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Cell handling using microstructured membranes

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Abstract

Gentle and precise handling of cell suspensions is essential for scientific research and clinical diagnostic applications. Although different techniques for cell analysis at the micro-scale have been proposed, many still require that preliminary sample preparation steps be performed off the chip. Here we present a microstructured membrane as a new microfluidic design concept, enabling the implementation of common sample preparation procedures for suspensions of eukaryotic cells in lab-on-a-chip devices. We demonstrate the novel capabilities for sample preparation procedures by the implementation of metered sampling of nanoliter volumes of whole blood, concentration increase up to three orders of magnitude of sparse cell suspension, and circumferentially uniform, sequential exposure of cells to reagents. We implemented these functions by using microstructured membranes that are pneumatically actuated and allowed to reversibly decouple the flow of fluids and the displacement of eukaryotic cells in suspensions. Furthermore, by integrating multiple structures on the same membrane, complex sequential procedures are possible using a limited number of control steps.

Introduction

Lab-on-a-chip technologies for cell-based, basic scientific or clinical applications promise to integrate all procedures from primary sample collection to data analysis in small, cheap, and versatile devices.^{1–3} One essential step in this complex process is sample preparation, when cells from primary samples are separated, washed and re-suspended in new buffer solutions, with or without specific stimulation steps, before they are made available for subsequent processing and analysis. However, most of the time this essential step is accomplished not on the chip but on the bench, by less glamorous procedures such as pipetting and centrifugation, because no common, easy-to-implement approaches exist today for on-chip sample preparation.

Handling of mammalian cells in microfluidic devices poses some non-trivial challenges. Eukaryotic cells in general, and human cells in particular, are mechanically more fragile and more deformable than other cells. They are also biologically more sensitive and quicker to respond to changes in their environment. While various methods for handling cells in suspensions have been proposed, each technique has draw-backs that limit its potential. Methods using electric fields for trapping and exposing mammalian cells to new reagents^{4–6} are dependent on the solution for cell suspension and on the cell type. Optical manipulation of relatively large mammalian cells⁷ can be laborious, expensive, and cannot be easily scaled up, while the use of mechanical structures^{8–10} is usually irreversible, since once cells

are mechanically trapped they cannot be easily released. Of these methods, however, mechanical manipulation methods for cell handling do not depend on the suspending media, or on the cell type, and have the potential to become universal methods for handling cells in suspensions, comparable with on-the-bench pipetting and centrifugation.

In this paper we present a general concept of microstructured membranes for reversibly decoupling the flow of fluids and the displacement of eukaryotic cells in suspensions for lab-on-a-chip applications. By using microstructures on mobile diaphragms we demonstrate precise control over the location of cells of interest, and new capabilities for controlling fluid and cell suspension flow. Blood sampling, cell enrichment, and sequential cell stimulation applications are presented to prove the versatility of the micro-structured membranes in microfluidic devices and their performance in sample preparation.

Materials and methods

Cells

Human monocytic leukemia cells (THP-1, American Type Culture Collection, Manassas, VA) were cultured in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM sodium pyruvate (Sigma–Aldrich, St. Louis, MO) and 50 μ M mercaptoethanol (Sigma–Aldrich). Cells from the culture were centrifuged and resuspended in phosphate buffered solution (PBS, Invitrogen) to a concentration of 10×10^6 cells mL^{-1} . Several dilutions were prepared in the range 10^3 to 10^7 cells mL^{-1} by the addition of PBS to aliquots of the original suspension. Capillary blood was collected after pricking the skin of the middle finger of a healthy volunteer and a volume up to 10 μ L placed at the inlet of the device.

Photoresist molds

Two separate molds were prepared on silicon wafers using standard photolithography techniques. The mold for the control channels used one 50 μ m thickness layer of SU8 epoxy (Microlithography Corp., Newton, MA), photopatterned through a Mylar mask (Fineline Imaging, Colorado Springs, CO) and processed according to the manufacturer's specifications. The mold for the fluidic network layer used either one layer (30 μ m) or two layers (3 and 30 μ m) of SU-8 epoxy. The single layer was photopatterned on a silicon wafer, using the same protocol as for the control layer. When two layers were employed, the thin layer was processed first and then the thicker layer was aligned and exposed in similar conditions on top of the first one. Small errors in mask alignment for the two layers could be tolerated by designing the masks such that smaller and larger structures were extended and partially overlapped.

Glass slides ($45 \times 50 \times 0.1$ mm; Fisher Scientific, Pittsburgh, PA) were sputtered with chrome (50 Å; Lance Goddard Associates, Foster City, CA). Subsequently, the thin metal film was patterned using standard microfabrication techniques. At the end, the glass slides were diced into smaller slides using a glass scriber.

Device assembly

Poly(dimethylsiloxane) (PDMS, Sylgard 184; Dow Corning, Midland, MI) was prepared according to the manufacturer's instructions. To create complementary microchannels in PDMS, a 4 mm thick layer, and separately a 100 μ m layer were cast over the control and network molds, respectively. The thickness of the network layer was controlled by spinning PDMS on the network mold at 500 rpm for 40 s. Holes were punched through the control layer using a sharpened 25-gauge needle (NE251PL, Small Parts Inc, Miami Lakes, FL). The two PDMS layers were exposed to oxygen plasma (50 W, 2% O_2 , 25 s) in a parallel

plate plasma asher (March Inc., Concord, CA) and bonded after contact and heating (75 °C, 5 min). Through holes, defining the inlets and outlets for the network layer, were subsequently punched using the same needle size. The bonding surfaces of the PDMS and the glass slides were treated with oxygen plasma. Precise alignment between the PDMS and the thin film metal patterns on the slides was achieved under a stereomicroscope (Leica MZ8, Leica, Heerbrugg, Switzerland). After the assembly of the device, the metal patch could be optionally removed using metal etchant solutions, to allow unobstructed view of the whole channel through the transparency of the glass or polymer. Extensive washing with distilled water from a syringe was employed to remove traces of the etching solution and avoid eventual toxic effects on cells.

Tygon tubing (TGY-010, Small Parts) was inserted in the inlet and outlet holes and connected through blunt syringe needles (NE301PL, Small Parts) to fluid reservoirs (network channels) or 1 mL syringes (control channels). Pressure changes in the control channels were accomplished by manual displacement of the syringe plunger.

Devices were mounted on a Nikon Eclipse TE2000-S inverted microscope (Nikon, Japan) and observed either using brightfield or fluorescence imaging. For the characterization of fluid mixing, devices were loaded with a solution of 2% fluorescein (Sigma–Aldrich) in distilled water and fluorescence evolution over time analyzed using the average light intensity scan feature in MetaMorph (Universal Imaging, Downingtown, PA).

Results

In this paper, we introduce a microstructured membrane valve concept and three examples of applications for cell handling in microfluidic devices. We built a two-layer PDMS device on glass, which incorporates a microfluidic network and a control layer with the purpose of handling small populations of suspended cells for sample preparation type applications (Fig. 1A). We first fabricated two layers of PDMS by casting the elastomer on separate photopatterned epoxy molds. The top, control layer contained a number of channels and the actuation chambers for the pneumatic valves. The bottom, network layer contained the circuitry of channels of different widths and heights for the manipulation of cells and fluids. The control and network layers were bonded together and a membrane was formed between the actuation chambers in the top control layer and the bottom network layer (Fig. 1B). The two-layer construct was then selectively bonded on the top of a glass slide. The microstructures on the diaphragm were aligned to thin film metal patterns, preventing the adhesion of the microstructure to the glass substrate (Fig. 1C). At the same time, the rest of the device was irreversibly bonded to unprotected glass. In the simplest configuration, the fluid flow in a system of channels in the network layer was controlled by the transversal microstructure on a membrane. In the inactive position, the transversal structure rested on the metal pattern and separated the single or multiple inlet channels from the outlet channel (Fig. 1D). Upon actuation, the transversal barrier was lifted by the upward deformation of the membrane into the control channel, allowing the simultaneous opening of the inlet channels into the outlet channel (Fig. 1E). The excursion of the membrane was large enough to allow passage of any objects up to 50 μm in size, either particles or cells, and larger displacements were possible by increasing the thickness of the control mold.

To underscore the flexibility of the valving concept, we present three arrangements of simple and complex microstructured membranes for applications in cell handling and flow control at the micro-scale. In the first example, four barriers on separate membranes were combined to realize a T-junction type structure for sampling precise volumes from cell suspensions (*e.g.*, whole blood). Two valves, their control chambers coupled, controlled the flow of the cell suspension along the vertical axis (Fig. 2A). A second pair of valves allowed

the displacement of the precisely metered sample by flow of buffer along the horizontal axis into the outlet sampling channel (Fig. 2B). The microstructures on the valves, in the form of curved barriers, defined the walls of a cylindrical chamber with a 30 nL volume (Fig. 2C). Repeated metering of whole blood was accomplished on the device by alternately operating the valves. The precision of the metering was verified by the absence of resident cells in the sampling chamber after the buffer wash (Fig. 2D).

In the second example, several microstructures were combined on the same membrane for partial decoupling of cell movement and fluid flow. Two barrier structures of different heights on the same membrane were used for concentrating a cell suspension, by splitting the liquid and the cells into distinct channels (Fig. 3A). A first leaky, barrier in resting position, allowed only the passage of fluid through $3 \times 10 \mu\text{m}$ channels while mechanically blocking the cells. A second, full barrier directed the leaked fluid into a drain channel. An additional microstructured membrane valve was used to control the flow in the drain channel independently (Fig. 3B). A cell suspension was introduced through the inlet channel and cells were trapped at the leaky barrier. With the accumulation of cells at the first barrier, the flow was obstructed, due to the blocking of the small leaky channels. The accumulated cells were then transported into the outlet channel by briefly lifting and then quickly releasing the microstructured membrane. Clusters of enriched cells were formed into the outlet channel (Fig. 3C) and the sieve was regenerated and ready to capture more cells. To avoid the waste of captured cells into the drain channel, the drain valve was closed during this step. Through this procedure, the density of a sparse cell suspension could be increased in the output channel by several orders of magnitude. Cell suspensions with concentrations ranging from 1×10^3 to 1×10^7 cells mL^{-1} were enriched to 1×10^7 cells mL^{-1} . The concentration increase was more dramatic, more than three orders of magnitude, in the case of sparse cell suspensions (Fig. 3D). With increasing concentration of the cell suspension, an increasing number of cells are trapped in the $30 \mu\text{m}$ space between the first and second microstructured barriers and wasted into the drain channel, limiting the yield of enrichment for cell suspensions above 1×10^7 cells mL^{-1} . For low concentration suspensions, the limiting factor for enrichment was the time required for draining the suspension liquid and trapping the maximum number of cells at the first barrier.

In the last example, one cell was exposed to a fast temporal sequence of chemicals. A microstructured membrane with two concentric structures was employed to trap the cells and the reagents in a configuration that allowed the rapid exchange of solutions around the cell (Fig. 4 A–D). While the inner microstructure was the same height as the inlet channel, the outer one was shorter and could only touch the bottom glass when moderate pressure was applied through the control channel and the membrane deformed downwards (Fig. 4C). The two concentric structures divided the chamber under the membrane in three distinct compartments. The inner compartment (S1) was the compartment where cells were trapped at the beginning of the experiment in their original suspension fluid. The middle compartment (S2) formed a ring around the inner compartment and was filled with the first solution of the sequence. The outer compartment (S3) was filled with the second solution of the temporal sequence. To load the device, the membrane was lifted by decreasing the pressure in the control channel and a cell suspension was introduced (Fig. 4A). One cell was trapped in the inner compartment (S1) by venting the control chamber and relaxing the membrane (Fig. 4B). Subsequently, the first reagent was introduced, filling the middle and outer compartments. The middle compartment was then isolated by pushing down the membrane and was sealed between the two concentric structures (Fig. 4C). The device was completely loaded after the filling of the outer compartment with the second reagent (Fig. 4E). Perfect sealing was conveniently available for as long as needed, before sequential mixing was accomplished at the time of choice by lifting the membrane (Fig. 4D). The change of concentrations of solutions in the compartments over time, estimated from the

quantified total fluorescence, is presented in Fig. 4F. We observed an initial exponential decay for the concentration in the inner compartment (S1), that reached an equilibrium level consistent with the dilution in the larger, limited space under the structured membrane. We also measured an initial fast increase, over approximately 30 s, followed by slower decrease of the first reagent (S2) at the level of the cell in the inner compartment. The fast concentration increase was consistent with a cumulative effect of diffusion and convection from the middle compartment, while the slow decay was representative for a diffusion-driven mixing. We recorded a 15 s delay in the increase of concentration at the cell level of the reagent from the outer compartment (S3). The concentration increase was slower than for S2, but still faster than expected by diffusion alone, suggesting at least a brief convective process immediately following the actuation. The axisymmetric configuration of the system assured that a cell in the middle of the inner compartment was exposed only to a temporal gradient, in the absence of a spatial gradient around the cell. We estimated less than 1% deviations from uniform conditions occurred on the circumference of a 20 μm diameter cell during exposure after loading the intermediate compartment with fluorescein solution and lifting the microstructured membrane.

Discussion

We developed a new concept of microstructured membrane for the control of eukaryotic cells and fluid displacement in networks of microfluidic channels. Cell position and displacement in channels of high aspect ratios could be precisely controlled by reversible decoupling of cell and fluid movement using the microstructured membranes. Throughout the handling processes cells were maintained in the same focal plane, allowing for easy observation using microscopy. Moreover, one unique feature of the microstructured membrane was the integration of multiple features on the same membrane with the possibility for simultaneous or sequential displacement, enabling complex actuation schemes with limited number of controls.

The microstructured membrane allowed the control of channels of any cross-section. High aspect ratios of 2:1 (height to width) or larger could easily be achieved, limited only by the SU8 photopatterning process or application requirements. For comparison, other PDMS valves are dependent on a rounded cross section of the channel^{11–14} and they could control channels with aspect ratios from 1:10, when fabricated using positive resist reflow,¹¹ to 1:1 when etched in a glass substrate.¹² Recently, a valve with lateral actuation on a rectangular cross-section channel has been demonstrated, although only partial closure of the channels was possible.¹⁵ The direct consequence of the controlled channel geometric features was the easy control of suspensions of mammalian cells. While the rounded cross section works well for manipulating fluids and even small size (few microns) particles (like bacteria), it is not appropriate for handling eukaryotic cells having average sizes between 10 and 20 μm . Such large cells would not use the entire cross section of the channel or worse, are trapped at the acute angles at the edge of the rounded channels.

The microstructured membrane could seal a channel in the absence of the actuation pressure, only by elasticity of the membrane pressing against the valve seat, a feature shared with the three-layer PDMS valves.^{12,16,17} In contrast, most of the other elastomeric mechanical valves would seal upon the application of pressure through a thin membrane, and may introduce the challenge of maintaining the homeostasis of a cell suspension in the controlled channel. Gasses can diffuse easily through the membrane under the actuation pressure¹⁸ and either diffuse in the cell suspension or, worse, form gas bubbles that can damage the cells. While a common solution for this problem is the filling of the control channels with liquid, recent results reported that the PDMS is permeable even to some commonly used liquids,¹⁹ and raise the concern of maintaining the homeostasis of the medium around cells during

pressure actuation. Such potential shortcomings are avoided by the vacuum actuation of the microstructured membranes. Owing to the elasticity of the PDMS, the microstructured membrane presses the microstructure against the valve seat and keeps the valve normally closed in the absence of actuation. The pressure that a valve could withstand without supplementary pressure in the control chamber was relatively low, in the range of few Pascals, but comparable to the pressures likely to drive very slow movement of cell suspensions.

Of practical importance is the unitary fabrication procedure, which allows the microstructured valve to be fabricated using soft lithography techniques based only on epoxy photoresists (*e.g.*, SU8). One significant challenge to overcome in the fabrication of the normally closed valve, has been how to selectively seal PDMS and glass components and simultaneously avoid irreversible valve bonding. Previous technical solutions involved mechanically clamping a PDMS membrane between the two glasses,¹² partial bonding in combination with mechanical sticking,¹⁶ or a water-soluble retardant²⁰ for patterned PDMS bonding. In our approach, the patterned control of bonding was achieved by a metal “patch” at the site of the valve seat. A thin layer of metal on the glass was patterned and then aligned to the valve seat, thus preventing the bonding of the PDMS to glass after exposure to oxygen plasma. The metal layer could eventually be etched at the end of the fabrication process, resulting in fully transparent devices.

The unitary fabrication procedure is also important in reducing the costs associated with the fabrication of these devices. While for certain experiments the devices can be cleaned with various solutions and re-used, in applications involving human blood or human cells, safety issues require these devices to be single use. Nonetheless, when translating the design into different materials, the implementation of unitary fabrication procedures becomes less challenging, ultimately resulting in cheaper, disposable devices.

We presented three examples that substantiate the complexity of the cell suspension handling procedures which can be implemented using microstructured membranes. Precise blood metering, augmentation of cell concentration in a sparse suspension, and circumferentially uniform exposure of cells to sequences of reagents were implemented using design variations of the microstructured membranes.

Precise metering of whole blood samples is essential for many clinical diagnostic applications, *e.g.*, the biochemical analysis of blood²¹ and blood cell counting and analysis.³ Volumes of blood as small as a few microliters can be precisely sampled using syringes and micropipettes. However, smaller volumes, like those used in microfabricated devices, require different approaches not always suited to complex cell-rich fluids like blood. Vented capillaries with a hydrophobic barrier^{22–24} could trap the air bubbles between fluid segments that need to be mixed. Microdroplets on electrowetting platforms²⁵ have to be formed outside the device by pipetting; their minimum size is limited to the microliter range, and stickiness of blood proteins and cells on the hydrophobic surfaces may be problematic. Finally, valves have been designed to sample sub-microliter volumes of fluid and perform biochemical assays,²⁶ although the geometry of the valves is not friendly for cells. Precise metering volumes are possible using valves in channels with vertical walls.¹⁶ In our design, the use of microstructured membranes for the implementation of four valves, in a chromatography T-junction-like design, allowed the precise metering of 30 nL of blood. Chambers with volumes from few nanoliters to several microliters were designed by changing the position of the barriers or by altering the height of the chamber. Because of the efficient removal of all blood cells from the chamber after a sample, volumes of blood equal to multiples of the chamber volume could also be precisely metered. To accomplish this,

repetitive, alternative opening and closing of the valves controlling the blood and the buffer could be used to load and unload the chamber.

Although widely used for handling cell suspensions, centrifugation methods are unlikely to be implemented in microfluidic devices which usually have no mobile parts that could accomplish rapid rotations. To circumvent this obstacle and realize enrichment of cell suspensions, we propose an alternative approach based on the use of microstructured sieves on membranes. Microstructured sieves can be quickly regenerated by the actuation of the supporting membrane and were implemented for controlled cell trapping and release. The new cell enrichment procedure was extremely effective for very low original cell concentrations when enrichment of three orders of magnitude was easily achieved. In addition, the device could efficiently handle very small samples, a situation when centrifugation would be ineffectual because of major cell losses while removing the supernatant. Such capabilities would be useful in microfluidic devices that are very likely to dilute the cell suspension by the addition of reagents and when the reconstitution of the initial cell concentration is important for later analysis. For example, selective destruction of cells in a blood sample during the preparation of the buffy-coat equivalent usually results in large-volume, low-cell concentration samples.²⁷ Subsequent cell analysis protocols would benefit from reducing the sample volume and increasing the cell concentration. In addition, on-chip processing is gentler and less likely to affect the cells of interest by exposure to mechanical stresses during centrifugation.^{28–30} The new cell enrichment procedure may also be useful for the isolation of cells from dissociated tissues and removal of debris,³¹ or for separating cells in particles based on size, in an approach comparable to mechanical filtering of blood cells.³

The study of cellular responses to chemical stimulation is of fundamental importance for many biology studies. While macroscopic techniques using pipettes and Petri dishes are still widely used in biology laboratories, there is increasing interest in the more precise methods available through microfluidics. Most often, when studying cells in suspensions, cells are trapped using flow and mechanical obstacles,^{9,32,33} centrifugation,³⁴ dielectrophoresis,^{6,35} or laser beams⁷ and the soluble stimulant brought to the cells by convective flow. Alternatively, suspended cells are contained in a no-flow environment³⁶ and solutions brought to cells by diffusion from short distances. Although comparable as a strategy, the use of co-axial compartments in the microstructured approach accomplished two performances unmatched by other current techniques. Uniform stimulation of cells along their circumference was, for the first time, possible, and may become a useful tool for studying the response of cells to temporal stimuli in the absence of spatial gradients. Additionally, precise temporal control of sequential stimuli was possible through a single actuation. The temporal profile and sequence of stimulation could be adjusted by changing the size of the ring compartment and the size of the barriers which, once incorporated into the physical device, could assure reproducibility of experiments in the absence of sophisticated equipment.

Conclusions

We have developed a microstructured membrane design for the purpose of cell handling on the chip. Important features of the microstructured membrane are the integration of several functions in one actuation step, the independence of the functioning on the cross section shape and aspect ratio, the restricted movement of cells in only one plane, and the capability for tight sealing when implemented as a valve. Through different applications we demonstrated the potential performances and underlined the likely impact on cell manipulation on chips, especially when a small number of cells are handled. Based on these results we believe that the flexibility in the design of microstructures that can be

implemented on mobile diaphragms will allow, in the future, the development of novel multifunctional microfluidic tools for the study of biology and physiology of mammalian cells for scientific and clinical applications.

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Fig. 1. Schematics and functioning of the microstructured membrane. A. Fabrication of the microstructured membrane device. Three main components, a control layer, a network layer, and a patterned glass substrate, are fabricated individually using standard microfabrication technologies. B–C. Side and top view of the assembled device. D. Scanning electron microscopy of a microstructured membrane functioning as a valve with multiple inlets and one outlet. The device is shown from the bottom with the underlying glass removed for clarity. The valve is shown in the closed position, with barrier pressing against the glass. E. In open configuration, the membrane is deformed and the barrier is lifted, allowing communication between inlet and outlet channels. Scale bar is 200 μm .

Fig. 2.

Precise sampling of a cell suspension. A. Pairs of barriers are used to sequester cells from suspensions. B. Cell suspension flows from inlet to outlet by the opening of the “cell suspension” inlet and outlet valves. Following the closing of both “cell suspension” valves a precise volume can be trapped in the sampling chamber. When the “buffer inlet” and “cell sample outlet” valves are opened, the sampled cell suspension is moved into the cell sample channel. Actuation chambers for corresponding valves are connected together for simpler actuation. Scale bar 300 μm . C. Sampling of 30 nL of whole blood is demonstrated. In the first step whole blood is introduced in the chamber. D. The precisely metered blood volume is pushed outside the chamber into the sample channel by the flow of a buffer solution. Metal valve seats were removed in this device. Scale bar 400 μm .

Fig. 3. Leaky valve for cell concentration. A. Schematics of a membrane combining two different microstructures: a barrier with small leak channels and a full barrier. B. Cells are trapped at the position of the small leakage channels on the first barrier. Leaked fluid with no cells is directed to the “drain channel” by a second barrier. An additional valve on the drain channel is used to stop the flow during the actuation of the main membrane and prevent losses of cells in the drain channel. Scale bar is 120 μm . C. Lifting of the dam structure releases all the trapped cells simultaneously into the “cell outlet” channel. Four clusters of cells, captured and released from the first microstructured membrane are outlined. D. Cell enrichment of up to three orders of magnitude can be achieved by repeated actuation of the device. Scale bar 200 μm .

Fig. 4. Sequential exposure of trapped cells to different solutions. A–D. Circular ridges of different heights are used to sequentially trap a cell in medium S1 in the central compartment and then solution S2 in the middle compartment. The external space is filled with solution S3. Lifting of the microstructured membrane leads to sequential exposure of the cell to the solutions in the middle and outer compartments. E. Brightfield image of the microstructured membrane with circular ridges. A cell is trapped in the central compartment. Scale bar 200 μm . F. Time change of the relative concentrations in the center of the inner compartment. Immediately after actuation, solution S1 (dotted line) will diffuse outwards in the outer compartments while solution S3 (broken line) will move and diffuse towards the center compartment. The concentration of solution S2 in the inner compartment will temporarily increase and then decrease slowly by outward diffusion (solid line).