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Molded polyethylene glycol microstructures for capturing cells within microfluidic channels

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Received 31st March 2004, Accepted 21st May 2004
First published as an Advance Article on the web 26th July 2004

The ability to control the deposition and location of adherent and non-adherent cells within microfluidic devices is beneficial for the development of micro-scale bioanalytical tools and high-throughput screening systems. Here, we introduce a simple technique to fabricate poly(ethylene glycol) (PEG) microstructures within microfluidic channels that can be used to dock cells within pre-defined locations. Microstructures of various shapes were used to capture and shear-protect cells despite medium flow in the channel. Using this approach, PEG microwells were fabricated either with exposed or non-exposed substrates. Proteins and cells adhered within microwells with exposed substrates, while non-exposed substrates prevented protein and cell adhesion (although the cells were captured inside the features). Furthermore, immobilized cells remained viable and were stained for cell surface receptors by sequential flow of antibodies and secondary fluorescent probes. With its unique strengths in utility and control, this approach is potentially beneficial for the development of cell-based analytical devices and microreactors that enable the capture and real-time analysis of cells within microchannels, irrespective of cell anchorage properties.

Introduction

The development of biological micro-electromechanical devices comprised of microfluidic channels could potentially revolutionize biological analysis and create new ways of analyzing cells *in vitro*.[1] These microdevices are advantageous in that they use very small volumes of reagents and can be potentially scaled up for high-throughput analysis. Although great progress has been made in the fabrication of cell-based biosensors and high-throughput screening approaches, more research in interfacing cells with such microdevices is of benefit.

Cells are typically interfaced with microfluidic channels using one of two schemes. In one scenario, cells are flowed through the channel, either hydrodynamically or through electroosmotic pumping to separate and transport cells.[2,3] As the cells flow through the channel, they are potentially lysed, and the intracellular components of the cells are analyzed.[4,5] This approach is, however, potentially limited to non-adherent cells and transient analysis since unanchored cells exit the system and elude follow-up characterization. An alternative approach to analyze adherent cells is to immobilize cells within channels.[6] For example, anchorage dependent cells have been attached to microfluidic channels by a number of groups.[7,8] To immobilize cells within particular regions of a microfluidic channel, laminar flow,[9] pre-patterning with adhesive ligands,[10] and immobilization inside hydrogels[11] have been used. Despite the success of these approaches, there are potential limitations. For example, the geometrical shapes of the patterned regions using laminar flow patterning is restricted to the flow of the laminar streams, while the UV induced immobilization of cells inside hydrogels requires the exposure of cells to potentially toxic photoinitiators and radiation.[12] Also, the direct immobilization of cells on the substrate of the channels could lead to shear induced modifications in cell behavior.[13]

An alternative approach to immobilize cells within microfluidic channels is to capture and localize cells within particular regions of a channel using physical structures. For example, a dam structure between two parallel channels can be used to constrict cells within microfluidic channels.[14] However, lack of control of the surface properties has hindered the widespread use of topographical heights in micro-scale analytical tools. Therefore, the development of simple and direct techniques for fabricating microstructures within microchannels with precise control over the surface properties of the microstructures could be of benefit.

Photolithography has been a useful tool to fabricate microstructures outside[12,15,16] or within microfluidic channels.[17,18] In photolithography, UV light is exposed through a mask to polymerize defined regions of a thin pre-polymer film. One potential disadvantage of photolithography is that it requires multiple steps to fabricate microstructures with multiple heights. Molding of polymer under PDMS stamps is an alternative method of creating microstructures.[19–21] A previous study on molding of polystyrene has shown that the microstructures correlate with the initial thickness of the polymer film. We hypothesized that the merger of our previously developed method of patterning inside microchannels[10] with polymer molding under a PDMS stamp could generate a unique approach in fabricating microstructures as integral components of microfluidic channels, with improved control over the surface properties of the channel.

In this report, we demonstrate a novel method of fabricating PEG microstructures within microchannels using a two step process. In the first step, microstructures are generated by molding photocrosslinkable PEG onto a small predefined region of a substrate. To allow for irreversible bonding of the microfluidic mold, the size of the PDMS stamp is restricted to a small region of the substrate. The stamp is left undisturbed on

DOI: 10.1039/b404842c

the molded regions to protect the patterns from the plasma cleaning of the substrate. The stamp is then peeled from the substrate and the microfluidic mold is aligned on the substrate to allow for the formation of microstructures capable of capturing cells. Additional control of these features could be achieved by obtaining exposed (*i.e.* regions containing bare substrate) or non-exposed substrates (*i.e.* substrate is completely covered by PEG), thus facilitating the patterned deposition of proteins and cells.

Methods and materials

Materials

Poly(ethylene glycol) dimethacrylate (PEGDM, MW = 330, 575, 1000), bovine serum albumin (BSA), carbon tetrachloride, 2-hydroxy-2-methyl propiophenone, and *n*-heptane were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). 3-(trichlorosilyl)propyl methacrylate (TPM) was obtained from Fluka Chemicals (Milwaukee, WI). Polydimethylsiloxane (PDMS) elastomer composed of pre-polymer and curing agent was purchased from Essex Chemical Sylgard 184 (Edison, NJ). For cell culture, Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), fibronectin (FN), trypsin, and other cell culture reagents were purchased from Gibco Invitrogen Corporation (Carlsbad, CA). MC-480, the antibody against SSEA-1 was purchased from Developmental Hybridoma Bank (Iowa City, IA). Phycoerythrin conjugated goat anti-mouse IgM antibody was purchased from Jackson ImmunoResearch (West Grove, PA). All cells were obtained from American Tissue Type Collection (Manassas, VA). Calcein AM, ethidium homodimer and Texas-red labeled BSA (TR-BSA) were purchased from Molecular Probes (Eugene, OR). 1 ml syringes and polyethylene tubing were purchased from Becton Dickinson(Franklin Lakes, NJ). Metal tubing and Y connectors were purchased from Small Parts Inc. (Miami Lakes, FL).

Cell culture

Cell culture was performed under sterile tissue culture hoods, and cells were maintained in a 95% air/5% CO_2 humidified incubator at 37 °C. NIH-3T3 fibroblasts were maintained in DMEM supplemented with 10% FBS. Mouse embryonic stem (ES) cells (R1 strain) were maintained on gelatin treated dishes in a medium comprised of 15% ES-qualified FBS in DMEM knockout medium. ES cells were fed daily and passaged every 3 days at a subculture ratio of 1:4. During the experiments, the medium specific to the cell type was used.

PDMS mold fabrication and substrate preparation

PDMS molds were fabricated by curing the pre-polymer on silicon masters patterned with SU-8 photoresist. The masters used for patterning had receding cylindrical features (ranging from 15 to 150 μm in diameter), 100 μm lanes or larger grids which resulted in PDMS replicas with the opposite sense. The masters used for microfluidics had protruding features with the impression of microfluidic channels (ranging from 50 to 800 μm in width and ~80 μm in height). To cure the PDMS prepolymer, a mixture of 10:1 silicon elastomer and the curing agent was poured on the master and placed at 70 °C for 2 h. The PDMS stamps (*i.e.* used for patterning) and the microfluidic molds were then peeled from the masters and cut. The PDMS stamps were cut into narrow strips (~0.3 cm × 2 cm) that were sufficiently large to pattern the entire width of the channels, while allowing the rest of the substrate to be plasma cleaned.[10]

Prior to patterning, glass slides were plasma treated for 2 min, immersed in a solution of 30% H_2O_2 and H_2SO_4 (3:1 ratio) for 5 min and washed in DiH_2O. The slides were then immersed in a 1 mM solution of 3-(trichlorosilyl)propyl methacrylate (TPM) for 5 min to enhance the adhesion of PEG microstructures to the surface[15] and washed with a mixture of heptane/carbon tetrachloride (80/20 v/v) and DiH_2O.

Scanning electron microscopy

To perform scanning electron microscopy (JEOL 6320FV) samples were mounted onto aluminium stages and sputter coated with gold to a thickness of 200 Å and analyzed at a working distance of 20 mm.

Protein adsorption

TR-BSA was dissolved in PBS (pH = 7.4) at 100 μg mL^{-1}. To test for substrate exposure through protein adhesion, a few drops of the protein solution were evenly distributed onto the patterned substrates and incubated at room temperature for 45 min. All patterned surfaces were then washed and analyzed using an inverted fluorescent microscope (Axiovert 200, Zeiss).

Fabrication of the microstructures within microfluidic channels

The microstructures were made using a solution of 99.5 wt.% PEGDM (MW 330, 550, or 50% 1000 dissolved in PBS) and 0.5 wt.% of a water soluble photoinitiator 2-hydroxy-2-methyl propiophenone photoinitiator. To fabricate the exposed and non-exposed microstructures on the substrate we used a technique called capillary force lithography.[21] Two different approaches were used to generate the substrates with varying features (Fig. 1). To generate features with non-exposed substrates, a few drops of the PEG polymer were evenly distributed onto the substrate, whereas to generate features with the exposed substrate a few drops of the pre-polymer were evenly spread on the PDMS stamp. The PDMS mold was then placed directly on the polymer film and exposed to 365 nm, 300 mW cm^{-2} UV light (EFOS Ultracure 100ss Plus, UV spot lamp, Mississauga, Ontario) for 30 s.

Once the microstructures were fabricated, the devices were completed by plasma cleaning the slide (without disturbing the

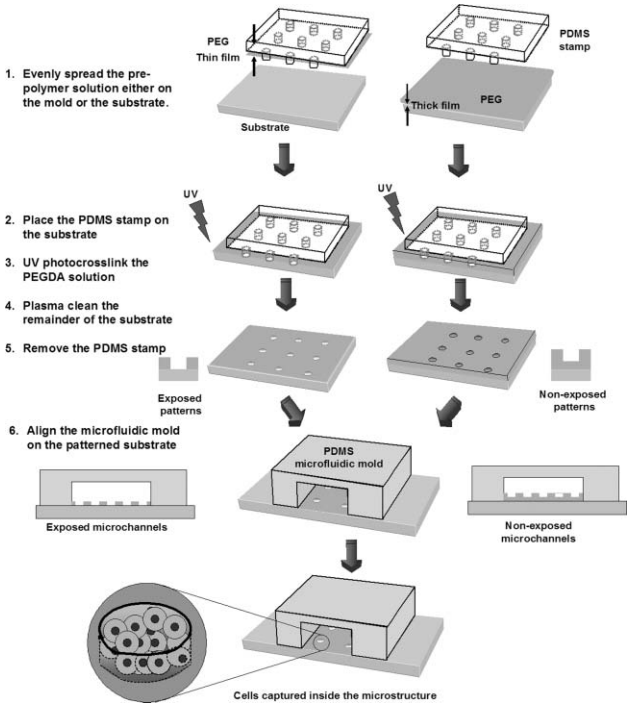

Fig. 1 Schematic diagram for the fabrication of exposed and non-exposed microstructures inside microchannels.

PDMS stamp) and the microfluidic mold for 2 min (60 W, PDC-32G, Harrick Scientific, Ossining, NY). After plasma treatment, the PDMS stamp was peeled from the substrate and the microfluidic mold was aligned and brought in conformal contact with the substrate and firmly pressed to form an irreversible seal. In some experiments the devices were further supported by clamping the mold to the substrate.

Fluids were driven through the channels using a SP200i syringe pump (World Precision Instruments, Sarasota, FL) that was connected to the device using polyethylene tubing. Transitions between different injections were facilitated with a Y connector that was used to redirect bubbles that were formed by changing the inlet solution.

Docking and analysis of cells in microchannels

All experiments involving cells inside the channels were carried out in a 37 °C, 5% CO_2 environment chamber (Zeiss, Germany) and visualized under a fluorescent microscope. To immobilize cells within the microstructures, cells were trypsinized and resuspended in medium at a concentration of $\sim 2 \times 10^7$ cells mL^{-1} and kept on ice. The channel was first treated with ethanol (95%) to clear potential air bubbles, followed by PBS for 10 min at a flow rate of 1 μL min^{-1}. For cell adhesion studies, fibronectin (25 μg mL^{-1}) was then flowed in the channel for 15 min.

Cells were introduced into the channel and the flow was stopped to sediment the cells into microwells. After 10 min the flow was restarted and maintained at 1 μL min^{-1}.

To analyze cellular viability, a live/dead assay was performed by flowing ethidium homodimer and calcein AM dissolved at 1 μg mL^{-1} in DMEM containing 10% FBS through the channel for 20 min. Staining of ES cells was performed by flowing MC-480/SSEA-1 (diluted 1:10 in a PBS solution with 1% BSA) for 20 min and then phycoerythrin conjugated goat anti-mouse IgM (diluted at 2:1000 in 1% BSA) for 20 min, both at the flow rate of 1 μL min^{-1}. PBS was then flowed through the channel to wash the channel and remove non-specific staining.

Results and discussion

Fabrication of exposed and non-exposed microstructures

Control over the features of microdevices including microfluidic channels is important for the development of analytical devices. We aimed to immobilize cells within microchannels by fabricating PEG microstructures that could facilitate the capture and analysis of cells with control over the adhesion of anchorage dependent cells. To fabricate PEG microstructures, PEGDM was molded beneath a PDMS stamp and subsequently photopolymerized. PEGDM was used due to its ability to crosslink at short exposure times and its low viscosity which allow for its use at high concentrations to fabricate structures with a high aspect ratio.[21,22]

To fabricate microstructures with a non-exposed substrate we molded the polymer onto the features of the PDMS stamp by placing a thick polymer film on the substrate and subsequently placing the stamp on the film (Fig. 1). As shown in Fig. 2a, the microstructures could be generated using this approach without the underlying substrate becoming exposed. Alternatively, to fabricate exposed substrates a layer of PEG was coated onto the PDMS stamp and subsequently molded onto the substrate. These thinner films resulted in the formation of microstructures with exposed substrates (Fig. 2b). The PEG microstructures were ~25 μm in height with good pattern fidelity (Fig. 2c–d).

To demonstrate that the approach could be used to generate microstructures with exposed or non-exposed substrates, the ability of the PEG microstructures to resist protein adsorption was examined. Since PEG networks are protein resistant, it is

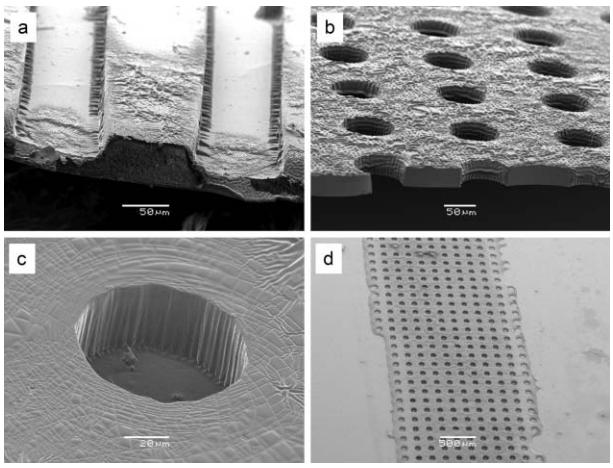

Fig. 2 Scanning electron micrographs of molded PEG lanes (a) or microwells (b–d). The underlying substrate could be either exposed (a) or non-exposed (b) based on the polymer film thickness. (c) Individual patterns of circular microwells demonstrate that the structures are ~25 μm in height.

anticipated that for the non-exposed patterns, the patterned regions will resist protein adhesion while for patterns with exposed substrates, proteins will adsorb onto the hydrophobic underlying substrate forming patterned regions. As shown in Fig. 3, microstructure patterns that were treated with TR-BSA could be fabricated either with exposed substrates or without the substrates depending on the fabrication process. Further quantification of the degree of protein adsorption onto the PEG-based microstructures showed that *ca.* 98% of the protein adsorption was reduced as compared to that of exposed surface of the substrate. In addition, the ability of the exposed substrate to allow for adhesion of cells within the microstructures was examined by dipping the patterned substrates in a solution of FN. After adhesion of the protein onto the substrate the solution was washed and NIH-3T3 cells were seeded on the substrate. After 6 h, the patterns were thoroughly washed to remove all non-adhered cells. As seen in Fig. 4, cells adhered and spread inside microwells with exposed substrates while the cells on the non-exposed microwells were completely washed away even though they had been patterned within the wells.

PEGDM ranging in molecular weight from 330 to 1000 Da was successfully used to fabricate non-biofouling microstructures. However, for the experiments reported here PEGDM

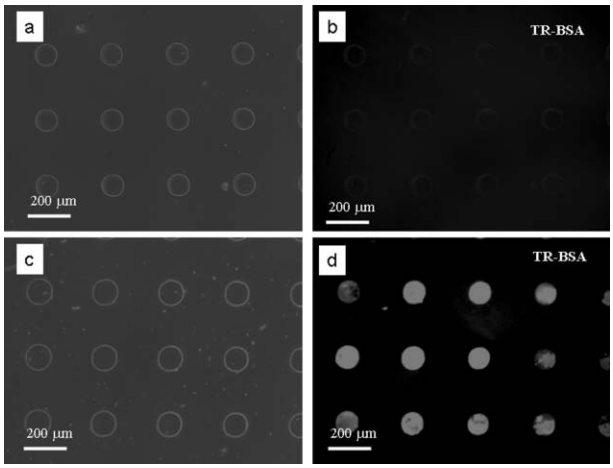

Fig. 3 Light (a, c) and fluorescent (b, d) images of microstructures with non-exposed (a–b) and exposed (c–d) underlying substrates. Substrate exposure was tested by testing the adsorption of TR-labeled BSA on the patterned surfaces.

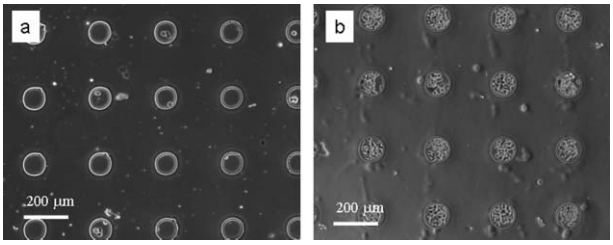

Fig. 4 NIH-3T3 cell adhesion of the non-exposed (a) and exposed (b) PEG microwells. NIH-3T3 cells were seeded on substrates patterned with PEG polymer for 6 h and subsequently washed and analyzed.

330 and 550 Da were routinely used due to their improved mechanical strength, low swelling properties and ability to resist cells and proteins.

Fabrication of the device

Although it is possible to UV crosslink PEG pre-polymer inside microchannels,[11,17,18] the direct crosslinking of the PEG polymer within microfluidic channels has not been shown to generate features with both exposed and non-exposed substrates. Therefore, we hypothesized that the molding of the PDMS stamp on a polymer film would allow for more control over the features of the microfluidic channel.

To fabricate PEG microstructures inside microwells, the microfluidic molds were aligned on substrates that had been pre-patterned with PEG microstructures. To ensure that the microfluidic mold could be irreversibly adhered to the substrate we patterned only a small region of the substrate to allow for plasma treatment of the remainder of the substrate. The PDMS stamp was left undisturbed after molding and the remainder of the substrate was plasma treated to allow for adhesion of the substrate to the PDMS mold (Fig. 1).

One of the observations obtained using this approach was the ability of the elastomeric microfluidic mold to seal the channels despite the topographical differences between the patterned region and the surroundings (Fig. 1). These microchannels were robust and could stand flow rates of > 5 μL min^{-1}. This could be attributed to the elastomeric properties of the mold.

Docking of cells within microstructures

To evaluate the ability of the microstructures within the channels to capture cells, NIH-3T3 and ES cells were used as model cell lines. Both these cells are anchorage dependent and thus they enable testing of the potential adhesion of these cells. In addition, trypsinized cells from both cell types can be used as a model for non-adherent cells. Initial experiments were performed using various shaped features including lanes, grids and circles. Although numerous conditions were tested, two specific conditions facilitated cell docking. In the first approach, the flow rate was tightly regulated to enable flow of the cells inside the channel but was slow enough to allow for a fraction of the cells to be captured by the microstructures. Within standard microchannels (800 μm in width and 80 μm in height) using the parameters used in these experiments, a flow rate of ~ 0.3 μL min^{-1} was found to be optimized in that it allowed for docking of the cells, yet did not clog the tubes due to excessive clumping and aggregation of the cells. However, the optimized flow rate is a function of channel dimensions and geometry (determining shear stress), cell phenotype and concentration. The second approach was to stop the flow briefly to allow for the cells to settle into the microstructures. In general, it took less time with the latter technique to deposit cells within structures and was overall preferred for our subsequent experiments. As shown in Fig. 5, cells successfully docked within features of various shapes. Furthermore, once the cells had settled within these regions, they remained in place and were not washed away even when the flow rate was increased to high values of > 5 μL min^{-1}.

Analysis of captured cells within microchannels

To test for the ability of the microchannels to act as potential bioreactors and analytical tools, cells were analyzed using a variety of techniques. To analyze cell viability and the ability to perform enzymatic reactions, ethidium homodimer and calcein AM were allowed to flow through the channel. Ethidium homodimer is a DNA binding dye that stains the membrane of compromised cells. On the other hand, calcein AM is a membrane permeable substrate that is converted within the cells to a green fluorescent molecule that is membrane impermeable. Therefore 'live' cells can be visualized as green, while cells with compromised cell membranes show up as red. As expected, $\sim 98\%$ of NIH-3T3 cells that were immobilized within the channels remained viable based on the expression of the green fluorescent dye. In addition, the cells did not stain red indicating that the membrane integrity had not been compromised during the process (Fig. 6 a–b).

A potential application for immobilizing non-adherent cells within microstructures is to analyze the cells for surface staining of various molecules. To examine the application of the microwells for cell surface staining, ES cells were docked within the channel and subsequently stained for the expression of an undifferentiated stem cell marker, SSEA-1. This was obtained by performing a two-step staining process in which medium containing the SSEA-1 antibody was flowed in the channel followed by a solution containing the secondary antibody, followed by a non-fluorescent medium to wash non-specific binding. As shown in Fig. 6 (c–d), ES cells could be directly stained within the microstructures. Approximately 95% of the cells could be seen expressing SSEA-1, which is similar to the results obtained when the cells are stained and flown through a flow cytometer. These results demonstrate the potential application of this technique to capture cells for a wide range of subsequent applications such as bioreactors and analysis including antibody staining.

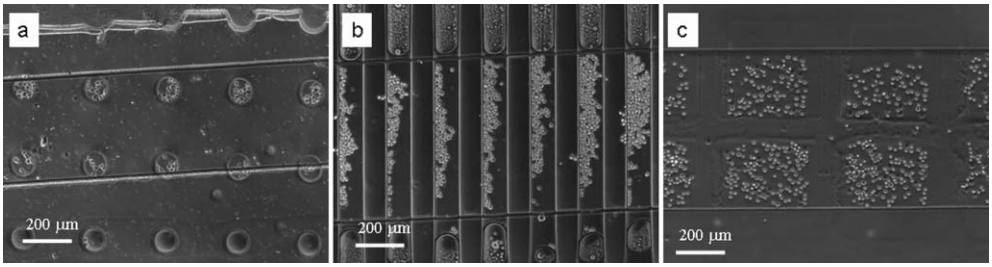

Fig. 5 Cells flowing through microchannels could be docked within microstructures of various sizes and shapes such as 100 μm microwells (a), perpendicular lanes (b) and grids (c).

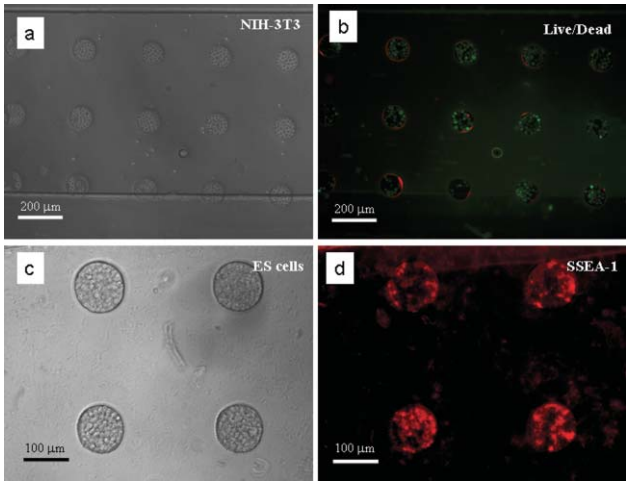

Fig. 6 NIH-3T3 cells were immobilized within microwells generated from PEG microstructures (a). These cells remained viable as measured by live-dead staining. Live cells express the green color representing the formation of calcein while the nucleus of membrane compromised cells (dead cells) was stained with the red color. (c–d) Murine embryonic stem cell patterning (c) and SSEA staining (d) within non-exposed microfluidic channels.

Cell adhesion onto exposed microwells within microfluidic channels

As demonstrated earlier, microwells could be generated with exposed substrates that allow for protein adsorption and adhesion of cells. To test the application of this process within microchannels, we generated patterned channels (with exposed substrate) and analyzed the ability of cells to dock and adhere within these wells. FN was allowed to flow through the channels to coat exposed surfaces and promote cell adhesion and spreading. NIH-3T3 fibroblasts were then flowed through the channel and their ability to adhere within the channels was examined using morphological characteristics of the captured cells with time. As expected, cells adhered to the bottom surface of the microwells and elongated within 6 h (Fig. 7). In addition, non-exposed patterns did not promote cell adhesion or elongation using the same experimental conditions (data not shown).

There are a number of potential advantages associated with this technique for patterning cells within microchannels. For example, both non-adherent and adherent cells can be immobilized, with tight control over the substrate properties while minimizing the effects of shear, therefore widening the potential application of cell-based microdevices. Also, the fabrication process used here is simple and could be applied without the use of masks and special equipment required for photolithography. This fabrication process also has a number of limitations. For example, currently the alignment procedure of the channels on the patterned substrates is facilitated by aligning the PDMS mold on the microstructures. This approach may be cumbersome for complicated patterns that require precise positioning. It is anticipated that the use of micromanipulators could be of benefit in alignment and adhesion of the microfluidic mold with the patterned substrate. Also, there is a potential height barrier for the microstructures since the approach is limited to the elastomeric properties of the PDMS to conform to the height of the polymeric features at the interface of the glass surface and the pattern edge. Therefore, the development of specifically designed patterning stamps that can construct microstructures that can directly fit in the channel may help alleviate this problem.

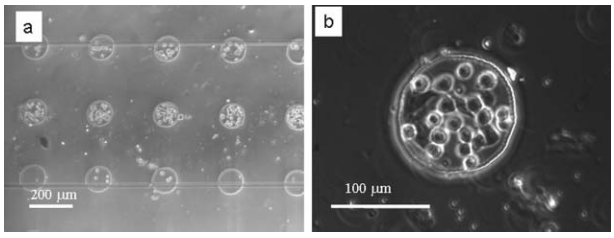

Fig. 7 NIH-3T3 cells were captured and adhered on channels with FN coated substrates. Images were taken after 6 h at low (a) and high (b) magnifications.

Conclusions

In conclusion, we have shown that PEG-based microstructures can be used to immobilize cells within microchannels, potentially an important tool for the development of cell-based biosensors and analytical devices. In addition, a method of generating microstructures inside robust microchannels was introduced that is capable of forming microwells with exposed or non-exposed substrates using simple modifications to the fabrication process. While both exposed and non-exposed microstructures allowed for stable docking and analysis of cells, only microwells with exposed substrates facilitated cell anchorage.

Acknowledgments

This research has been supported by the US Army (through the Institute for Soldier Nanotechnology under the Contract DAAD-19-02-D0002 with the US Army Research Office), Draper laboratory and the NSF (through the Bioprocess Engineering Research Center).

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