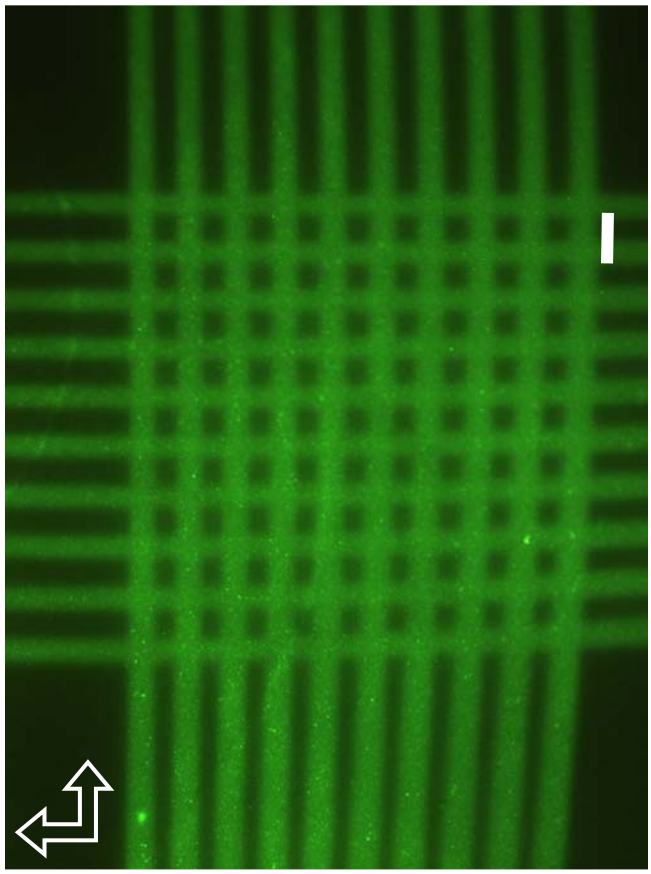

Figure 2
Analysis of the lane variation using the streptavidin-biotin affinity reaction. A microfluidic chamber with the same design and dimensions as described in Figure 1, but etched into a silicon chip and closed by bonding, was coated with streptavidin. Fluorescein-labeled biotin was then hydrodynamically guided in 50-μm-wide lanes over an area of 1000 × 1000 μm (arrows indicate the directions of flow). Ten lanes were applied evenly, and the procedure was repeated in a perpendicular direction, resulting in a grid of 100 intersections. The bended shape of the lanes in the lower and left part of the photomicrograph resulted from the hydrodynamic bending of the flow due to the quadratic shape of the microfluidic chamber. Scale bar, 100 μm.

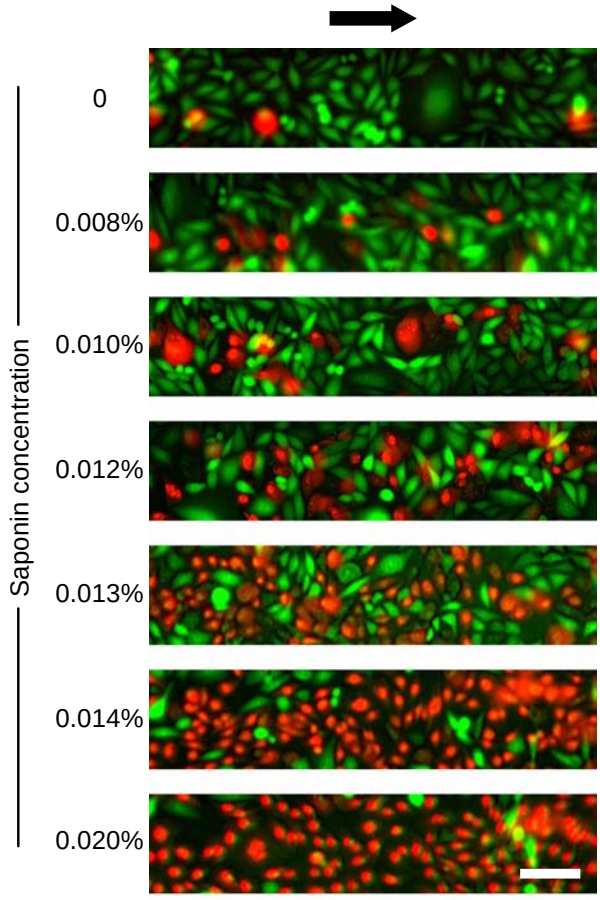

Figure 3
Cell-membrane integrity of chinese hamster ovary cells on exposure to saponin. Immobilized cells were exposed for 30 s to saponin guided hydrodynamically into 200-μm-wide lanes (arrow indicate the direction of flow). Calcein AM and ethidium homodimer-1 were then applied in the same positions of the flow chamber. Calcein AM enters all cells, and is enzymatically converted to green-fluorescent calcein in the cytoplasm. Cells with an intact plasma membrane (viable cells) retain calcein, and thus fluoresce green. Only cells with a compromised plasma membrane (dead cells) take up ethidium homodimer-1. The red fluorescence of ethidium homodimer-1 is strongly enhanced once it interacts with the nucleic acids of the cell. The ratio of dead (red) to viable (green) CHO cells increased with increasing concentration of saponin. Scale bar, 100 μm.

non-activated HUVEC nor to the supporting polystyrene slides (not shown).

Concentration of C-reactive protein measured in whole blood

In order to determine whether whole blood could be injected in the microfluidic system and analyzed for individual components, a sandwich-immunoassay for human CRP was chosen. Whole blood was spiked with CRP (antigen) to give final concentrations in the range of 5–80 mg/liter above the endogenous level (which is typically less than 2 mg/liter for a healthy individual). The spiked blood was guided over antibodies against CRP and insulin (capture antibodies), which had been covalently

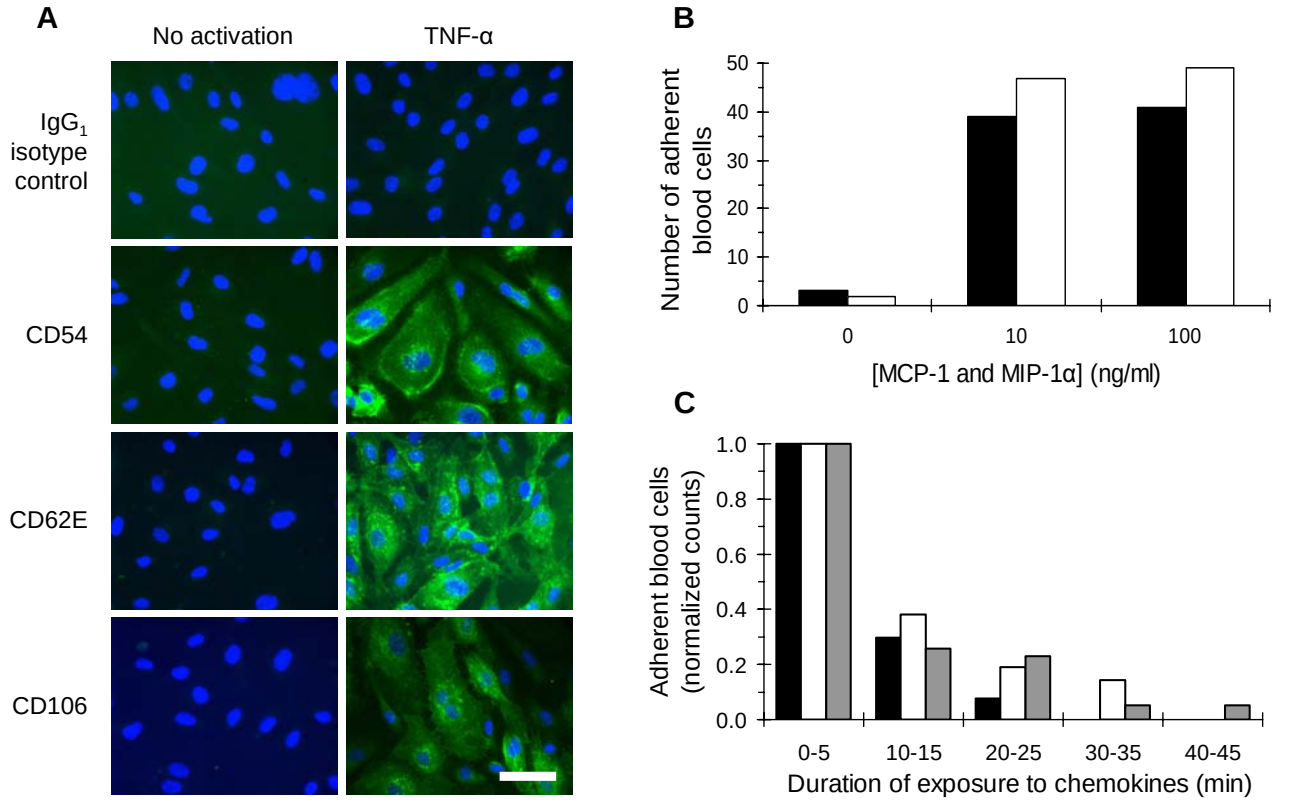

Figure 4

Adhesion of mononuclear leukocytes to activated human umbillical vein endothelial cells. (A): HUVEC were incubated with TNF-α for 6 hr and then stained for CD54, CD62E and CD106 by secondary immunofluorescence (FITC-conjugated secondary antibody). CD54, CD62E and CD106 were all induced by TNF-α, and were present in the plasma membrane and around the nuclei (stained blue by DAPI). IgG_1 with an irrelevant specificity (isotype control) did not give staining of the cells. Scale bar, 50 μm. (B): Mononuclear leukocytes from two individuals (black bars: individual 1; white bars: individual 2) were stained with BCECF-AM, given 10 or 100 ng/ml of both MCP-1 and MIP-1α, and then hydrodynamically guided in 500-μm-wide lanes at 10 nl/s (0.75 dyn/cm^2) for 10 min over a confluent monolayer of TNF-α-activated HUVEC. HUVEC were washed (15 dyn/cm^2), and the number of adherent blood cells was counted. Chemokine-activated mononuclear leukocytes did not adhere to non-activated HUVEC or to the supporting polystyrene slides (not shown). (C): In three different experiments, mononuclear leukocytes from individual 1 were mixed with MCP-1 and MIP-1α (both at 10 ng/ml), and aliquots of this leukocyte-chemokine mixture were applied to TNF-α-activated HUVEC immediately or after a 10, 20, 30 or 40 min preincubation at 37°C. The leukocytes were guided over HUVEC in 200-μm-wide lanes at 4.0 nl/s (0.75 dyn/cm^2) for 5 min, and had thus been in contact with the chemokines for 0–5, 10–15, 20–25, 30–35 or 40–45 min, as indicated on the x-axis. HUVEC were washed (15 dyn/cm^2), and the number of adherent blood cells was counted (counts are normalized because different counting frames were used). Mononuclear leukocytes activated with chemokines for any of the exposure times did not adhere to non-activated HUVEC (not shown).

immobilized on an activated glass slide. Bound CRP was subsequently detected with a Cy3-labeled antibody. No clotting of the system occurred, and a linear relationship between fluorescence signal (area under average intensity curve) and CRP concentration was obtained (Figure 5). The capture antibody against insulin was included to determine the specificity of the assay, and all lanes were guided over both capture antibodies. No fluorescence signals were detected in the area of the slide coated with capture antibodies against insulin.

Discussion

The many diverse biological applications of microfluidic systems, including analysis [14,15] and sorting [16,17] of cells and molecules, reflect the broad potential in biological sciences of working with sample in small volumes under dynamic laminar-flow conditions [1,14]. Microfluidic systems for bioanalytical applications take advantage of the small distances between analytes in sample fluids and cells or molecules immobilized in the system. The unique feature of the microfluidic system presented in this article is the ability to position sample in streams of adjustable width and flow rate over the surface of standard slides (and other formats that fit in the docking station). Patterning of the slide surface, for example, by immobilizing cells and molecules onto it, does not have to be performed in the microfluidic system, but can be done under standard static conditions.

In order to study the variation within and between lanes produced by hydrodynamic guiding, a two-dimensional grid was made by guiding fluorophore-labeled biotin over immobilized streptavidin in two dimensions sequentially. Although the lanes were equally spaced and uniform, and the grid thus appeared regular, the fluorescence intensity decreased slightly along the length of the lanes, indicating that the binding sites for biotin were not saturated. In most assays, saturation is not desirable for at least one of the components involved. Decreasing binding or activity along the length of lanes should, therefore, be taken into consideration when designing/evaluating assays based on hydrodynamic guiding. In the analyses of cell-membrane integrity, cell adhesion, and CRP concentration presented in this article, regions for analysis were determined by shifting a frame laterally between lanes, and not along lanes. This approach also avoids the bias introduced if regions of interest are chosen on the basis of visual inspection.

Analyses based on microfluidics are fast and require only a small amount of sample material. In the microfluidic system described in the present article, the feeding capillary held 10 μl of sample, which is the minimum volume of sample needed for producing a lane by hydrodynamic guiding in this system. Shorter feeding capillaries can be used if less sample is available, and longer feeding capillaries should be used if high flow rates and/or wide lanes are needed for prolonged exposures. The time required for completing a single lane depends on how many substances that the lane should be sequentially exposed to, and the duration of these exposures. In the analyses of cell-membrane integrity, cell adhesion, and CRP concentration each lane was completed in approximately 2 min, 6–11 min, and 2 min, respectively (does not include time to prepare sample and time to record and analyze the image). For completing a series of 5–7 lanes, including setup and analysis, but not preparation of cells, 30–90 min was typically required.

In-vitro toxicology testing has gained attention during the last decade, as regulatory issues are limiting the use of animals for this purpose, and because many different mammalian cell lines have become available for screening purposes [18,19]. A microfluidic format of a standard assay for cell-membrane integrity was established, in which a set of dilutions of a compound can be guided over cells immobilized on a surface, followed by a staining for cell viability. In this microfluidic format of the assay, the membrane damaging effect of a given compound, for example, a drug candidate from a combinatorial library or a natural source, can be determined by using only a few microliters of sample material (10 μl in the system described here) and a minimum processing time (two minutes per sample/lane). As an example of this, the membrane damaging effect of saponin was shown on CHO cells.

Microfluidics can also be used to study cell-cell interactions, for example, to simulate the early events of transendothelial migration of leukocytes, which is pivotal for both acute and chronic inflammation, and also involved in the atherosclerotic process. Leukocytes enter tissues through the post-capillary venules, a process that requires recognition of and firm adhesion to the endothelium: CD54, CD62E and CD106, which are membrane-bound ligands for leukocyte LFA-1/Mac-1, CD162 and VLA-4, respectively, are induced on endothelium overlying inflamed tissues. In chronic inflammation, cytokines from tissue macrophages, for example, TNF-α, are responsible, at least in part, for the endothelial-cell activation required for adhesion of mononuclear leukocytes (monocytes and lymphocytes). For firm adhesion, mononuclear leukocytes require activation of receptors already present in the cell membrane. This conformational change might be induced by MCP-1 and MIP-1α, and increased expression levels of these chemokines have been demonstrated in joints of individuals suffering from rheumatoid arthritis [20,21].

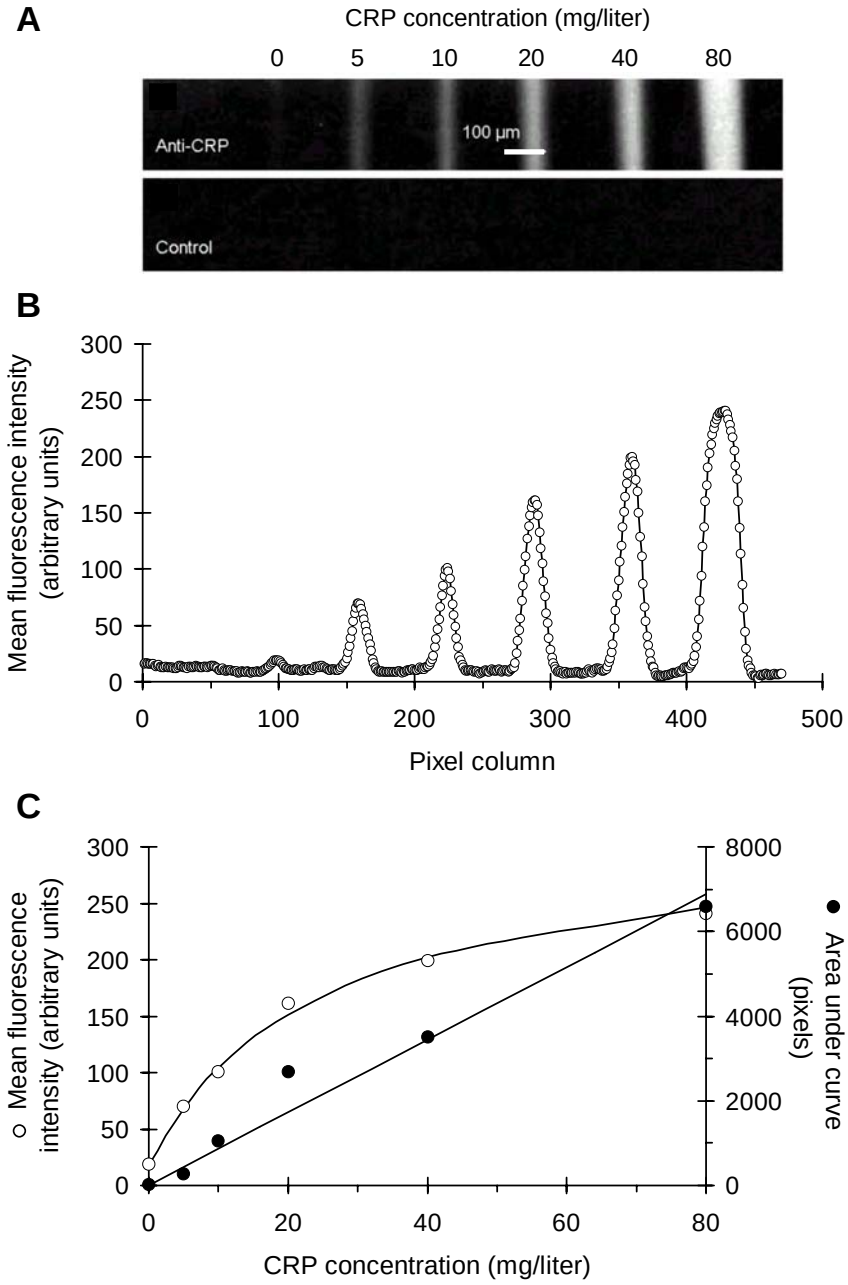

Figure 5

Immunoassay for C-reactive protein in whole blood. Capture antibodies against CRP and insulin (control) were covalently immobilized on an activated glass slide. Whole blood spiked with CRP to give a concentration of 0, 5, 10, 20, 40 or 80 mg/liter above the endogenous level was hydrodynamically guided over both capture antibodies for 2.0 s. The lane was then washed, and Cy3-labeled secondary antibody was applied to the lane. After all lanes were completed, the entire slide was scanned in a fluorescence scanner. (A): Segments of this scan (each 68 × 470 pixel), showing a fluorescence signal from the area with anti-CRP capture antibody, but not from the area with anti-insulin capture antibody (control). The width of the lanes increased with increasing concentration of CRP, possibly due to diffusion of the antigen. Scale bar, 100 µm. (B): The mean fluorescence intensities of the 470 columns of pixels of the upper segment in (A) plotted against pixel column number. (C): The fluorescence signal from the area with anti-CRP capture antibody increased with increasing CRP concentration. The relationship between CRP concentration and area under the mean fluorescence intensity curve in (B) was linear (● $R^2 = 0.97$), whereas the relationship between CRP concentration and peak in the mean fluorescence intensity curve in (B) was better described by a model of saturation (○ $MFI_{peak} = K_a + \mu \times [C_{CRP} / (C_{CRP} + K_s)]$).

In the present study, a microfluidic model of the recognition events that occur between leukocytes and endothelial cells in chronic inflammation was established. In this model, firm adhesion of mononuclear leukocytes required that HUVEC had been treated with TNF-α, which induces the expression of CD54, CD62E and CD106. By contrast, monocytes adhere even to non-activated HUVEC in static models of leukocyte-endothelial cell adhesion [22]. This basal level of adhesion has been shown to be CD18 dependent, and in static assays, a pretreatment of the mononuclear leukocytes with antibodies against CD18 may be necessary [23]. In the microfluidic system, adhesion of mononuclear leukocytes also required the presence of chemokines: MCP-1 and MIP-1α increased the number of adherent mononuclear leukocytes 14–25 fold. This effect of the chemokines was instant and decreased with time of preincubation with the blood cells. Because adhesion in the dynamic *in-vitro* model described here is TNF- and chemokine-dependent, it may be a good alternative or supplement to static assays for leukocyte-endothelial cell interactions.

CRP, an acute phase reactant synthesized by the liver, is widely used as a marker of inflammation and has been shown to correlate with the risk of coronary vascular disease [24]. Whereas the commercially available assays for detection of CRP in serum are based on conventional immunoassays [25], assays that can be performed on less sample or on crude sample material have been developed, for example, a microchip-based system for measurement of CRP in serum [26], and a micro-particle based assay for CRP detection in whole blood [27].

The present study demonstrates that CRP can be detected in a dose-dependent manner in unprocessed whole blood in a microfluidic system. The relationship between fluorescence signal and CRP concentration was linear in the range tested (5–80 mg/liter of CRP) when the width of the lanes was taken into consideration. The analysis is fast (one sample completed in two minutes) and has a high degree of specificity, as no signal was detected over the area coated with an irrelevant capture antibody. In addition to the specificity of the antibody, the short reaction time between antigen and capture antibody might also contribute to the specificity of the assay.

Conclusions

Microfluidic systems combine the advantages of performing bioassays under laminar flow with the possibility of having multiple experimental conditions on a small surface area. By contrast to microfluidic systems based on physically separated reaction chambers, the microfluidic system described here achieves this by hydrodynamic guiding of sample over an open surface area. In the three biomedical applications demonstrated, the use of hydrodynamic guiding made multiple and dynamic experimental conditions on a small surface area possible. The described microfluidic system is widely applicable, and allows the use of surfaces patterned in the micro- and nanometer scale under static conditions, and even analysis of slides with thin tissue sections. The ability to change the direction of flow, in order to produce two-dimensional grids, adds to the flexibility of the system, and increases the number of possible reactions per surface area even further.

Methods

Microfluidic system

The microfluidic system consisted of a microfluidic chip, a docking station, and a liquid-controlling unit. The microfluidic chip was produced in poly(methyl methacrylate) using standard mechanic machining technology, mainly milling, turning and drilling. The flow chamber of the chip was quadratic with rounded corners (radius = 200 μm) and had the following dimensions (length × width × depth): 5000 × 5000 × 40 μm. Each of the two flow directions held drillings for three influent streams (Ø = 200 μm for the sample fluid, and Ø = 300 μm for the guiding streams) and one effluent stream (Ø = 300 μm). A T-junction was placed upstream the flow chamber in order to bypass surplus sample fluid or to flush the flow chamber. All influent and effluent channels were glued with ultra-violet curable glue (Threebond, Germany) to non-polar fused silica capillaries (Sigma Chemical Co., St. Louis, MO) with an inside diameter of 250 μm for guiding and effluent flows, and an inside diameter of 100 μm for sample flows. The docking station, which provided the mechanical force for sealing between the microfluidic chip and the slide with the biological material, was manufactured using metal-cutting technology. The docking station was placed on the stage of an Axioskop 2 epifluorescence microscope (Zeiss, Switzerland) on which a CCD camera (Princeton Instruments RTE/CCD-1317-K/1; Roper Scientific, Germany) was mounted.

The microfluidic chip was connected via the capillaries to a liquid-controlling unit with three high-precision syringe pumps (XL3000; TECAN Systems, San Jose, CA) and three solenoid valves (Lee, Westbrook, CT). Multiport injection valves (ValcoVICI; Switzerland) were used for sample introduction and change of flow direction from one dimension to the other. Fluidics was controlled with LabView 6.0i (National Instruments, Austin, TX) software installed on a Pentium-class computer, allowing command transmission between the communication ports and the pumps and valves.

Lane-variation analysis

A custom-made microfluidic chamber with the same design and dimensions as described above was etched

into a silicon chip (SLS μ-Technology, Germany) and closed by bonding pyrex glass directly onto the structure. For coating of the chamber with streptavidin, the following protocol was followed: the chamber was washed with phosphate-buffered saline (PBS) with 0.05% Tween 20 (v/v; Merck, Germany); incubated with 0.01% poly-L-lysine (w/v; Sigma Chemical Co.) in PBS for 10 min at room temperature; flushed with PBS; incubated with 12.5% glutaraldehyde (v/v; Sigma Chemical Co.) in PBS for 45 min at room temperature; flushed with PBS; incubated with 40 mM ethanolamine (Merck) in PBS for 15 min at room temperature; and finally flushed with PBS. The chip was then connected to the pumps and valves, via the non-polar fused silica capillaries glued to the structure, and placed on the stage of the microscope. Fluorescein-labeled biotin (1.0 μM in PBS; Sigma Chemical Co.) was hydrodynamically guided in 50-μm-wide lanes over an area of 1000 × 1000 μm. The lanes were produced at a guiding-stream flow rate of 6.0 μl/s and a sample flow rate of 60 nl/s for 10 s. When 10 lanes had been completed, the flow direction was changed, and another 10 lanes were positioned perpendicularly to the first set of lanes. An image (8 bit, RGB) of the green fluorescence (excitation at 495 nm and emmission at 515 nm) was recorded on the CCD camera.

Cells

Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were grown in Ham's F-12 medium (with 2.0 mM L-glutamine) supplemented with 10% heat-inactivated (56°C, 30 min) newborn calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from BioWhittaker, Belgium). The cells were seeded in fibronectin-coated (20 μg/ml, 37°C for 2 hr; Invitrogen, Carlsbad, CA) glass slide flasks (Nunc, Denmark), and incubated until confluency at 37°C in a humidified atmosphere of 5% CO_2 in air.

Buffy coat from two healthy human individuals was obtained from the local blood bank and diluted 1:1 in RPMI 1640 (BioWhittaker). Mononuclear leukocytes were isolated by density centrifugation (800 × *g*, 30 min) of 25 ml diluted buffy coat on 20 ml Lymphoprep (Axis-Shield, Norway). Cells in the interphase were harvested, washed twice in RPMI 1640 (500 × *g*, 5 min), resuspended in RPMI 1640 with 50% heat-inactivated fetal bovine serum (FBS, v/v; BioWhittaker) and 10% dimethylsulphoxide (v/v; Sigma Chemical Co.), and frozen in the vapor phase over liquid nitrogen. Mononuclear leukocytes for flow assays were thawed, washed once in RPMI 1640, and incubated in Hanks' balanced salt solution (HBSS; Sigma Chemical Co.) with 10 μM 2',7'-bis-2-carboxyethyl-5 (and -6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) for 30 min at room temperature. The cells were then centrifuged (500 × *g*, 5 min), resuspended in RPMI 1640, and kept at 5°C. Just prior to use in flow assays, cells were heated to 37°C, then given MIP-1α and MCP-1 (to a final concentration of 10 or 100 ng/ml of each chemokine; both from R&D Systems, UK). Aliquots of the leukocyte-chemokine mixture were applied to the endothelial-cell layer immediately or after a 10, 20, 30 or 40 min preincubation at 37°C.

HUVEC were obtained from PromoCell (Germany), and grown in Endothelial cell growth medium (PromoCell) at 37°C in a humidified atmosphere of 5% CO_2 in air. HUVEC in passage 4–6 were plated at 5000 cells/cm^2 and grown until confluency in polystyrene slide flasks (Nunc). In order to activate HUVEC, TNF-α (10 ng/ml; R&D Systems) was added to the medium 6 hr before use in flow assays.

In order to demonstrate TNF-α-mediated induction of CD54, CD62E and CD106, HUVEC were stained by secondary immunofluorescence. The cells were washed with PBS, fixed in 4% paraformaldehyde (w/v) in PBS for 20 min (room temperature), air dried, washed with PBS, incubated with absolute ethanol for 2 min, washed with PBS, incubated with PBS with 5% horse serum (v/v) and 0.1% Triton X-100 (v/v) (both from Sigma Chemical Co.) for 60 min, and then incubated with PBS with 1% horse serum (v/v), 0.1% Triton X-100 (v/v), and 10 μg/ml of mouse IgG_1 against human CD54, CD62E, CD106 or an irrelevant antigen (isotype control) (all antibodies were from R&D Systems) for 2 hr at room temperature. HUVEC were then washed with PBS, incubated for 30 min (room temperature) with FITC-conjugated goat anti-mouse IgG (30 μg/ml; Sigma Chemical Co.), washed with PBS again, counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.2 μg/ml; Molecular Probes), washed in distilled water and finally mounted in FluoroGuard (Bio-Rad, Hercules, CA).

Cell-membrane integrity assay

A slide with CHO cells, prepared as described above, was inserted in the docking station, and the docking station was placed on a heating plate (37°C; Linkam Scientific Instruments, UK) on the stage of the microscope. HBSS was applied to the cells at 2.0 μl/s (corresponding to a shear stress of 15 dyn/cm^2) for 5 min to test adherence. Solutions of saponin (0.008%, 0.010%, 0.012%, 0.013%, 0.014%, and 0.020% in HBSS; w/v; Sigma Chemical Co.), and HBSS only (to determine the background level of cell death), were addressed one at a time at 80 nl/s for 30 s in 200-μm-wide lanes (15 dyn/cm^2 shear stress). After each lane, HBSS with 2.0 μM calcein AM (green fluorescence; Molecular Probes) and 4.0 μM ethidium homodimer-1 (red fluorescence; Molecular Probes) was applied at 80 nl/s for 60 s in the same position of the flow chamber. After completion of each lane, images of the green and red

fluorescence (excitation at 495 nm and emmission at 515 or 635 nm, respectively) of the same part of the lane were recorded on the CCD camera. The images were overlaid and saved in 8-bit RGB format.

Leukocyte-endothelial cell adhesion model

A slide with a confluent monolayer of HUVEC was inserted in the docking station, and the docking station was placed on the heating plate (37°C) on the stage of the microscope. Endothelial cell growth medium was applied to the cells at 2.0 μl/s (corresponding to a shear stress of 15 dyn/cm^2) for 5 min to test adherence. BCECF-AM-treated mononuclear leukocytes, prepared as described above, were hydrodynamically guided over the HUVEC monolayer at 4.0 nl/s or 10 nl/s for 5 or 10 min in 200- or 500-μm-wide lanes, respectively (both corresponding to a shear stress of 0.75 dyn/cm^2). Each lane was washed with Endothelial cell growth medium immediately (2.0 μl/s for 60 s), and an image (8 bit, RGB) of the green fluorescence (excitation at 495 nm and emmission at 515 nm) of the straight part of the lane was recorded on the CCD camera.

Detection of C-reactive protein in whole blood

A custom-made FlexiPerm silicone stamp (two chambers of 2150 × 5000 μm placed side-to-side and separated by 700 μm; In Vitro Systems & Services, Germany) was mounted on a SpotOn glass slide coated with an acid chloride functional polymer for covalent attachment of amines (Scandinavian Micro Biodevices, Denmark). The two chambers confined by the stamp and the slide were incubated at 4°C over night with polyclonal rabbit antibodies against human CRP or insulin (0.1 mg/ml in PBS; Dako, Denmark). The chambers were washed with PBS and incubated for 30 min at room temperature with a blocking solution consisting of 5% skim milk powder (w/v; Merck) and 0.2% Tween 20 (v/v) in PBS. The blocking solution was replaced with PBS, the stamp was removed, and the slide was washed for 30 min in 100 ml PBS (gentle stirring).

The polyclonal antibody against CRP was labeled with Cy3 monofunctional dye according to the manufacturer (Amersham Biosciences, UK). Excess dye was removed by gel filtration on a Superdex 75 column (Amersham Biosciences). The labeling gave a Cy3/IgG molecular ratio of 3.

The slide was mounted in the docking station with both capture antibodies represented in the flow chamber. Heparinized whole blood from a healthy human individual was spiked with Human serum CRP calibrator (Dako) to give concentrations of 0, 5, 10, 20, 40 and 80 mg/liter above the endogenous level (which is typically less than 2 mg/liter for a healthy individual). The CRP calibrator was at a concentration of 160 mg/liter. In order to avoid different degrees of dilution of the blood when spiked to obtain low and high concentrations of CRP, the CRP calibrator was diluted in PBS, and then mixed 1:1 with the blood. For each concentration of CRP, the blood was injected immediately after spiking and guided into a lane at a sample flow rate of 20 nl/s and a total flow rate of 2.0 μl/s. After 2.0 s, the lane was washed with PBS at 2.0 μl/s for 30 s. Cy3-labeled detection antibody (0.3 μM in PBS) was then applied to the same lane for 30 s (sample flow rate, 20 nl/s; total flow rate, 2.0 μl/s), and the lane was finally washed with PBS. The assay was performed at room temperature. When all lanes were completed, the entire slide was scanned in an ArrayWorx fluorescence scanner (Applied Precision, Issaquah, WA). Intensity measurements were performed using Matlab 6.0 software (The MathWorks, Natick, MA).

Authors' contributions

U.K., M.B. and T.A. developed the microfluidic system. T.B., U.K., R.A.K. and L.H.P. designed and performed the described experiments. T.B. drafted the manuscript. All authors read and approved the final manuscript.

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